The role of interleukin-17 and RANKL in the regulation of bone destruction in arthritis

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For Mum and Dad

Abstract

Arthritis is a common disease characterised by chronic inflammation as well as bone and cartilage destruction. Proinflammatory cytokines such as interleukin-17 (IL-17), a T cell-derived cytokine, and Oncostatin M (OSM) a microphage cytokine, are elevated in rheumatoid arthritis (RA). The aim of this study was to characterise the effects of IL-17 and OSM on the expression of receptor activator of NF-kappaB ligand (RANKL) and its decoy receptor osteoprotegerin (OPG) by mesenchymal lineage cells in vitro, in particular synovial fibroblasts and the human osteosarcoma cell line (MG-63 and SaOS-2) that are potentially involved in this process as they regulate osteoclastogenesis. In addition, the ability of IL-17 to support osteoclastogenesis in vitro was assessed.

Human synovial fibroblasts (SFB) from RA and OA patients were contrasted with cell lines MG-63 and SaOS-2 and were treated with IL-17 and/or OSM for up to 48 hr. The expression and production of RANKL and OPG over a time course was assessed in these cells. To investigate osteoclastogenesis, peripheral blood mononuclear cells (PBMCs) were cultured with IL-17 either alone or with Macrophage colony stimulating factor (M-CSF). Osteoclastogenesis was analyzed after 21 days by tartrate-resistant acid phosphatase (TRAP), positive multinucleated cell counts, and resorption of ivory slices.

This study demonstrated a direct role for IL-17 in bone and cartilage catabolism through the induction of RANKL and OPG mRNA levels at 6 hr and 48 hr in SFB RA and OA, MG-63 and SaOS-2. Furthermore the combination of IL-17 and OSM showed that the expression of RANKL and OPG was down-regulated. Co-incubation of IL-17 with or without M-CSF significantly enhanced TRAP+ve multinucleated cell formation. Furthermore, cultures of PBMC with IL-17 on ivory slices showed significantly increased resorption and when co-cultured with synovial fibroblasts from RA and OA patients further significant resorption. The addition of OPG to IL-17 cultures significantly inhibited the formation of resorption lacunae. The effect of IL-17 on bone resorption in vitro is mostly RANKL-dependent. This finding may lead to the conclusion that IL-17 significantly induces osteoclastogenesis in vitro. Therefore, IL-17 represents a key cytokine involved in the exacerbation of inflammatory joint disease, and is an important target for anti-cytokine therapies. Also these results provide proof of the concept that OPG production can result in effective therapies.

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Authors' declaration

This thesis is based on research performed in the Musculoskeletal Research Group (Rheumatology and Orthopaedics), the Medical school, University of Newcastle Upon Tyne, UK. Except for commonly held concept, and where specific reference is made to other work, the content of this thesis is original. Furthermore, no part of this thesis has been submitted for the award of any other degree.

Publications

Abstract

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Abbreviations

AA	Amino acid
ALP	Alkaline Phosphatase
AKt	protein kinase B
ANOVA	Analysis of Variance
AP-1	Activated protein 1
APS	Ammonium persulphate
∝-MEM	Alpha minimal essential medium
bp	Base pair
BMP	Bone morphogenic protein family
BSA	Bovine serum albumin
Ca2+	Calcium ion
cAMP	Cyclic adenosine monophosphate
c-cb1	proto-oncogene
Cbfa1	Core binding factor alpha 1
cDNA	Complementary DNA
c-FOS	
CNS	central nervous system
CO_2	Carbon dioxide
Coll I	Collagen I
C_T	Threshold cycles
CTR	calcitonin receptor
COX	Cyclooxygenase
DAPI	4, 6-Diamidino-2-phenylindole
ddH_2O	Double-distilled water
dNTPs	Deoxynucleotide triphosphates
DEPC	Diethyl pyrocarbonate
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline

DTT	D, L-1, 4-dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
g	Relative centrifugal force
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp130	Glycoprotein 130
GRO	Growth related oncogene
H_2O	Water
HRP	Horseradish peroxidase
ICAM	intercellular adhesion molecule
IkB	Inhibitor of NF-kB
IKK	IkB kinase
IL	Interleukin
IGF-I	insulin-like growth factor I
IFN	Interferon
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK	Jun N-terminal kinase
kb	Kilobase
KDa	Kilodaltons
LIF	Leukaemia inhibitory factor
mAB	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase

M-CSF	macrophage-colony stimulating factor
MEK	MAP kinase/extracellular signal-regulated kinase kinase
MEM	Minimum essential Medium
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinase
MOPS	3-(N-morpholino) propanesulphonic acid
Mr	Relative molecular mass
mRNA	Messenger RNA
NF-kB	Nucler factor kB
NIK	NF-kB inducing kinase
NaOH	Sodium hydroxide
NO	Nitric acid
NSE	non-specific esterase
OA	Osteoarthritis
OC	Osteocalcin
OPN	Osteopontin
OSM	Oncostatin M
OPG	Osteoprotegerin
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PI3K	Phosphatidyinositol-3 kinase
PKB	Potein kinase B
PS	Penicillin and Streptomycin solution
PTH	Parathyroid hormone
PTHr	Parathyroid hormone-related protein
PYK2	a calcium-dependent tyrosine kinase
RA	Rheumatoid arthritis
Raf	
RANK	Receptor activator of NF-kB
RANKl	Receptor activator of NF-kB ligand

RNA	Ribonucleic acid
RNase	Ribonuclease
Ras	
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyaceylamide gel electrophoresis
SEM	Scanning electron microscopy
Ser	Serine
SFC	Fibroblast-like synoviocytes
SH2	Src homology 2 domain
sIL-6R	Soluble IL-6 receptor
SMCs	vascular smooth muscle cells
SSC	Saline-sodium citrate
STAT	Signal transduction and activator of transcription
Src	
TACE	TNF-α converting enzyme
Tak	TGF-β-activated kinase
TBS	Tri buffered saline
Thr	Threonine
TEMED	NNN'N'-tetramethylethylene-daimine
TF	Tissue factor
TGF	Transforming growth factor
T_H	T helper cell
T_H0	Naïve T helper cell
T_H1	T helper cell type 1
T_H2	T helper cell type 2
TIMP	Tissue inhibitor of metaloproteinasis
TNE	Tris, EDTA and sodium chloride buffer
TNF	Tumour necrosis factor
TNFRSF	TNF-receptor-superfamily

TRAF	TNF receptor associated factor
TRAP	Tartrate-resistant acid phosphatsae
TRANCE	TNF-releated activation-induced cytokine
Tyr	Tyrosine
V-CAM	vascular cell adhesion molecule
V-ATPase	vacuolar proton- translocating
VEGF	Vascular endothelial growth factor
v/v	Volume per Volume
w/v	Weight per Volume

CHAPTER-1

Introduction

1 General introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease that links the immune system with bone and cartilage destruction. RA is a chronic, systemic, inflammatory disease that mainly affects the synovial membranes of multiple joints of the body. As the disease is systemic, there are many extra-articular features of the disease as well. The skeletal abnormalities in RA are characterised by progressive articular cartilage destruction and bone erosions as well as local and systemic osteoporosis. There is also major development of new vessels and indications of systemic inflammation, for example, up-regulated acute phase proteins. In more severe cases there is contribution of vessels and other organs (Feldmann M. et al., 1996). A diseased joint showing the major cell types and sites of joint destruction is illustrated in Figure 1.1

Characteristic histopathological features of RA are hyperplasia of synovial lining cells with a dense microvasculature and infiltration by blood-derived cells, essentially memory T cells, plasma cells and macrophages, all cells that show signs of activation (Rehman Q. and Lane NE 2001; Suzuki Y. et al., 2001; Janossy G. et al., 1981; Cush JJ. and Lipsky PE. 1988). RA, in common with many autoimmune diseases, is more often diagnosed in women, suggesting a role for sex hormones in this disease (Feldmann M. et al., 1996). This leads usually to progressive destruction of cartilage and bone, which occurs after attack of these tissues by the cellular synovial tissue and is believed to be mainly mediated by cytokine induction of destructive enzymes, predominantly matrix metalloproteinases (MMPs).

It has long been hypothesized that proinflammatory cytokines excessively produced in areas of active inflammation may transfer signals from immune cells to osteoclasts, which constitute the specialised cells of bone resorption (Rehman Q. and Lane NE. 2001). Human osteoclast (hOC) and human osteoclast-like cells (hOCL) synthesize and secrete high constitutive and inflammation-stimulated levels of the chemokine interleukin-8 (IL-8). Consequently, hOC-derived IL-8 could act as an important regulatory signal for bone, vascular, and immune cell recruitment and activation during normal and pathological bone remodeling (Rothe L. 1998; Hofbauer LC. et al., 2001). A recent study demonstrated that the bone-resorbing activity of multinucleated cells

derived from rheumatoid synovial tissue was much higher than that of osteoarthritic synovial tissue, and was related to disease activity (Suzuki y. et al., 2001). In addition, multinucleated giant cells are uncommon in the rheumatoid synovium, but some of the synovial lining cells appear to become multinucleated (Muller-Ladner U. et al., 1998). Human macrophages obtained at arthroplasty and human monocytes cultured with RA synovial fibroblasts have been shown to differentiate into osteoclastic bone-resorbing cells (Kotake S. et al., 1996; Suzuki. Y. 2001; Toyosaki-Maeda T. et al., 2001). However, the pathogenesis of tissue destruction in RA and the cellular and molecular context of this process, in particular the details of cross-talk between the immune system and bone metabolism, remain elusive. Functional interdependence between the immune and bone systems is reflected in a number of regulatory molecules such as receptor activator of NF-kB ligand/receptor activator NF-kB/osteoprotegerin (RANKL/RANK/OPG) (see table 1-1 for pseudonyms of these agents). These cytokines have crucial roles in the development and function of osteoclasts, dendritic cells, and T and B lymphocytes, as well as in thymus and lymph node organogenesis. The cytokines produced by immune cells may affect bone cell function and vice versa, but the full complexity of these interactions awaits further investigation. Therefore, disturbances of the immune system may affect bone metabolism, and vice versa (Kotake S. et al., 2001; Grevice D. et al., 2001).

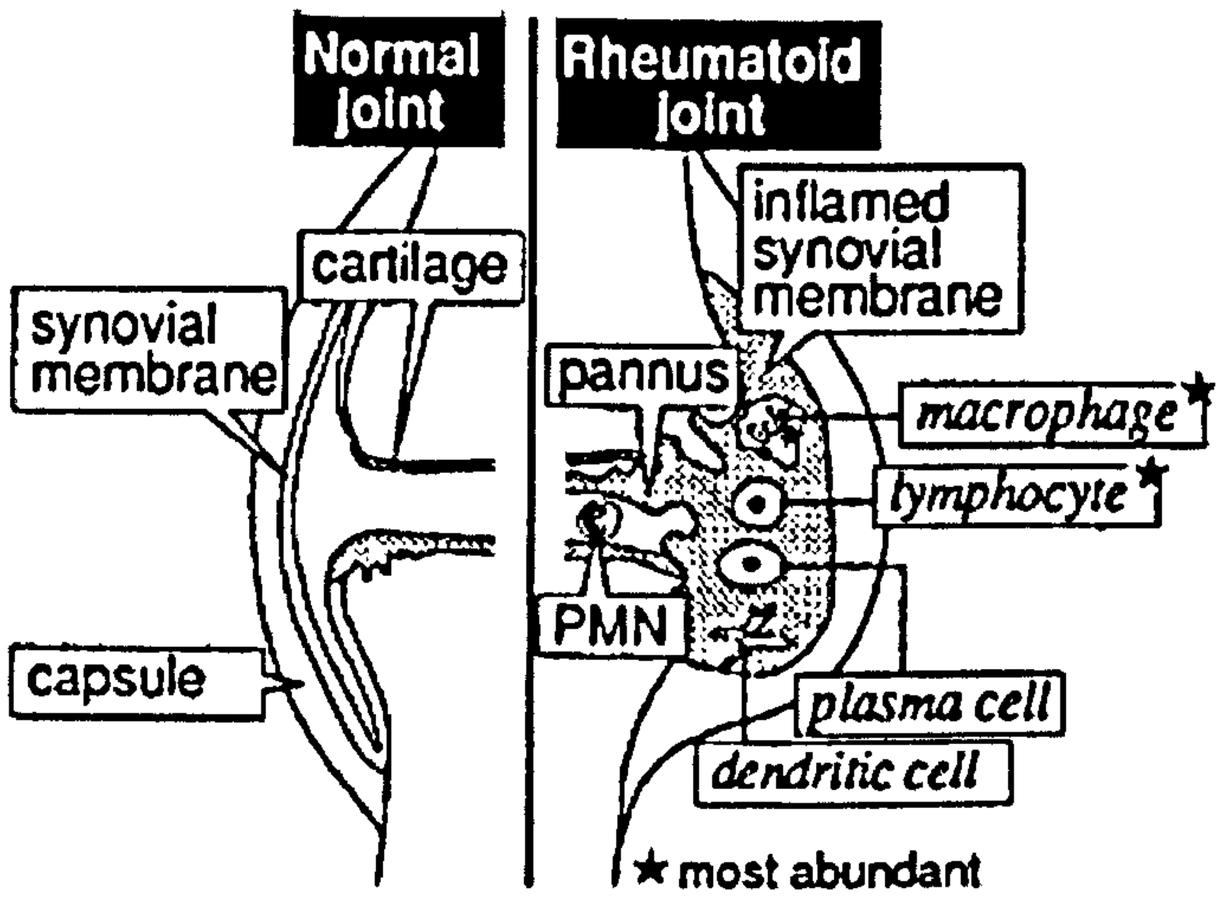


Figure 1.1. Rheumatoid joint showing cell types and sites of joint destruction. (Hammer et al., 1995).

OPG	**Osteoprotegerin (OPG) or** **Osteoclastogenesis inhibitory factor (OCIF)**
RANK	**Receptor activator of NF-kB (RANK)or** **Ostoclast differentiation and activation receptor (ODAR)**
RANKL	**Receptor activator of NF-kB ligand (RANKL) or** **Osteoclast differentiation factor (ODF) or** **Osteoprotegerin ligand (OPGL) or** **TNF-related activation-induced cytokine (TRANCE)**
TRAF	**Tumor necrosis factor receptor- associated factor**
TRAIL	**TNF-related apoptosis-inducing ligand**

Table 1.1. Commonly used synonyms of bone regulating factors (Hofbauer, et al., 2000).

1.2 Bone

In life, bone is a rigid yet dynamic organ that is continuously remoudelled. Bone microstructure is patterned to provide maximal strength with minimal mass, as determined by the mechanical needs of the organism. Once formed, bone undergoes a remodelling process that involves breakdown (resorption) and build-up (formation) of bone, and this occurs in microscale throughout the skeleton. Imbalances of remodelling can result in gross perturbations in skeletal structure and function, and potentially to morbidity and shortening of lifespan (Boyle W, J., 2003).

Bone can develop by one of two routes. Mesenchymal cells can differentiate directly to bone, as occurs in the flat bones of the craniofacial skeleton; this process is termed intramembranous ossification. Alternatively, cartilage can provide a blueprint or

template for bone morphogenesis, as occurs in the majority of the 200 + bones in the human skeleton. The cartilage blueprint is ephemeral and is replaced by bone in a process termed endochondral ossification (Reddi AH. 1981). Bone is also continuously modelled during growth and development and remodelled throughout the life of the organism in response to physical and biochemical signals. The physical signals include, but are not limited to, gravity, electricity, magnetism and ultrasound. Bones are a superb example of design architecture and engineering. They consist of cortical or compact bone and cancellous or trabecular bone. The outer cortical and inner trabecular bone network permits mechanical function and adaptation to changing mechanical environment and signals. Osteoporosis is a systemic skeletal disease of low bone mass, loss of the network of trabeculae, and deterioration of microarchitecture in bone trabeculae especially in postmenopausal women (Riggs and Melton, 1992). In developing bone, osteoblasts of mesenchymal lineage initially form woven bone that is subsequently modeled by osteoclasts of the hematopoietic monocyte-macrophage lineage (Marks SC. 1989), leading to the formation of lamellar bone with its characteristic concentric rings with a central blood vessel. The osteocytes and osteoblasts in the bone form syncytium by gap junctions and connecting canalicular cell processes (Vukicevic et al., 1990). The primordial signals for the lineage of bone-forming osteoblasts are the family of bone morphogenic proteins (Reddi, 1997).

Further studies have demonstrated that bone modelling during development and remodelling throughout life are dependent upon factors that regulate the number and activity of both bone-forming osteoblasts and bone-resorbing multinucleated osteoclasts (Chambers, T. et al., 2000; Teitelbaum S. L. 2000; Boyle W, J., 2003). Deciphering the biochemical and molecular mechanisms that manage bone cell precursor recruitment, differentiation, and activity is, therefore, important for understanding both normal and pathological processes in bone. Bone remodelling is tightly regulated by local (ie. autocrine and/or paracrine) and endocrine factors. The endocrine regulation of bone resorption has been well known for many years. Many osteotropic hormones such as 1∝, 25 dihydroxy vitamin D3 (vitD3), parathyroid hormone (PTH), and calcitonin preferentially modulate the process of bone resorption and control bone remodelling (Teitelbaum SL. 2000).

Many adult skeletal diseases are due to excess osteoclastic activity, leading to an imbalance in bone remodelling which favours resorption (Rodan GA. and Martin T. J.

2000). Excessive osteoclast activity is observed in many osteopenic disorders characterized by increased bone resorption and crippling bone damage, Such diseases include osteoporosis, periodontal disease, multiple myeloma, metastatic cancers, post-menopausal osteoporosis, Paget's disease, lytic bone metastases and RA. Local or generalized bone loss has also been reported in chronic infections [hepatitis, human immunodeficiency virus (HIV)], leukamia, auto-immune and allergic disease, and RA, suggesting that an activated immune system can affect bone physiology (Reddy SV and Roodman GD. 1998; Kotake S et. al., 1999). However, it is only recently that bone formation has been shown to be under endocrine control (Ducy P. et al., 2000).

For individuals with osteoporosis, bone fractures represent life-threatening events, and today there are enormous numbers at risk worldwide. Recent breakthroughs in our understanding of osteoclast differentiation and activation have come from the analysis of a family of biologically related tumour necrosis factor (TNF) receptor (TNFR)/TNF-like proteins: osteoprotegerin (OPG), receptor activator of nuclear factor (NF)-kB (RANK) and RANK ligand (RANKL), which together regulate osteoclast function (Khosla, S. 2001). The study of this pathway is providing a deeper understanding of how diverse physiological and pathophysiological signals exert their effects on the RANK signalling pathway to induce osteoclastogenesis, bone resorption and skeletal remodelling, and so control bone mass.

1.3 The osteoblast

Osteoblasts are believed to be derived from mesenchymal cells, which further differentiate into osteocytes once embedded in calcified tissues (Rodan GA. 1992; Suda T. et al., 1992; Aubin JE. 1998). Functionally, osteoblasts are the cells within bone that lay down the extracellular matrix and regulate its mineralization. Morphologically, these cells are cuboidal in shape and located at the bone surface together with their precursors, where they appear as a tight layer of cells. Osteoblasts are highly anchorage-dependent and rely on a number of cell–matrix and cell–cell contacts, via a multiplicity of transmembrane proteins (connexins, cadherins, integrins) and specific receptors (for growth factors, hormones, cytokines) to continue cellular function and responsiveness to metabolic and mechanical stimuli (Ferrari SL. et al., 2000, Lecanda F. et al., 1998).

The lifespan of an osteoblast ranges between 3 days in young rabbits up to 8 weeks in humans, during which time it lays down 0.5–1.5 μm of osteoid per day (Jowsey J 1977, Owen M. 1972). Ultimately, some osteoblasts may become "trapped" in their own calcified matrix, changing their phenotype and developing into osteocytes. These cells continue to survive, but considerably reduce their cell organelles and the production of matrix proteins. They remain attached with other similar cells but also with bone-lining cells (inactive osteoblasts) at the bone's surface, creating an extensive network of intercellular communication. There is accumulating evidence for a functional role of these cellular connections in sensing the need for, and directing the site of, new bone turnover (Donahue HJ. et al., 1995; Mosley JR. 2000).

In cell culture, osteoblasts are almost identical to fibroblasts. The only morphological feature specific to osteoblasts is positioned outside the cell, in the form of a mineralized extracellular matrix. However, there are two genes expressed in osteoblasts selectively as evidence to date that bone matrix mineralization is orchestrated by osteoblasts, and conversely, only osteoblast-specific transcription factors have been identified: one encoding cbfa1, a transcription factor and the other encoding osteocalcin, a secreted molecule that inhibits osteoblast function (Ducy P. et al., 1996, 1997; Zambotti A et al., 2002). A recent study revealed that bone morphogenic protein-7 (BMP-7), part of the transforming growth factor beta (TGF-β) protein superfamily, is a growth and differentiation factor that causes a shift in the differentiation pathway from myoblastic to osteoblastic phenotype in C2C12 mouse myoblast precursor cells in vitro. BMP-7 induces the expression of osteoblastic differentiation markers (Gu K. et al. , 2004).

1.4 The osteoclast

1.4.1 The origin, differentiation and molecular characteristics of the osteoclast

Osteoclasts are bone-resorbing polykaryons (often in the range 4-20 nuclei) that form fusion of mononuclear precursors and are capable of resorbing mineralised tissues. They are morphologically characterizied by well-developed Golgi complexes around each nucleus, abundant lysosomal vesicles, vesicular trafficking, and a highly polarized structure. Perhaps the most recognisable osteoclast features are the ruffled border

(striking finger-like protrusions formed by membrane and cytoplasmic undulations) and the sealed zone (the region of close apposition of the osteoclast ventral membrane to the bone surface) (Aubin JE. 2000).

The osteoclast progenitors are of hemopoietic origin, such as colony forming unit-granulocyte macrophage (CFU-GM) or more differentiated monocytic cells (Udagawa, N. et al., 1990). The precursor cells differentiate into mature osteoclasts at the site of bone resorption under the control of osteotropic hormones, local factors produced in the microenvironment, and osteoblastic stromal cells (Suda, T. et al., 1992; Suda, T. et al., 1995). Osteoclast progenitors are present in hemopoietic tissue. Bone marrow-derived osteoblastic stromal cells play an important role in the modulation of the differentiation of osteoclast progenitors in two different ways: one is through cell-to-cell recognition, and the second through production of soluble mediators such as cytokines and growth factors which are produced in the microenvironment mainly by osteoblast lineage cells but also by immune cells, endothelial cells and megakaryocytes (Chambers TJ. 1992; Suda T. et al., 1992; 1995; 1999; Takahashi N. et al., 1988). In addition, Feuerbach, D. and Feyen JH. (1997) suggested that vascular cell adhesion molecule 1 (V-CAM-1) is involved in the interaction between stromal cells and osteoclastic precursor cells during osteoclastogenesis, presumably most importantly during early stages of the formation of osteoclasts. Moreover, co-culturing of B9/BM1 cells and bone marrow-derived endothelial cells (BMECs) promotes osteoclast differentiation, and this effect was blocked when these cells were separated from one another as the BMECs expressed factors on their surface.

Addition of OPG to the co-culture system of B9/BM1 murine bone marrow metastasis and bone marrow-derived endothelial cells (BMECs) in the presence of vitamin D3 and M-CSF promoted differentiation of primary osteoclast progenitors to osteoclasts. OPG completely blocked osteoclast induction as did addition of anti-CD44 antibody (Okada T. et al., 2003). In other words, osteoblastic cells are supporting cells that are able to provide a suitable microenvironment for osteoclast differentiation in bone (Suda T. et al., 1992).

1.4.2 Osteoclast phenotype

The phenotype of osteoclasts is quite different from that of macrophages. Macrophages are non-specific esterase (NSE) positive, Fc receptor positive, C_3 receptor positive, and they phagocytose foreign bodies. In contrast, osteoclasts are tartrate-resistant acid phosphatase (TRAP) positive, express the calcitonin receptor (CTR), the vitronectin receptor ($\propto v\beta 3$, a member of the integrin family of cell adhesion receptors), carbonic anhydrase II, cathepsin K and vacuolar proton-translocating ATPase (V-ATPase) (Rehman Q and Lane NE 2001). However, the remarkable essential feature of the active osteoclast is its ability to resorb bone. Moreover, numerous vacuoles, endoplasmic reticulum and mitochondria are found in the cytoplasm of osteoclasts with a ruffled border-like structure and peripherally oriented filaments. V-ATPase and cathepsin K have also been demonstrated in multinucleated-like cells from synovial lining cells (Okahashi N. et al., 1997; Hummel KM. et. al., 1998; Gravallese EM. et. al. 2000; Suzuki Y. et al., 2001).

1.4.3 Mechanism of bone resorption

The osteoclast is responsible for bone resorption, which involves several cellular and molecular processes. When osteoclasts transform from the resting (non-polarised) state to the polarised (resorbing) state, a striking alteration occurs in the organisation of their cytoskeleton. The osteoclasts adhere to the surface of bone via integrin molecules, mostly through the vitronectin receptor and develop specialised structures called podosomes in the clear zone that consists mainly of F-actin. After these cytoskeletal changes, osteoclasts exhibit a specific bone-resorbing activity that involves acid demineralisation of bone by a proton transporter ATPase (V-ATPase) and degradation of the bone matrix by proteinases. While a number of proteases are known to exist in osteoclasts, recent studies have demonstrated that the acidic cysteine proteinase, cathepsin K, and a neutral proteinase (MMP-9) play a crucial role in osteoclast-mediated matrix degradation. Both protons and these enzymes are secreted into the sealed space beneath the ruffled border to degrade bone (Flanagan AM. et al., 1992; Blair HC. 1998; Suzuki Y. et al., 2001; Boyle WJ. et al., 2003). The survival of the mature osteoclast, and its participation in successive rounds of bone resorption, is regulated in part by

hormones and cytokines (Rodan, G. A. 1998). RANKL and IL-1 increase the survival time of the mature osteoclast in vitro and in vivo, and this may be due to the ability of these factors to induce nuclear factor kB (NF-kB) activity (Jimi E. et al., 1999; Lacey D. et al., L 2000).

1.4.4 Regulation of osteoclast formation (osteoclastogenesis)

Osteoclast formation has been studied intensively in rodent cell systems, in which cells of the stromal cell/osteoblast lineage have been shown to promote the differentiation of osteoclast precursors (Flanagan AM. et al., 1992; Chambers TJ. and Collins DA. 1992; Suda T. et al., 1992). Recently, culture systems have been devised for the study of human osteoclast differentiation. Together, these experimental systems have been invaluable tools in investigating the activity of molecular promoters or inhibitors of osteoclast formation and function. Addition of a number of hormonal and cytokine factors has been found to promote formation and differentiation to fully functional murine osteoclasts from precursor cells (Anderson DM. et al., 1997; Wong BR. et al., 1997a,b; Yasuda H. et al., 1998; Itonaga I, et al., 2000; Boyle WJ. 2003).

Many local factors as well as hormones play a role in osteoclast differentiation. These can be classified into three categories in terms of their signal transduction: vitD_3 receptor-mediated signals; protein kinase A-mediated signals: parathyroid hormone, parathyroid hormone-related protein and prostaglandin E2 (PTH, PTHrP, PGE_2 and IL-1); and gp130 -mediated signals [IL-6, IL-11, Oncostatin-M (OSM), and leukaemia inhibitory factor]. In addition, certain adhesion molecules such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), can act as osteoclast-inducing factors. Moreover M-CSF/CSF-1, a product of osteoblast/stromal cells, regulates differentiation of osteoclast progenitors into mature osteoclasts (Chambers TJ. And Collins DA.1992; Feuerbach D and Feyen JH. 1997; Suda T. et al., 1997; Suda T. et al., 1998; Jimi E. et al., 1999; Nakashima, et al., 2000; Norgard M. et al., 2003; Boyle WJ. et al., 2003). In contrast, calcitonin, interferon-γ, GM-CSF, IL-4, IL-13, TGF-β1 and 3-methylcholanthrene (3MC) inhibit osteoclast formation (Hattersley G and Chambers TJ. 1991; Rodan GA. 1992; Kasono K. et al., 1993; Kudo O. et al., 2002; Ikeda T. et al., 1998; Ura K. et al., 2000; Naruse M. et al., 2004). However, another

report suggested that TGF-β enhances osteoclast formation by abrogating the suppressive effect of the non-adherent PBMCs, therefore maintaining the osteoclast forming potential of the osteoclast precursor population. The effect of TGF-β is context-dependent on the cytokine milieu of the microenvironment and/or the state of activation of the cell being targeted by TGF-β. The recent discovery of RANKL has led to a greater understanding of the mechanisms that regulate osteoclast formation (Massey HM, et al., 2001).

Cellular interactions between osteoblast/stromal lineage cells and hematopoietic osteoclastic progenitors maintain osteoclastogenesis (Suda T. et al., 1999). In addition, Nakagawa N. et al., (1998) reported the essential role of RANKL and RANK in the development of osteoclasts. RANKL is preferentially expressed on osteoblast/stromal lineage cells whereas its receptor RANK is preferentially expressed on osteoclast lineage cells and dendritic cells. However, RANK is the signaling receptor essential for RANKL-mediated osteoclastogenesis (Nakagawa, N. et al., 1998). Binding of RANKL to RANK activates NF-kB and c-jun N-terminal protein kinase (JNK), which are associated with osteoclastic differentiation and activation (Jimi, E. et al., 1999, Nakashima, et al., 2000). Osteoclastogenesis also occurs under the control of bone marrow stromal cells (Suda T. et al., 1992). Most of these cytokines produced by stromal cells in bone marrow appear to be essential for osteoclastogenesis.

At least 24 genes loci have been shown to positively and negatively regulate osteoclastogenesis and osteoclast activation, based on naturally occurring mutations or targeted knockout mutations in rodents and humans (McLean W. et al., 2001). Disruption of these genes blocks the development and/or function of the mature osteoclast, resulting in abnormally high levels of mineralized bone and cartilage, a condition called osteopetrosis. As a class of mutations, these genes exert their effects at various stages of osteoclast development and activation. Some genes act during the formation and/or survival of the osteoclast precursor cell (PU-1 and op/CSF-1), whereas other genes mediate either the ability of the precursor cell to undergo differentiation (RANK, p50/p52 rel and c-fos) or the adherence and lytic function of the mature osteoclast (src, oc/Tcirg and CATK). Continued analysis of the unknown loci in this group of genes is likely to further define important control mechanisms

within the osteoclast and the osteoclast regulatory networks (Marks SC and Popoff SN. 1989; Wagner EF. et al., 2001).

1.5 Cytokines

All cytokines have certain properties in common. They are all small molecular weight peptides or glycopeptides. Many are produced by multiple cell types such as lymphocytes, monocytes/macrophages, mast cells, eosinophils, even endothelial cells lining blood vessels. The immune system has many different types of cells acting together to counteract unwanted infections and altered cells. Cytokines play a central role in the modulation of the immune system. They orchestrate the cellular and humoral adaptive immune responses and also play a significant role in innate immunity. They are produced by a variety of cells, have varied functions and form a complex interactive system. Cytokines often act together and enhance the effects of one another (synergy) or may also act as antagonists. Each individual cytokine can have multiple functions depending upon the cell that produces it and the target cell(s) upon which it acts via specific receptors (called pleiotropism). Binding initiates signal transduction and can lead to gene activation, mitosis and cell division, cell growth and differentiation, cell migration or programmed cell death (apoptosis). Also, several different cytokines can have the same biological function (called redundancy). Cytokines can exert their effect through the bloodstream on distant target cells (endocrine), on target cells adjacent to those that produce them (paracrine) or on the same cell that produces the cytokine (autocrine). Physiologically, it appears that most cytokines exert their most important effects in a paracrine and/or autocrine fashion. Their major functions appear to involve host defence or maintenance and repair of the blood elements. Thus cytokines may induce, amplify, prolong and terminate inflammation (Rogler G. and Andus T. 1998). The effects of cytokines are not limited to immune cells but include cells of the epithelium, endothelium, mesenchyme and extracellular matrix including bone and cartilage cells.

Cytokines are categorized by their major specific function(s). There are four major categories of cytokines. **Interferons** are so named because they interfere with virus

replication. The **colony stimulating factors** are so named because they support the growth and differentiation of various elements of the bone marrow. The **tumor necrosis factors (TNF)** are so called because injecting them into animals causes a hemorrhagic necrosis of their tumors. The largest group is the **interleukins**, so named because their fundamental function appears to be communication between **(inter-)** various populations of white blood cells (leucocytes **-leukin)**. They are produced by a variety of cell types such as monocytes/macrophages, T cells, B cells and even non-leucocytes.

1.5.1 RANKL, RANK and OPG

1.5.1.1 RANKL

1.5.1.1.1 Identification and characterization

RANKL expression is restricted primarily to stromal/osteoblastic cells and lymphocytes and has been demonstrated in $CD3^+$ T cells. The ability of activated T lymphocytes to support osteoclast formation may provide a mechanism for the potentiation of osteoclast formation and bone resorption in disease states such as RA (Horwood NJ. et al., 1999; Anderson DM. et al., 1997; Lacey DL. et al., 1998; Yasuda H. et al., 1998). In addition, RANKL expressed by synovial fibroblasts is believed to be involved in rheumatoid bone destruction by inducing osteoclastogenesis (Takayanagi H. et al., 2000). In extra-skeletal tissues, RANKL mRNA and protein is detected in brain, heart, kidney, skeletal muscle and skin during mouse development, suggestive of the possibility of various other functions of the molecule. RANKL is also developmentally regulated, as evidenced by its expression in the intestine, liver and lung in newborn mice but not in the adult (Kartsogiannis. V. et al., 1999). Additionally, RANKL has been detected in various stromal cell lines (ST-2, MC3T3-E1, hMs, primary human marrow stromal cells), osteosarcoma cell lines (ROS, MG-63), primary murine osteoblasts and a murine monocytic cell line RAW 264.7 (Anderson MD. et al., 1997; Wittrant Y. et al., 2004).

RANKL is a stimulatory factor for dendritic cells and is an essential cytokine signal for various osteoclast functions. A recent study demonstrated that both B and T lymphocytes might act through the RANKL/RANK/OPG cytokine system, which has

been independently discovered within immune and bone systems. These cytokines have crucial roles in the development and function of osteoclasts, dendritic cells, and T- and B-lymphocytes, as well as in thymus and lymph node organogenesis (Wong BR. et al., 1997; Anderson DM. et al., 1997; Yasuda H. et al., 1998; Lacey DL. et al., 1998; Grevice D. et al., 2001). Moreover RANKL, RANK, and OPG were detected in the superficial zone of normal cartilage. However, RANKL does not activate human articular chondrocytes (Komuro, H. et al., 2001).

Human RANKL is a polypeptide of 317 amino acids that is a type II transmembrane glycoprotein which possesses a long extracellular stalk region followed by a receptor-binding core domain as shown in Fig 1.2 (Yasuda H. et al., 1998). Murine RANKL encodes a 316 amino acid, 35kDa protein (45kDa after N-terminal glycosylation) that shares 83% sequence homology with human RANKL (Wong BR. et al., 1997). RANKL is related to the TNF-related apoptosis inducing ligand (TRAIL; 20-34% homology), the CD40 ligand (38% homology), and to the Fas ligand (19% homology) (Lacey, et al., 1998). RANKL lacks a signal peptide and has a cytoplasmic domain at the N-terminus (residues 1-48), a transmembrane domain (residues 70-317), and an active ligand site (residues 158-317) sequence that are present in TNF ligand family members (Anderson DM. et al., 1997; Yasuda H. et al., 1998).

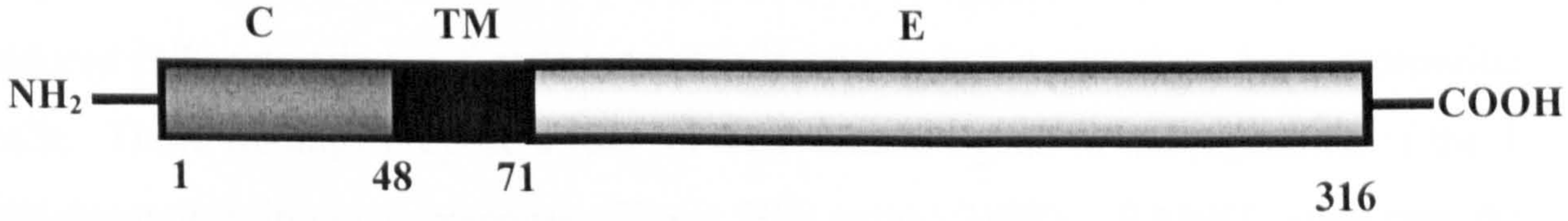


Fig 1.2. Schematic structure of RANKL. RANKL is an OPG-binding protein. Domains: C, cytoplasmic; TM, transmembrane; E, extracellular. (Yasuda, H., et al., 1998).

RANKL exists in two biologically active forms; a cellular membrane bound form (40-45 kDa) and a soluble form (31 kDa) derived post translationally by cleavage at position 104 or 145 (Lacey DL. et al., 1998). A variety of transmembrane proteins are synthesised as a membrane-bound protein, which are cleaved into a soluble form by ectodomain shedding (Schlondorff J. and Blobel CP. 1999; Walsh MC. and Choi Y. 2003). Since an inhibitor for metalloproteases prevents the conversion of membrane-bound RANKL to its soluble form, metalloproteases appear to play a role in cleaving

membrane-bound RANKL (Lum L. et al 1999). TNF-∝ converting enzyme (TACE), a sheddase that cleaves membrane-bound TNF-∝, has been identified as a member of the disintegrin and metalloprotease (ADAM) family (Black R. et al., 1997). Membrane bound RANKL is cleaved into its soluble form by TACE (also called ADAM-17) (Black R. et al., 1997 and Nakashima T. et al., 2000). RANKL is more potent when membrane-bound, but further investigation will be required to elucidate any true physiological difference. The protein in its soluble form may have different functional properties from that of the membrane-bound one, which is exemplified in Fas-mediated apoptosis elicited by Fas l. Membrane-bound Fas l efficiently induces target cell apoptosis, whereas the apoptotic activity of soluble Fas l (sFas l) was significantly lower than that of membrane-bound Fas l (Nakashima T. et al., 2000). However, the functional difference of both soluble and nonsoluble RANKL is not entirely clear (Walsh MC. and Choi Y 2003). Characterization of the functions of RANKL and its receptors have contributed significantly to the emergence of a new field of study, osteo-immunology, directed at examining the interplay between active immunity and maintenance of bone homeostasis (Walsh MC. and Choi Y. 2003; Jones DH. et al. , 2002).

Human RANKL was mapped to chromosome 13q14 while mouse TRANCE was located to the portion of mouse chromosome 14 syntenic with human chromosome 13q14. A recombinant soluble form of RANKL composed of the entire ectodomain induced JNK activation in T cells but not in B cells or in bone marrow-derived dendritic cells. These findings propose a role for TNF-related ligand in the regulation of the T cell-dependent immune response (Wong BR. et al., 1997). RANKL augments the ability of dendritic cells to excite naive T-cell proliferation in a varied lymphocyte reaction, and enhance the survival of RANK+ T cells generated with IL-4 and TGF-β. Therefore, RANK and RANKL seem to be necessary regulators of interactions between T cells and dendritic cells (Anderson DM. et al., 1997).

1.5.1.1.2 Expression of RANKL

RANKL is an essential factor for osteoclast formation, fusion, activation, and survival. In addition to the cell-bound RANKL form, activated T lymphocytes produce the secreted RANKL form (Fig1.3). Manabe, et al., (2001) demonstrated that B-

lineage cells are a major source of endogenous RANKL in bone marrow and support osteoclast differentiation in vitro. Moreover, the levels of RANKL mRNA are highest in skeletal (trabecular bone, bone marrow) and in lymphoid tissues (lymph node, thymus, spleen, fetal liver, peyers patches) that are active in mediating the response. However, lower levels of RANKL are found in heart, skeletal muscle, lung, stomach, placenta and the thyroid gland (Lacey DL. et al., 1998). In addition, a recent study with animal models suggests the involvement of RANKL in the pathogenesis of periodontal disease (Mogi M. et al., 2004).

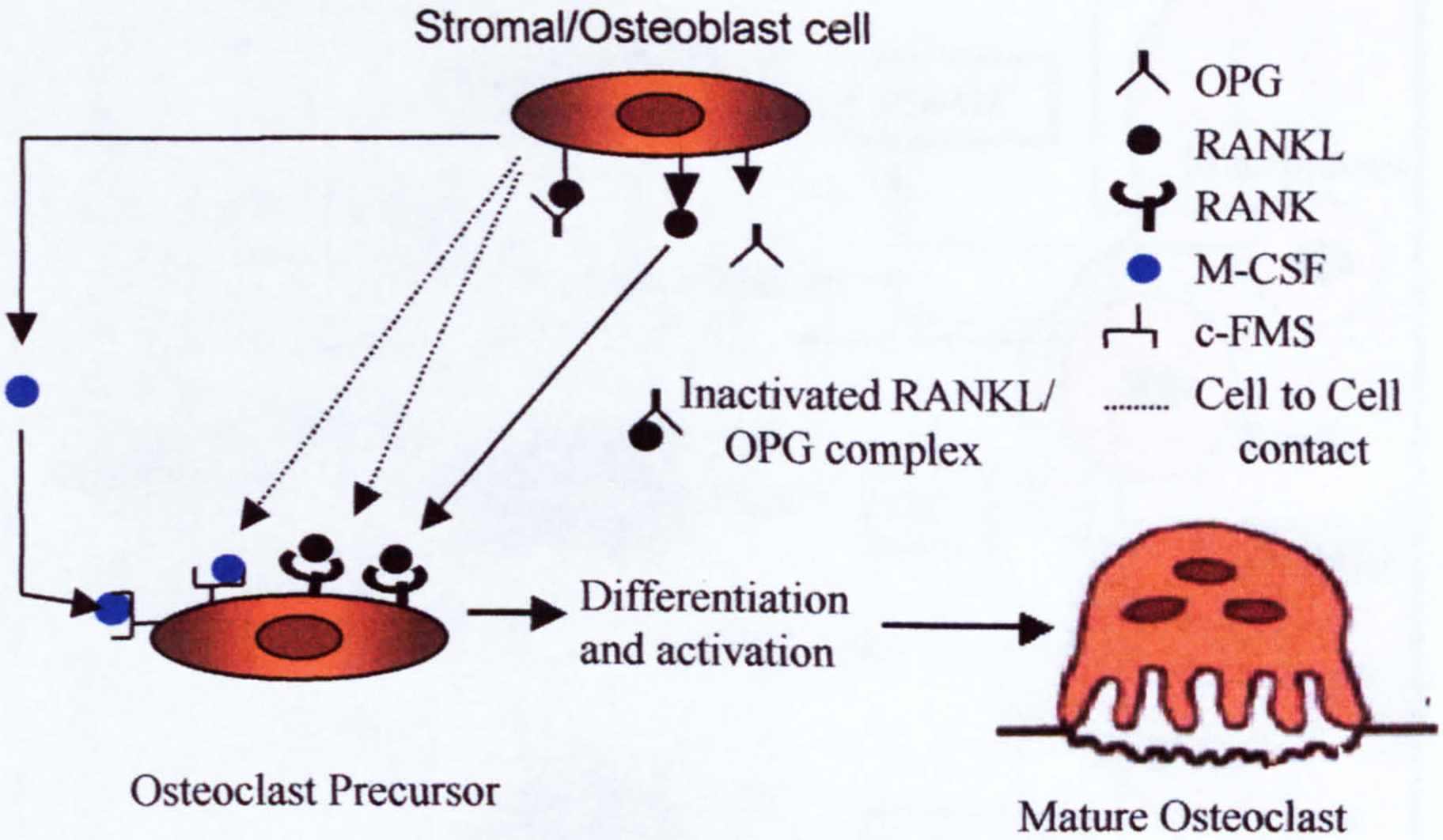


Fig-1.3 Regulation of osteoclastogenesis by the interaction of RANKL, OPG and them receptor RANK and M-CSF and its receptor c-FMS in the bone microenvironment. RANKL can bind to the receptor in a soluble sRANKL or cell-bound (cRANKL) form (Feng X et al., 2002; Hofbauer LC. et al., 2000).

1.5.1.1.3 Biological effects of RANKL

Both forms of RANKL act through binding to and activating RANK. This is a cell-bound receptor of the TNFR superfamily that is located on osteoclast precursor cells, mature osteoclasts, and dendritic cells (Kong Y-Y. et al., 1999). Similarly to other cytokine systems [IL-1, TNF-∝], the stimulatory effects of RANK by RANKL are

through binding to it (Lacey DL. et al., 1998). The up-regulation of RANKL protein production capable of regulating aspects of early uncommitted precursor differentiation, fusion and activation, leads to osteoclast maturation and functions, while up-regulation in OPG protein production leads to osteoclast apoptosis as shown (Fig-1.4).

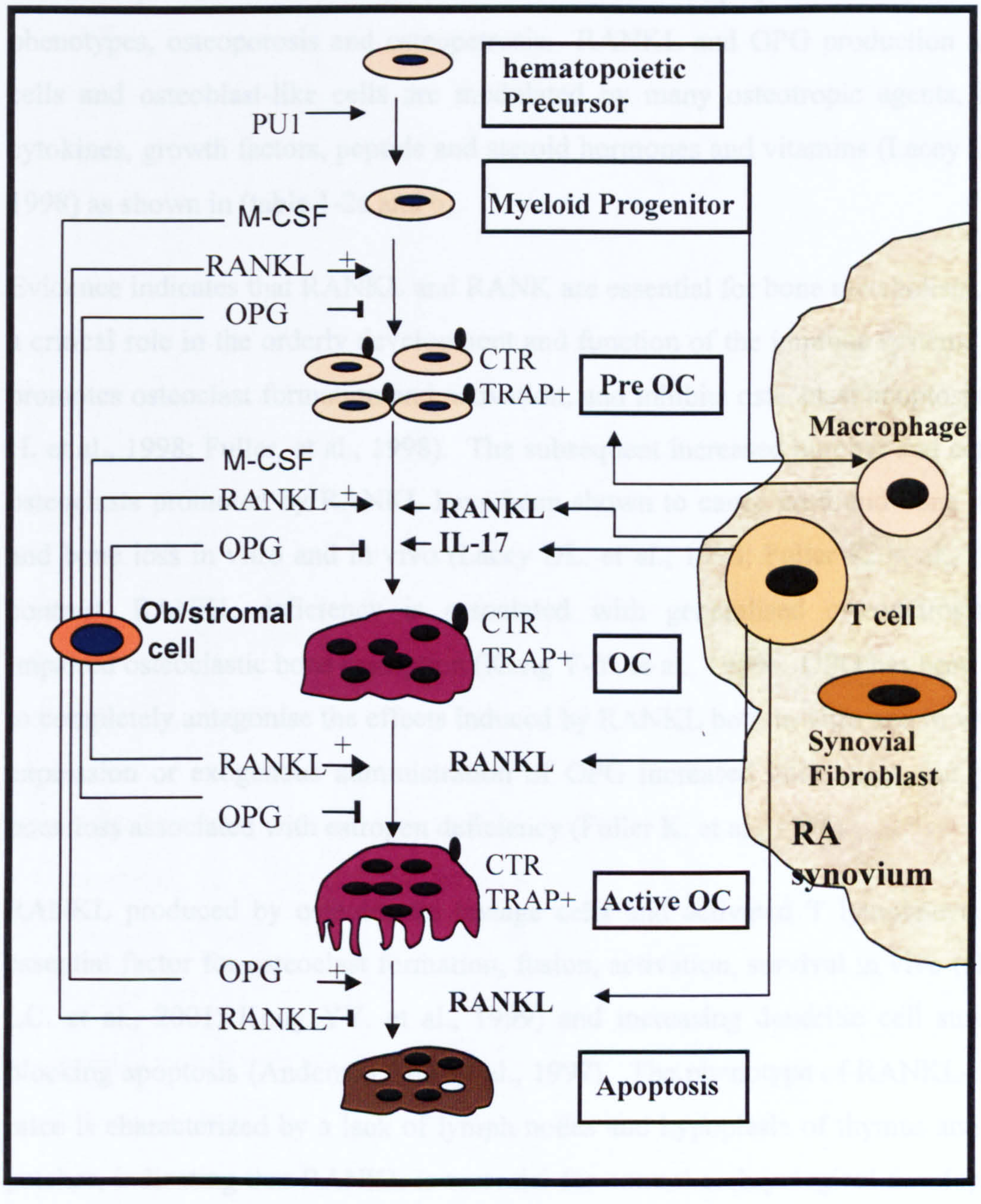


Fig-1.4 Osteoclast formation and function are regulated by RANKL/OPG. Including initiation of differentiation, fusion of preosteoclasts to form active mature osteoclasts; M-CSF is required only for initiation of differentiation of osteoclast precursors (Hofbauer LC. et al., 2000).

The balance of bone resorption depends on the local RANKL:OPG ratio, which is enhanced in bone metastases and humoral hypercalcemia of malignancy. Abnormalities of the RANKL/OPG system have been implicated in the pathogenesis of various primary and secondary bone malignancies (Hofbauer LC. et al., 2001), and excessive or defective production of RANKL, RANK and OPG display the extremes of skeletal phenotypes, osteoporosis and osteopetrosis. RANKL and OPG production in stromal cells and osteoblast-like cells are modulated by many osteotropic agents, including cytokines, growth factors, peptide and steroid hormones and vitamins (Lacey DL. et al., 1998) as shown in (table 1-2a and b).

Evidence indicates that RANKL and RANK are essential for bone metabolism, and play a critical role in the orderly development and function of the immune system. RANKL promotes osteoclast formation and activation, and inhibits osteoclast apoptosis (Yasuda H. et al., 1998; Fuller, et al., 1998). The subsequent increased number and activities of osteoclasts promoted by RANKL have been shown to cause profound bone resorption and bone loss in vitro and in vivo (Lacey DL. et al., 1998; Fuller K. et al., 1998). In contrast, RANKL deficiency is associated with generalised osteopetrosis due to impaired osteoclastic bone resorption (Kong Y-Y. et al., 1999). OPG has been observed to completely antagonise the effects induced by RANKL both in vitro and in vivo. Over expression or exogenous administration of OPG increased bone mass and prevented bone loss associated with estrogen deficiency (Fuller K. et al., 1998).

RANKL produced by osteoblastic lineage cells and activated T lymphocytes is the essential factor for osteoclast formation, fusion, activation, survival in vivo (Hofbaure LC. et al., 2001; Kong YY. et al., 1999) and increasing dendritic cell survival by blocking apoptosis (Anderson MD. et al., 1997). The phenotype of RANKL-deficient mice is characterized by a lack of lymph nodes and hypoplasia of thymus and peyer's patches, indicating that RANKL is essential for normal embryological development of lymphoid tissue (Kong YY. et al., 1999).

Factor	*Effect on RANKL*	*Effect on OPG*
IL-1α	-	↑
IL-1β	↑	↑
TNF-α	↑	↑
TNF-β	-	↑
PGE_2	↑	↓
IL-11	↑	↑
IL-6	-	-
IL-17	↑	-
IL-4 + ∝-CD3 (T Cells)	-	-
CD40L (dendritic cells)	-	↑
OSM	↑	↑
BMP2	-	↑
TGFβ	↓	↑
TGFβ + α-CD3 (T Cells)	↑	-
Calcium	↑	↑
IGF-I	-	↓
Cyclosporin A	↑	↓
Tacrolimus	↑	↓
Ionomycin (T Cells)	↑	-
PMA (T Cells)	-	-
Vasoactive intestinal peptide	↓	↑
Indian hedgehog	↑	-
LPS	-	↑

Table 1.2a Shows the regulation of RANKL and OPG by regulatory factors and cytokines based on (Hofbauer LC, 2000; Kong Y-Y, 2000 and Walsh M. and Choi Y. 2003). ↑ up-regulates; ↓ down-regulates; – not determined.

Factor	*Effect on RANKL*	*Effect on OPG*
PTH	↑	↓
Vitamin D3 1α, 25-$(OH)_2D_3$	↑	↑
17β-Estradiol	-	↑
Glucocorticoid	↑	↓
Estrogen	↑	↑

Table 1.2b Shows the regulation of RANKL and OPG by Calcitropic Hormones based on (Hofbauer LC, 2000; Kong Y-Y, 2000 and Walsh M. and Choi Y. 2003). ↑ up-regulates; ↓ down-regulates; – not determined.

1.5.1.2 RANK

RANK belongs to the TNFR superfamily. Since the molecular identification of RANK, an abundance of reports have been published documenting its biological function and signalling mechanisms.

RANK is a type I transmembrane protein and human RANK (616 amino acids) has a signal peptide (28 amino acids), an N-terminal extracellular domain (184 amino acids), a short transmembrane domain (21 amino acids), and a large C-terminal cytoplasmic domain (383 amino acids). The gene encoding RANK has been localised to human chromosome 18q22.1 (Anderson DM. et al., 1997; Hofbauer, et al., 2000; Walsh MC and Choi Y. 2003). The extracellular domain of RANK contains four cysteine-rich pseudorepeats and two N-glycosylation sites, a characteristic feature of members of the TNFR superfamily, including OPG. RANK has a 40% homology with CD40, although RANK was originally described as a receptor. on osteoclasts and dendritic cells (Anderson DM. et al., 1997). RANK mRNA expression has been detected in a variety of tissues including skeletal muscle, liver, small intestine, colon, thymus, and adrenal gland, as well as osteoclastic, lymphocytic, and nonlymphocytic cells. The distribution of RANK is restricted to osteoclasts, dendritic cells, B- and T-cell lines. Among bone cells, RANK is found only in osteoclast lineage cells. In dendritic cells RANK expression is stimulated by CD40 ligand (Anderson DM. et al., 1997; Hofbauer, et al., 2000).

The important role of RANK and its ligand RANKL in the formation of osteoclasts was clearly recognized by genetic studies in which both RANK and RANKL knockout mice displayed a prominent osteopetrotic phenotype and inflammation that may explain part of the abnormal phenomena in diseases such as RA characterized by both inflammation and destruction (Dougall WC. et al., 1999). Cytokines play a central role in the perpetuation of synovial inflammation. The persistence of the chronic inflammatory response in conjunction with ongoing joint destruction (a finding in many patients with RA despite the use of effective anti-inflammatory agents and disease-modifying drugs) probably appears as a direct result of sustained recruitment, inappropriate retention and impaired apoptosis (Morovic-Vergles J. 2003). Osteoclast-like cells are formed in co-culture of peripheral blood mononuclear cells and rheumatoid synovial fibroblasts obtained by continued sub-cultures in the presence of vitD3 and M-CSF. The multinucleated cells showed all the phenotypical and functional characteristics of osteoclasts including the expression of TRAP, vitronectin receptors, receptors for human calcitonin and the ability to resorb bone. Synovial macrophages are capable of differentiating into osteoclasts in the presence of rheumatoid synovial fibroblasts which can support differentiation of monocytes/macrophages, implicating that osteoclasts generated within the synovial membrane are probably involved in bone destruction in RA (Takayanagi H. et al., 1997). Synovial cells are capable of differentiating to osteoclast-like cells under some circumstances, including culturing with M-CSF and RANKL. This suggests that bone erosion seen in RA may result from RANKL/RANK system activation by activated T cells. This opens up the possibility that OPG may have therapeutic effects mediated by blockade of the RANKL/RANK system (Saidenberg Kermanac'h N. et al., 2002). An initial study demonstrated that short term Fc-OPG treatment has powerful anti-erosive effects, principally on bone, even though synovitis is not affected. These findings indicate the potential utility of disrupting RANK signaling to preserve skeletal integrity in inflammatory arthritis (Romas E. et al., 2002). In addition, RANK and RANKL along with OPG have functions to do with lymph node organogenesis, dendritic cell survival and early lymphocyte differentiation (Takahashi N. 1999).

Moreover, RANKL and RANK are expressed in mammary gland epithelial cells, and play a role in lactation (Fata, J.E et al 2000). Modulation of these systems provides a unique opportunity to design novel therapeutics to inhibit bone loss in arthritis,

periodontal disease, and osteoporosis (Theill LE. 2002). While the detailed molecular mechanism for each of the diverse functions of RANK is expected to be unravelled in the near future, there has already been extensive progress in elucidating RANK signalling pathways in a few specific cell types, especially osteoclasts and osteoclast precursor cells (Blair HC. and Athanasou NA. 2004).

1.5.1.2.1 Biological effects and signal transduction

RANKL binds to its receptor, RANK with high specificity and affinity. This activates a cascade of intracellular events, including interaction with TRAF family members, activation of the transcription factor NF-kB, and stimulation of the JNK pathway (Suada, T. et al., 1999). RANK interacts with various TRAFs through distinct motifs and activates NF-kB via a novel TRAF6 interaction motif, which then activates NF-κB-inducing kinase (NIK), thus leading to NF-kB activation. RANK most likely activates JNK through a TRAF2-interacting region (Darnay BG. et al., 1999; Darnay BG and Aggarwal BB. 1999). Moreover, RANKL activates the antiapoptotic serine/threonine kinase (protein kinase B Akt/PKB)) (via a signaling complex involving c-Src and TRAF6. An insufficiency in c-Src or accumulation of Src family kinase inhibitors blocks RANKL-mediated PKB activation in osteoclasts. c-Src and TRAF6 interact with each other and with RANK upon receptor engagement. TRAF6 enhances the kinase activity of c-Src leading to tyrosine phosphorylation of downstream signaling molecules, for example c-Cbl. These data provide evidence of cross-talk between TRAF proteins and Src family kinases (Wong BR. et al., 1999). RANKL also induces the expression of c-Fos, a component of the dimeric transcription factor activator protein-1 (AP-1). AP-1 is composed mainly of Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun proteins (c-Jun, JunB and JunD). c-Fos has an essential role with TRAF6 in osteoclastogenesis in both the differentiation and maturation of osteoclasts by establishing a link between RANK signalling and the expression of AP-1 proteins in osteoclast differentiation (Matsuo K. et al., 2000).

TRAF adaptor proteins appear to play an important role in the signal transduction pathway induced by RANK. TRAFs 1, 2, 3, 5 and 6 were shown to bind through the conserved TRAF domain to RANK in vitro and in transfected cells. TRAF binding domains are functionally important for the RANK-dependent induction of NF-kB and

JNK activities. Site-directed mutagenesis demonstrated that these TRAF binding sites exhibited selective binding for different TRAF proteins. In particular, TRAF6 interacts with membrane-proximal determinants distinct from those binding TRAFs 1, 2, 3 and 5. Among the TRAF proteins, TRAF6 appear to be crucial for RANK signalling in osteoclasts (Galibert L. et al., 1998; Kim HH. et al., 1999). Furthermore, results from experiments where RANK knockout cells were manipulated to express RANK mutants lacking the TRAF6-binding region provided evidence for TRAF6 in RANK-directed cytoskeletal association and the resorptive functions of osteoclasts (Darnay BG. et al., 1999; Armstrong A. P. et al., 2002). TRAFs may act by transmitting the RANK signal to downstream targets that consist of NF-kB and JNK. Indeed, over-expression of TRAFs 2, 5 and 6 stimulate the actions of NF-kB and JNK and the activation of JNK and extracellular signal-regulated kinase (ERK), but not that of p38, and appear to be involved in AP-1 activation by RANK. Thus, RANK may utilize both JNK and ERK pathways to signal to the AP-1 transcription factor as shown in Fig 1.5 (Hofbauer LC. and Heufelder AE. 2001; Lee Z. H. et al., 2000; Walsh MC, Choi Y. 2003).

1.5.1.2.2 RANK signalling by way of NF-kB activation

RANK signaling leads to a strong increase in the activation of NF-kB. NF-kB activity seems to be critical for osteoclast differentiation (osteoclastogenesis) as shown in mice deficient in both NF-kB proteins p50 and p52. The surprising result was that these animals developed osteopetrosis because of a defect in osteoclast differentiation, suggesting redundant functions of NF-kB1 and NF-kB2 proteins in the development of this cell lineage (Iotsova,V. et al., 1997). .NF-kB is retained in the cytoplasm as a complex with the inhibitory kB (IkB) protein in unstimulated cells. Stimuli that activate NF-kB induce the activation of IkB kinases (IKK), resulting in the phosphorylation and subsequent proteasome-mediated degradation of IkB.

NF-kB then enters the nucleus where it binds to DNA target sites and exerts its transcriptional activity. Events that have been shown to occur after RANK stimulation in differentiated osteoclasts or osteoclast precursor cells include the phosphorylation and degradation of IkBα and the nuclear translocation and DNA binding of the NF-kB proteins p50, p52 and p65 (Jimi E. et al., 1999; Abu-Amer Y. et al., 2001). IKK functions as a complex composed of two catalytic components, IKKα and IKKβ, and a

regulatory protein, IKKγ. IKKα and IKKβ are essential for the proinflammatory cytokine-induced activation of NF-kB (Li Z. W. et al., 1999). Therefore, IKKβ might mediate RANK activation of NF-kB through IkB degradation. The activation of NF-kB inducing kinase (NIK) and IKKα is essential for the processing of the NF-kB2 precursor (p100) to p52 (Senftleben U. et al., 2001) and for the phosphorylation of Ser536 in the transactivation domain of p65 (Jiang X. et al. , 2003). It has been shown that TRAF6-deficient spleen cells were defective in the RANKL-induced degradation of IkBβ (Kobayashi N. et al., 2001). Furthermore, the recruitment of TAK1 to TRAF6 in RANK-transfected 293 cells and the interference of NF-kB activation by kinase-defective TAK1 in RANKL-treated RAW 264.7 cells were recently demonstrated. Thus, TAK1 appears to be a likely downstream target of TRAF6 in the RANK to NF-kB signaling pathway in osteoclasts (Mizukami J. et al., 2002).

1.5.1.2.3 RANK signalling and MAPK cascades

The activation of mitogen-activated protein kinases (MAPKs) through RANK signalling has been well characterized. It has been established that members of all three MAPK families, ERK, JNK and p38, are activated by RANK in osteoclasts or osteoclast precursors (Jimi E. 1999; Lee SE. et al., 2002). Additionally, p38 and ERK play roles in osteoclast differentiation at least in art by participating in RANKL signalling (Lee SE. et al., 2002). Spleen cells derived from TRAF6-defcient mice failed to activate JNK and p38 in response to RANKL, suggesting that TRAF6 is critical to RANK signalling through these MAPKs (Kobayashi N. et al., 2001). In differentiated osteoclasts, association of ERK1/2 and NF-kB regulate different aspects of osteoclast activation: ERK is responsible for osteoclast survival, whereas NF-kB regulates osteoclast activation for bone resorption (Miyazaki T. et al., 2000). Recently, osteoclast progenitor cells derived from JNK1 knockout, but not from JNK2 knockout, mice were shown to have a reduced (but not ablated) level of RANKL-stimulated osteoclastogenesis, suggesting that JNK1, and not JNK2, is important for efficient osteoclast differentiation (David J. P. et al., 2002). The inhibition of the TRAF2-mediated activation of JNK by a kinase-inactive form of mitogen-activated protein kinase 1 (MEKK1) may suggest the potential involvement of this mitogen-activated protein kinase kinase kinase (MAPKKK) in RANK signalling (Song HY. et al., 1997). Ras has been implicated in

the activation of ERK via a signal transduction pathway involving CD40, a TNF family receptor that shares similar signalling characteristics as RANK (Kashiwada M. et al., 1998). The TAK1/TAB2 activity that induces JNK and p38 activation was shown to be located downstream of TRAF6 in the RANK signalling pathway (Mizukami J. et al., 2002). Downstream targets of ERKs and JNKs include AP-1 transcription factor, a dimer composed of a Fos and a Jun family protein. ERK can induce and activate c-Fos, while JNK can increase AP-1 transcriptional activity through the phosphorylation of c-Jun (Cano E. and Mahadevan LC., 1995). Mice deficent in c-Fos developed osteopetrosis due to a failure to commit to osteoclast lineage, underscoring the fundamental role of AP-1 in osteoclast development (Grigoriadis AE. et al., 1994).

1.5.1.2.4 RANK signalling through the PI3K/Akt pathway

In osteoclasts, RANK induces the activation of Akt, which is blocked by the PI3K inhibitor LY294002 (Wong B. R. et al., 1999, Lee S. E. et al., 2002). Furthermore, LY294002 reduces the RANK-mediated survival response of osteoclasts (Wong BR. et al., 1999). The PI3K inhibitor also displays a potent inhibitory effect on osteoclast differentiation (Lee SE. et al., 2002), which may result from a reduced survival of osteoclast precursor cells during differentiation. It has been shown in osteoclasts that RANK elevates Src kinase activity and that RANK activation of Akt is blocked by a Src family kinase inhibitor or the genetic deletion of c-Src (Wong BR. et al., 1999). Thus, Src may play a role as mediator of RANK via PI3K/Akt signals. Among the many molecules downstream of Src, PYK2 and c-Cbl have been implicated in osteoclast adhesion signalling and bone resorption function. Src and PI3K may function at the point where RANK and adhesion signals converge, transmitting the signals for proper actin cytoskeletal organization that facilitates the resorptive activity of osteoclasts (Duong LT. et al., 1998, Tanaka S. et al., 1996).

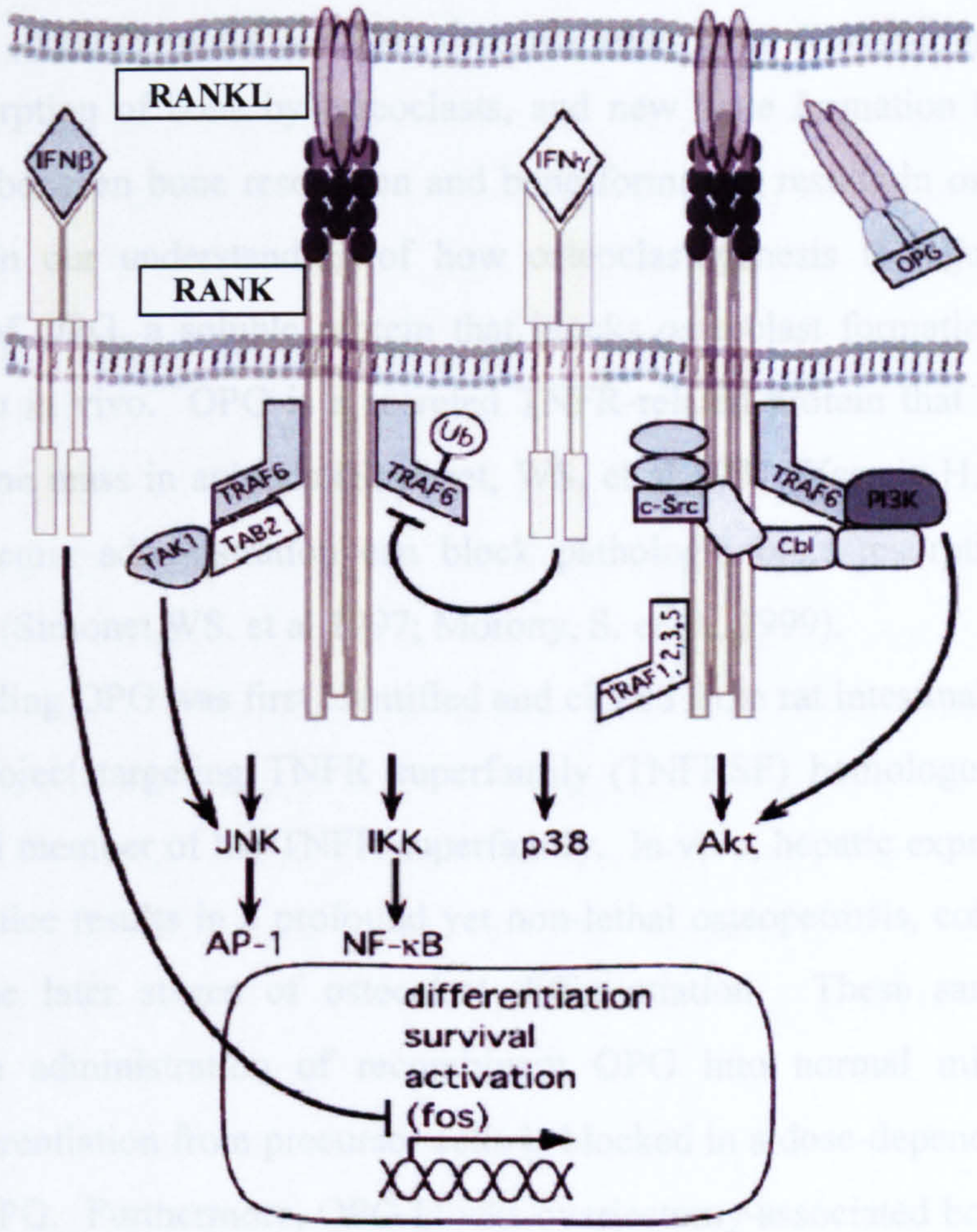


Fig 1.5 A model for RANKL-mediated signal transduction. Soluble or membrane-bound RANKL binds its soluble decoy receptor, OPG, or its functional receptor, RANK. Ligation of RANK leads to binding of the adapter TRAF6 at the proximal portion of the RANK intracellular domain. RANK signalling activates the mitogen-activated protein kinase (MAPK) JNK pathway, and the transcription factor AP-1 through the kinase TGF-β activated kinase (TAK1). The PKB/Akt is activated by RANK through a distinct TRAF6-dependent mechanism. TRAF6 forms a complex with the scaffolding protein Cbl, and phosphoinositide 3-kinase (PI3K), which recruits c-Src to the receptor. Activation of PKB/Akt through phosphorylation of membrane phosphatidylinositides by PI3K requires the kinase activity of c-Src. Additional downstream targets of RANK include NF-kB and p38. Activation of RANK downstream targets results in upregulation of various pro-survival and activating factors important for osteoclasts, as well as differentiating factors for osteoclast precursors, including c-Fos. Adapted from (Walsh MC, Choi Y. 2003).

1.5.1.3 OPG/OCIF

The functional integrity of the skeleton depends upon bone remodelling, the cycle of controlled resorption of bone by osteoclasts, and new bone formation by osteoblasts. An imbalance between bone resorption and bone formation results in osteoporosis. A step forward in our understanding of how osteoclastogenesis is regulated was the identification of OPG, a soluble protein that blocks osteoclast formation in vitro and bone resorption in vivo. OPG is a secreted TNFR-related protein that regulates bone density and bone mass in animals (Simonet, WS. et al 1997; Yasuda H. et al., 1998), and upon systemic administration can block pathologic bone resorption in various animal models (Simonet WS. et al 1997; Morony, S. et al., 1999).

The gene encoding OPG was first identified and cloned from rat intestinal cDNA during a screening project targeting TNFR superfamily (TNFRSF) homologues. However, OPG is a novel member of the TNFR superfamily. In vivo, hepatic expression of OPG in transgenic mice results in a profound yet non-lethal osteopetrosis, coincident with a decrease in the later stages of osteoclast differentiation. These same effects are observed upon administration of recombinant OPG into normal mice. In vitro, osteoclast differentiation from precursor cells is blocked in a dose-dependent manner by recombinant OPG. Furthermore, OPG blocks ovariectomy-associated bone loss in rats. OPG can act as a soluble factor in the regulation of bone mass and imply a utility for OPG in the treatment of osteoporosis associated with increased osteoclast activity (Simonet WS. et al., 1997).

OPG is synthesized as a propeptide (401 amino acids) from which the signal peptide (21 amino acids) is cleaved, thus generating the mature protein [380 amino acids (44 kDa)]. In contrast to all other TNFR superfamily members, OPG lacks transmembrane and cytoplasmic domains and is secreted as a soluble protein (Hofbauer LC. et al., 2000).

The N-terminus of OPG contains four domains (D1-D4) with cysteine-rich motifs involved in the formation of tethered loops and is most closely related to TNFR-2 and CD40. The C-terminus contains two death domain-homologous (DDH) tandem regions (D5 and D6) that are closely related to Fas and TNFR-1, and a domain (D7) with a heparin-binding site and a cysteine residue (position 400) required for homodimer formation. The integrity of all four cysteine-rich motifs (D1-D4) appears to be required for the biological activity of OPG. Furthermore, when fused to the transmembrane

region of Fas, the C-terminus (D5-D7) can mediate cytotoxicity directly; see (Fig 1-6) (Yasuda H. 1998; Yamaguchi k. et al., 1998; Hofbauer LC. et al., 2000).

OPG is synthesized as a monomer (55-62 kDa), assembled, and then secreted as a disulfide-linked homodimeric glycoprotein with 4-5 potential N-glycoslation sites (110-120 kDa) (Tsuda E. 1997; Simonet WS. et al., 1997; Kwon BS. et al., 1998). Tomoyasu A. et al., (1998) suggested that monomeric OPG is derived from the homodimeric OPG by lacking several amino acids including Cys^{379}, which is involved in intermolecular disulfide bone, in the C-terminal region. The human OPG gene has been mapped to chromosome 8q23-24 and contains five exons distributed over 29 kilobases (kb). Of interest, exon4 that encodes most of the first DDH region, is a partial duplication of exon5. Three major OPG mRNA species exist; there is one abundant 2.2 to 3.0-kb species and two minor species of 4.2-4.4 kb and 6.5-6.6 kb, respectively. The two largest species are splice variants that contain either the entire intron 2 (6.5-to 6.6-kb species) or the 3' half of intron 2 (4.2- to 4.4-kb species) (Hilton MJ. et al. 2001; Morinaga T. 1998; Yamaguchi K. et al., 1998; Hofbauer LC. et al., 2000).

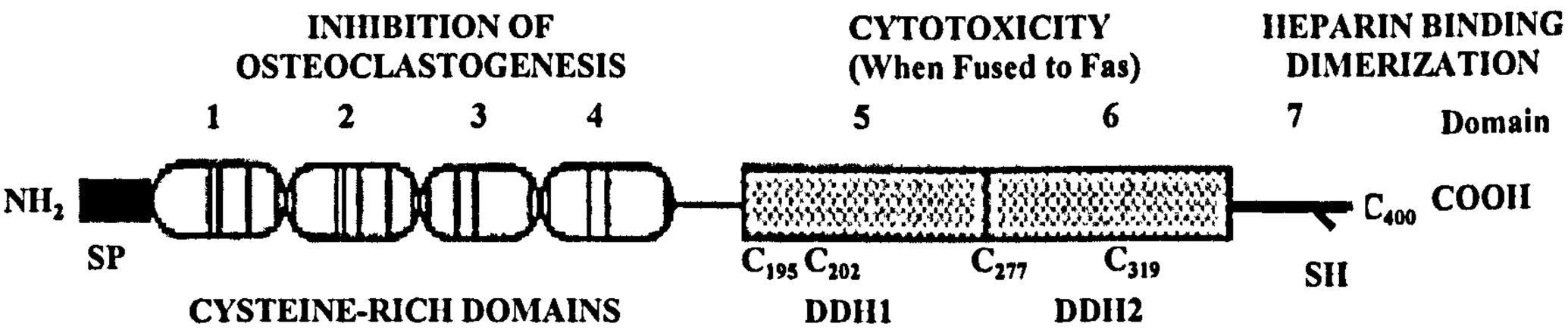


Fig 1.6 Representation of structure-functional relationship of OPG. OPG domains and their biochemical and functional properties are indicated. Closed box, signal peptide (SP); open boxes with vertical bars, cysteine-rich domains; hatched box, DDH regions; open square, domain 7. OPG is present as a monomer with a molecular mass of 60 kDa and a homodimer of 120 kDa in conditioned media of human fibroblasts. Monomeric OPG is derived from the homodimer by lacking several amino acids including Cys379 which is involved in intermolecular disulfide bond in C-terminal region (Tsuda E. et al., 1997; Yasuda, H. et al 1998; Tomoyasu, A. et al., 1998; Schoppet, M. et al., 2002; Yamaguchi, K., et al., 1998.

Another identical form to OPG which inhibits osteoclast-like cell formation was found in condition media of human embryonic lung fibroblasts IMR-90, and has been termed osteoclastogenesis inhibitory factor (OCIF). It is a heparin-binding basic glycoprotein

and has been isolated as a monomer with an apparent molecular weight of 60 kDa and a homodimer of 120 kDa. The N-terminus of OCIF is blocked and the determination of internal amino acid sequences revealed that OCIF has no homology to other known proteins. OCIF inhibited in a dose-dependent manner osteoclastogenesis elicited through three distinct signalling pathways stimulated by vitD3, PTH and IL-11, respectively. OCIF neither inhibits bone resorption by mature osteoclasts nor exerts any other biological activities (Tsuda E. et al., 1997). Additionally, TNF receptor-related molecule 1 (TR1) identified as a member of TNFR superfamily shows activities associated with osteoclastogenesis inhibition and fibroblast proliferation. TR1 was identified from a search of an expressed sequence tag database, and encodes 401 amino acids with a 21-residue signal sequence. Unlike other members of TNFR, TR1 does not contain a transmembrane domain and is secreted as a 62 kDa glycoprotein. TR1 gene maps to chromosome 8q23-24.1 and its mRNA is abundantly expressed in primary osteoblasts, osteogenic sarcoma cell lines and primary fibroblasts. The receptors for TR1 were detected on a monocytic cell line (THP-1) and in human fibroblasts. Recombinant TR1 induced proliferation of human foreskin fibroblasts and potentiated TNF-induced proliferation in these cells. In a co-culture system of osteoblasts and bone marrow cells, recombinant TR1 completely inhibited the differentiation of osteoclast-like multinucleated cell formation in the presence of several bone-resorbing factors. TR1 also strongly inhibited bone-resorbing function on dentine slices by mature osteoclasts and decreased Ca release in fetal long-bone organ cultures. Anti-TR1 monoclonal antibody promoted the formation of osteoclasts in mouse marrow culture assays. These results indicate that TR1 has broad biological activities in fibroblast growth and in osteoclast differentiation and its functions (Kwon BS. et al., 1998).

OPG has a wider tissue distribution and is expressed more abundantly than RANKL. High levels of OPG mRNA have been detected in lung, heart, kidney, liver, stomach, intestine, skin, brain and spinal cord, thyroid gland and bone (Simonet WS. et al., 1997; Yun TJ. et al. 1998). OPG mRNA levels have been detected in a variety of osteoclastic lineage cells, including marrow stromal cell lines, osteoblastic cell lines, and the osteosarcoma cell line MG-63, fibroblastic cells and human periodontal ligament cells (Anderson DM. et al., 1997; Lacey DL. et al., 1998; Hofbauer LC. et al., 1999; Zhang D. 2004). In addition, high OPG mRNA levels have also been detected in endothelial cells and aortic smooth muscle cells. Also, OPG is expressed in arterial smooth muscle

cells and endothelial cells, whereas RANKL and RANK are not expressed in vascular tissues under physiologic conditions (Simonet WS. et al., 1997; Min H. et al., 2000). Moreover, RANKL and OPG are differentially expressed in calcific aortic stenosis (AS). In cultured human aortic valve myofibroblasts, RANKL promotes matrix calcification and induces the expression of osteoblast-associated genes, indicating a transition towards an osteogenic phenotype which, suggests that the RANKL-OPG pathway may regulate valvular calcification in calcific AS (Kaden JJ 2004).

OPG mRNA and protein in osteoblastic lineage cells are increased by the cytokines IL-1∝, IL-1β, TNF-∝, TNF-β, and by the antiresorptive agents estrogen and TGF-β. OPG mRNA levels of murine marrow stromal cell lines are increased by treatment with calcium chloride. By contrast, OPG mRNA and protein levels are decreased by glycocorticoids, PGE_2 and by the estrogen receptor antagonist ICI182,780. Transcriptional regulation of the OPG gene, specifically in response to TGF-β, appears to be mediated by Smad1 and the osteoblast-specific transcription factor, cbfa1 (Thirunavukkarasu K. et al., 2001; Wan M. et al., 2001). However, both RANKL and OPG are regulated by various hormones (glucocorticoids, $vitD_3$, estrogen) and cytokines TNFα, IL-1, -4, -6, -11 and -17) see (table 1-2a and 1-2b). Various mesenchymal transcription factors such as cbfa-1, peroxisome proliferetor-activated receptor gamma, and Indian hedgehog have also been implicated (Hofbauer LC. et al., 2001).

1.5.2 Interleukin-17 and its receptor (IL-17R)

1.5.2.1 IL-17

Interleukin-17 (IL-17) is a novel proinflammatory cytokine, secreted exclusively by human [CD4+ RO+] T cells (TH1 and TH2) in healthy subjects [as well as by CD4+ CD8+ cells from mice] (Yao Z. et al., 1995; Shin HC. et al., 1999). IL-17 is a mixture of disulfide linked, homodimeric glycosylated and nonglycosylated polypeptides. Its gene has been mapped to a single site on human chromosome 2q31 (Yao Z. et al., 1995; Rouvier E. et al., 1993). IL-17 cDNA has been isolated and cloned from cytotoxic T lymphocyte antigen 8 (CTLA-8) (Rouvier E. et al., 1993; Yao Z. et al., 1996) and exhibits an overall sequence identity of 72% at the a level with open reading frame 13

from the T lymphotropic Herpesvirus saimiri and 63% with mouse IL-17 (mIL-17). The human IL-17A gene product is a protein of 155 amino acids with a molecular weight ranges from 28 (the glycosylated form) to 15 kDa (the nonglycosylated form), and is secreted as a disulfide linked homodimer of 30–35 kDa (Fossiez F. et al., 1996; Yao Z. et al., 1995).

Five related cytokines were identified, through database searches and degenerate RT-PCR, that share 20–50% homology to IL-17 and a conserved C-terminal region, but have N-terminal segments that differ IL-17B, C and E have significantly different biological profiles from IL-17A (Li H. et al., 2000; Shi et al., 2000; Lee J. et al., 2001). IL-17A has been identified as the original member of the IL-17 cytokine family. The mutual features of the IL-17 cytokine family include conserved cysteines which, in IL-17F, have been shown to exhibit the features of a classic cysteine knot structural motif found in BMPs, TGF-β, nerve growth factor (NGF) and platelet-derived growth factor BB (PDGF-BB) (McDonald NQ. and Hendrickson WA., 1993). IL-17F, like IL-17A, is produced primarily in activated T-cells (Hymowitz SG et al., 2001). In contrast, IL-17B is expressed in pancreas, small intestine and stomach. IL-17C is very rarely expressed. IL-17D is preferentially expressed in skeletal muscle, brain, adipose tissue, heart, lung, and pancreas (Starnes T. et al., 2002) whereas IL-17E mRNA was detected at very low levels in several peripheral tissues. IL-17E induces activation of NF-kB and stimulates production of the proinflammatory chemokine IL-8 (Lee J. et al., 2001). Their functions partially overlap those of IL-17A, although they have not been as thoroughly investigated (Shi Y. et al., 2000; Starnes T. et al., 2001, Spriggs MK. et al., 1997; Moseley TA. et al., 2003). IL-17B and IL-17C have ~27% related amino acid identity and stimulate the release of TNF-∝ and IL-1 β from the monocytic cell line, THP-1 (Shi Y. et al., 2000; Spriggs MK. et al., 1997). Li H, et al., (2000) demonstrated that IL-17B and IL-17C differ in their patterns of expression and proinflammatory responses, and may be transduced through a similar set of cell surface receptors.

IL-17 stimulates cells such as epithelial, endothelial and fibroblastic cells to secrete a large variety of proinflammatory molecules, including IL-1β, TNFα and inducible nitric oxide synthase (Miljkovic Dj et al., 2003; Jouzeau JY et al., 2002; Miljkovic D. et al., 2004). In addition, IL-17 has been found to induce the expression of potent modulators of bone resorption through NF-kB, and enhance the expression of ICAM-1, and

granulocyte macrophage colony-stimulating factor (GM-CSF) in fibroblasts, skeletal myoblasts and muscle tissue (Yao Z. et al., 1995; Chevrel G. et al., 2003). Thus, IL-17 can be found in the pannus of inflamed synovium in rheumatoid suffers (Chabaud M. et al., 1998; 1999). IL-17 appears to provide a direct linkage between T-cell activation and the inflammatory response. IL-17 can, alone and synergistically in combination with other proinflammatory cytokines, promote chondrocyte mediated MMP dependent type II collagen release from cartilage. Furthermore, in vivo, IL-17 has a direct catabolic effect on cartilage in addition to stimulatory effects on macrophages and synoviocytes, making it a potentially important cytokine in the pathogenesis of arthritis. The raised levels of proinflammatory cytokines in rheumatoid synovial fluids suggest that IL-17 may act as a potent upstream mediator of cartilage collagen breakdown in inflammatory joint diseases (Dudler J et al., 2000; Koshy PJ et al., 2002; Chabaud M. et al., 2001). In addition, IL-17 may affect bone metabolism in pathological conditions characterized by the presence of activated T cells and TNFα production such as RA and loosening of bone implants (Van bezooijen RL. et al., 1999 Chabaud M. et al., 2001; Kotake S. et al., 1999; Chabaud M and Miossec P. 2001). However, in vivo blockade of IL-17 by muIL-17R:Fc treatment attenuated AIA and reduced joint damage, suggesting that IL-17 plays an important role in the inflammation and joint destruction of AIA. IL-17 may be a potential therapeutic target for inflammatory diseases in humans, such as rheumatoid arthritis (Bush KA. Et al., 2002).

1.5.2.2 IL-17 Receptor

The cell surface receptor for IL-17 is widely expressed in many tissues and cell types. The presence of IL-17R has been demonstrated on the surface of various cells of human origin, such as MG-63, HOS osteosarcoma cell lines, cells from peripheral blood mononuclear cells (Honorati MC. Et al., 2003) and human foreskin fibroblast cells (Thiele K. et al., 2000). In addition, synovial and chondrocytes expressing IL-17R are found in the majority of patients with different type of arthritis (Honorati MC. et al., 2001). Also, IL-17R mRNA was expressed by mouse osteoblastic cells (Fossiez F. et al., 1998; Van bezooijen, et al., 1999) and were found in chondrocytes and synovial fibroblasts from OA patients (Honorati MC. et al., 2002).

The open reading frame for IL-17R has been characterised as an extremely long type 1 transmembrane glycoprotein of approximately 130 kDa 864 amino acids in total. It includes an N-terminal signal peptide with a cleavage site after amino-acid 31 followed by a 291-amino acid extracellular domain, a 21-amino-acid transmembrane domain and 521 amino-acid cytoplasmic tail. IL-17 Receptor is not a member of the immunoglobulin family of receptors and is unique in possessing a long cytoplasmic domain that has no sequence homology with any other growth factor receptors. IL-17R has tyrosine kinase (TK) activity associated with it. The extracellular domains are not related to any of the known cytokine receptor families. Through human chromosomal mapping techniques, the IL-17R is localised to chromosome 22q11.22-q11.23. Expression of IL-17R is almost ubiquitous (Yao Z. et al., 1995).

1.5.2.3 The biological function of IL-17

Although limited in number, studies now suggest that IL-17 may be a major vehicle by which T cells communicate with other cells of the hematopoietic system. In particular, fibroblasts, when cultured in the presence of IL-17, are able to sustain CD34+ hematopoietic progenitor cells and direct their maturation towards neutrophils (Fossiez, F. et al., 1996). The exact pathway(s) involving IL-17 is not clear. However, IL-17 has been demonstrated to induce IL-6, IL-8, G-CSF and PGE_2 production by fibroblasts, epithelial and endothelial cells, and some of these cytokines are known to impact on hematopoiesis (Fossiez, F. et al., 1996). IL-6, for instance, induces hematopoietic progenitor cells to form granulocyte/macrophage colonies (Ikebuchi, K. et al., 1987), while G-CSF, both in vitro (Berliner, N. et al., 1995) and in vivo (Roberts, A. W. and D. Metcalf 1994) accelerates the formation of neutrophils. IL-8, in contrast, seems to down modulate the effects of myelopoietic-promoting cytokines, suggesting that IL-17 may ultimately be found to have the ability to modify or impact all general phases of a hematopoietic response. Thus, it appears that IL-17 can now be considered the newest T-cell-derived hematopoietic cytokine, joining IL-3 (Dilloo D. et al., 1996; Huhn, R. D. et al., 1996), IL-4 and IL-5 (Takatsu K. et al., 1994; Kim MR. et al., 2002), as either regulators or co-regulators of hematopoiesis (Fossiez, F. et al., 1996).

It is not yet clear how widespread the expression of IL-17 is. Early reports implicated T-lymphocytes as the major source of IL-17 (Fossiez, F. et al., 1996; Yao, Z. et al.,

1995), particularly activated memory CD4+ T cells (Fossiez, F. et al., 1996). More recent studies have suggested a different and more restricted expression pattern in mice. Expression of mouse IL-17 by various activated T cells could only be detected using PCR analysis. Using Northern analysis only a subset of T cells, abTCR+CD4-CD8- T cells were found to express IL-17 at physiologically significant levels (Kennedy, J. et al., 1996). These cells are among the first to be activated during immune responses and are capable of producing large amounts of cytokines characteristic of Th2 type responses (IL-4, IL-5, IL-10 and IL-13) as well as factors usually associated with CD8+ T cells (IFN-γ, TNF-β, RANTES and Fas ligand) (Kennedy, J. et al., 1996). Although these results suggest that IL-17 is not expressed by a wide variety of cell types, it is possible that other cells may express higher levels of IL-17 when appropriate activation conditions are found.

IL-17 is as a pro-inflammatory cytokine, and has been implicated in a number of diseases including RA (Chabaud M. et al., 2001; Miossec P. et al., 2000), allergic skin immune responses (Albanesi C. et al., 2000), psoriasis (Teunissen MB, et al., 1998), organ transplant rejection (Antonysamy MA. et al., 1999; Van Kooten C. et al., 1998), multiple sclerosis (Matusevicius D. et al., 1999) asthmatic airways (Molet S. et al., 2001) and the inflammation process in strokes (Li HL. et al. , 2001) As well as being produced by tumor infiltrating lymphocytes, and increasing tumorogenicity of human cervical tumors (Fridman WH. and Tarour E., 1998; Tartour E. et al., 1999). IL-17 reportedly induces hematopoietic cytokines such as GM-CSF, LIF and IL-6 (Yao Z. et al., 1997). IL-17 can, therefore, be said to act as an element of the cytokine network that bridges the immune system to hematopoiesis (Fossiez F et al., 1996).

It is apparent that IL-17 has a pleiotropic effect upon a number of cell types associated with inflammation and bone destruction. Proliferation of T cells is enhanced in the presence of IL-17 co-stimulated with suboptimal amounts of phytohemagglutinin (PHA), and IL-17 has also been shown to stimulate the maturation of bronchial cells and recruitment of neutrophils (Fossiez F. et al., 1996). IL-1β and TNFα are Th-1 type cytokines that stimulates IL-17 production by human macrophages and corneal fibroblasts and also increase the effects of these cytokines on synoviocytes through synergistic mechanisms (Maertzdorf J. et al., 2002). IL-17 is known to up-regulate the production of MMPs, such as gelatinase-3 (MMP-9) in macrophages, IL-6 and PGE2. In addition, IL-1β and TNFα mRNA are also controlled by IL-17. The anti-

inflammatory cytokines IL-4 and IL-10 completely reverse IL-17-stimulated IL-1β release in human macrophages. IL-10 exerts a significant suppressive effect on IL-17 inducing TNF-α release that suggests a pivotal role for IL-17 in initiating and/or sustaining an inflammatory response in human macrophages (Jovanovic DV. et al., 1998). In contrast, Shin HC, et al., (1999) demonstrated that IL-17 and IFN-γ mRNA were both inhibited in the presence of PGE_2 or the adenylate cyclase (cAMP) analogue (dibutryl-cAMP), while the anti-inflammatory cytokine IL-10 was highly increased in human memory T cells.

1.5.2.4 Signal transduction by IL-17

IL-17 activates the transcription factor NF-kB and JNK. The upstream signalling events are largely unknown but Schwandner R. and Yamaguchi K. (2000) reported that the requirement of TRAF6 in IL-17-induced NF-kB and JNK activation in embryonic fibroblasts (EFs). In addition, IL-17 promotes cartilage breakdown (bovine articular chondrocytes) by inducing MMPs and aggrecanases by stimulating the phosphorylation of ERK, p38 and JNK via MAP kinases, AP-1 and NF-kB mediators (Sylvester J. et al., 2004). IL-17 induces NF-kB protein-DNA complexes consisting of P65/P50 heterodimers in the rat intestinal epithelial cell line IEC-6, correlating with the induction of CXC and CC chemokine mRNA expression. In IEC6-cells, synergism of IL-17 with IL-1β induces NF-kB by IL-17 through TRAF6, but not TRAF2. In addition, to activation of NF-kB, IL-17 regulated the activation of ERK, JNK, and p38 MAPK in IEC-6 cells (Awane M. et al., 1999), whereas the IL-17-mediated activation of ERK MAPK was mediated through Ras and JNK activation was dependent on functional TRAF6. However, TRAF-6 and IL-17R are coexpressed in 293 cells and TRAF-6 coimmunoprecipitates with IL-17R. Therefore, IL-17R and TRAF-6 are crucial components in IL-17 signalling leading to proinflammatory responses (Schwandner R et al., 2000).

IL-17F (ML-1) is able to induce the expression of IL-6, IL-8 and ICAM-1 in primary bronchial epithelial cells (Kawaguchi M. et al., 2001) and the activation process is mediated, via the phosphorylation of ERK1/2, but not p38 (Kawaguchi M. et al., 2002). Another study indicates a role for Raf1-MEK-ERK1/2 pathway in IL-17F (ML-1)

induced C-X-C chemokine expression (Kawaguchi M. et al., 2003). These signalling cascades can all be triggered by IL-17, TNF-alpha and IL-1 beta in (intestinal epithelial cells, fibroblasts, and bronchial cells) as shown by Fig-1-8 (Awane M. et al., 1999; Schwander R. et al., 2000). In addition, the effect of IL-17 on p38 MAPK appears to be necessary for the regulation of IL-18, growth-related protein GRO-alpha and GRO-beta expression from fibroblast-like synoviocytes (SFC) isolated from rheumatoid arthritis (RA) patients. These data support the hypothesis that IL-17/IL-17R may play a significant role in the pathogenesis of RA contributing to an unbalanced production of cytokines as well as participating in connective tissue breakdown (Kawaguchi M. 2003; Kehlen A. et al., 2002).

1.5.3 Oncostatin M (OSM)

Oncostatin M (OSM) is an approximately 28 kDa glycoprotein produced by activated monocytes and macrophage/T lymphocytes and is related to the family of cytokines that include leukaemia inhibitory factor (LIF), IL-6, IL-11, cardiotrophin-1 and ciliary neurotrophic factor. These cytokines share a common signal transducer receptor component, gp130, and have overlapping biological activities (Rose TM. and Bruce AG 1991). It is a pleiotropic cytokine that exerts numerous effects in many different cell types, and modulates the growth and differentiation of several normal and tumour cell lines. It also stimulates expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) and plasminogen activator in synovial fibroblasts (Wang H. et al., 1997) and up-regulates MMP-9 in human smooth muscle cells through the MEK-ERK but not STAT3 pathway (Nagata T. et al., 2003).

Activated T lymphocytes and macrophages are able to produce OSM and the level of OSM mRNA is higher in RA patients than OA patients (Wang H, 1997; Hirano, et al., 1994). In addition, Lisignoli G. et al. , (2000) reported that OSM was found only in mononuclear cells in RA. OSM has potentially important functions in the modulation of chemokine and MMP production by synovial cells of the joint (Langdon, et al., 1997).

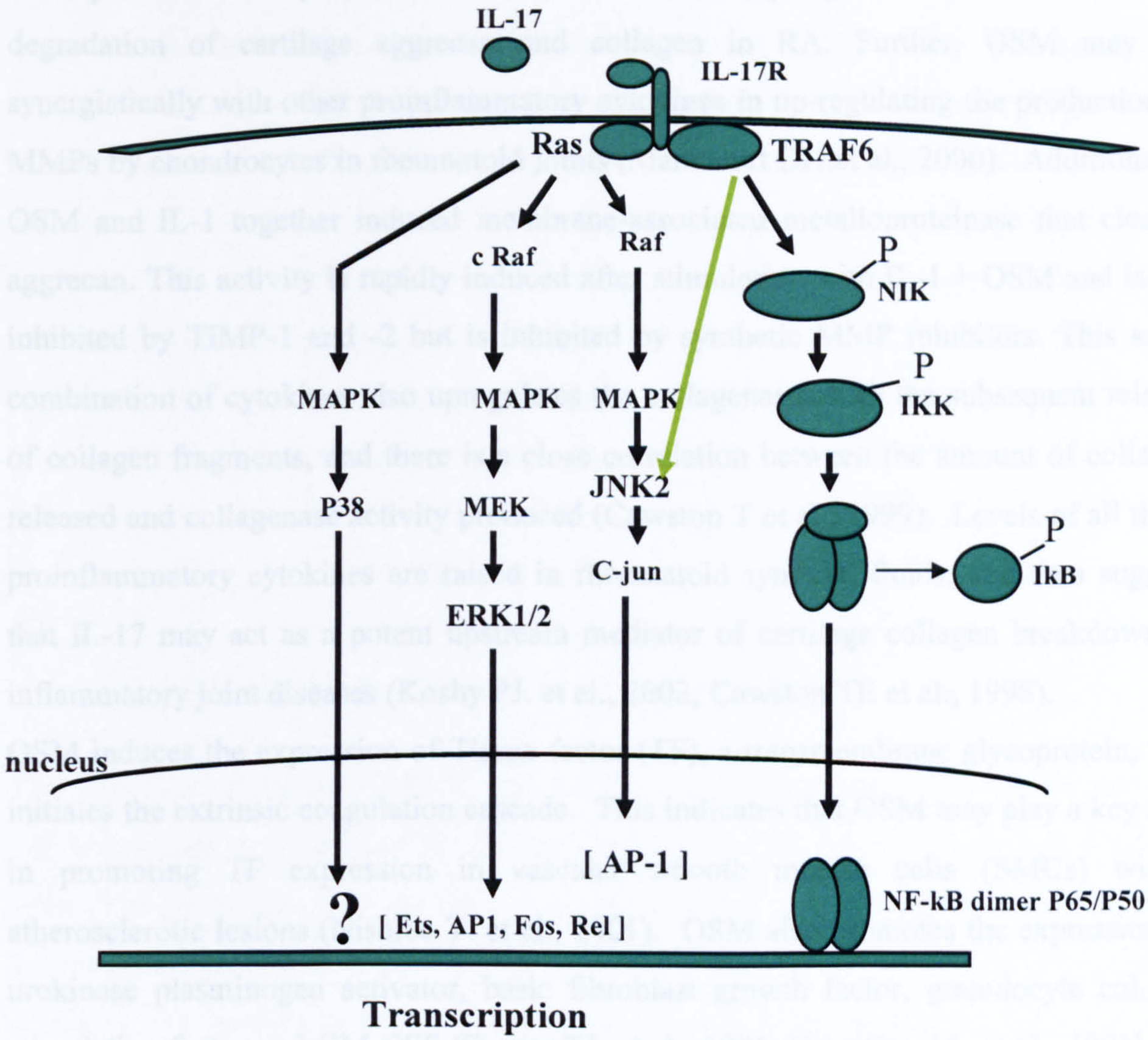


Fig 1.8 The signal transduction pathway of IL-17 through its receptor. Ligation of IL-17R leads to binding of the adapter TRAF6 at the proximal portion of the IL-17R. IL-17R signalling activates the Ras MAPK JNK pathway, and the transcription factor AP-1 through the MAP-kinase and JNK which interacts with TRAF6. Additional downstream targets of IL-17R include NF-kB and p38 through activation of IKB and MAPK. Activation of IL-17 downstream targets results in upregulation of various pro-survival and activating factors important for osteoclasts, as well as differentiating factors for osteoclast precursors.

Studies have demonstrated that IL-17 and other proinflammatory cytokines can, in combination with OSM, promote a synergistic, chondrocyte-mediated MMP-dependent release of type II collagen from cartilage (Koshy PJ et al., 2002). Recently Hui W et al., (2003) demonstrated that OSM + TNF-α represents a potent proinflammatory cytokine combination that markedly induces MMP production in both cartilage and synovium, thus promoting joint destruction. Another in vivo study suggests that TNFalpha and

other proinflammatory cytokines are involved in the up-regulation of the coordinated degradation of cartilage aggrecan and collagen in RA. Further, OSM may act synergistically with other proinflammatory cytokines in up-regulating the production of MMPs by chondrocytes in rheumatoid joints (Manicourt DH.et al., 2000). Additionally, OSM and IL-1 together induced membrane-associated metalloproteinase that cleaves aggrecan. This activity is rapidly induced after stimulation with IL-1 + OSM and is not inhibited by TIMP-1 and -2 but is inhibited by synthetic MMP inhibitors. This same combination of cytokines also upregulates the collagenases with the subsequent release of collagen fragments, and there is a close correlation between the amount of collagen released and collagenase activity produced (Cawston T et al., 1999). Levels of all these proinflammatory cytokines are raised in rheumatoid synovial fluids, and data suggest that IL-17 may act as a potent upstream mediator of cartilage collagen breakdown in inflammatory joint diseases (Koshy PJ. et al., 2002; Cawston TE et al., 1998).

OSM induces the expression of Tissue factor (TF), a transmembrane glycoprotein, that initiates the extrinsic coagulation cascade. This indicates that OSM may play a key role in promoting TF expression in vascular smooth muscle cells (SMCs) within atherosclerotic lesions (Nishibe T. et al., 2001). OSM also promotes the expression of urokinase plasminogen activator, basic fibroblast growth factor, granulocyte colony-stimulating factor and GM-CSF (Brown TJ. et al., 1991; Hamilton JA. et al., 1991). In human fibroblasts, OSM modulates not only MMPs but also tissue TIMPs (Nemoto O. et al., 1997; Korzus E et al., 1997). In addition, Rowan AD (2001) reported that OSM synergizes with IL-1alpha to induce chondrocyte-mediated cartilage collagen breakdown and collagenase production. Furthermore, OSM was demonstrated to stimulate angiogenesis in vivo and was shown to be present in human aortic aneurysms (Modur V. et al., 1997; Vasse M. et al., 1999). OSM is produced by activated monocytes/macrophages and T lymphocytes, cell types found frequently at sites of inflammatory wound repair (Nishibe T. et al., 2001).

OSM also stimulates osteoclast formation and function (Richards CD, et al., 2000). The role of OSM and its interactions with RANKL, RANK and OPG were studied in neonatal mouse calvaria. Human OSM and murine OSM (mOSM) enhanced the mRNA expression of RANKL and OPG in mouse calvaria, but had no effect on the expression of RANK (Richards CD. et al., 2000; Palmqvist P. et al., 2002). In addition, human LIF, hOSM, and mOSM also stimulated Ca^{2+} release and enhanced the mRNA

expression of RANKL and OPG in mouse calvaria. Also, hIL-6 plus shIL-6R and mOSM increased RANKL and OPG proteins in mouse calvaria cells (Palmqvist P et al., 2002). In contrast, another study described OSM as a bone-remodelling factor stimulating osteoblast activity. OSM induced proliferation, collagen synthesis, and IL-6 secretion by osteoblasts, whereas it inhibited alkaline phosphatase activity and bone resorption. In vitro, OSM enhanced the effect of BMP-2 on osteoblast differentiation. Immunohistochemistry demonstrated the expression of RANKL and RANK in the periosteum but osteoclasts were not detected at sites of bone apposition. OSM favours bone apposition at periosteal sites instead of resorption in vivo. This effect was not dependent on, or inhibited by, IL-6 (Jay PR. et al., 1996; de Hooge AS. Et al., 2002).

1.6 Mechanism of bone destruction in RA

The paralleled roles of RANKL and OPG in regulating bone metabolism and the immune system are mediated by the expression of RANK by osteoclasts and dendritic cells. The cross-talk of T lymphocyte-derived RANKL and osteoclasts within the bone microenvironment have made RANKL an obvious candidate cytokine that promotes the skeletal complications of RA. Several in vivo studies using established animal models of RA provide support for this hypothesis (Kong YY. et al., 1999; Romas E. et al., 2000; Haynes DR. et al., 2001). Kong and associates have clearly demonstrated that RANKL mRNA is produced by synovial fibroblasts and activated T lymphocytes in affected lesions of rats with adjuvant arthritis. These animals displayed severe bone loss due to enhanced osteoclast formation and joint erosions within one week after subcutaneous administration of *Mycobaterium tuberculosi* (Kong YY. et al., 1999).

In another rodent model of RA (induced by intradermal injection of type II collagen) RANKL expression was detected in mononuclear cells and chondrocytes within areas of inflammation, and actively resorbing osteoclasts were located at sites where RANKL production was most abundant (Romas E. et al., 2000). In addition, targeted over expression of the antiresorptive cytokine IL-4 markedly suppressed RANKL production in collagen-induced arthritis, and protected these mice from developing bone loss and cartilage damage (Lubberts E. et al., 2000).

There is mounting evidence supporting an essential role of RANKL in human RA. Gravallese EM. et al., (2000) demonstrated that both RANKL mRNA and protein are abundantly produced by activated T lymphocytes infiltrating the synovium, but are absent in synovial tissue derived from patients with other forms of arthritis and in healthy subjects. Expression of RANKL mRNA and protein was confirmed in vitro with cultured fibroblasts and T lymphocytes derived from synovial specimens of patients with RA (Gravallese EM. et al., 2000). Therefore, the systemic bone sparing effects of immunosuppressants such as cyclosporin A in patients with RA may be through inhibition of T cell activation, resulting in decreased RANKL production (Ferraccioli G. et al., 1996). In addition, Kong YY. et al., (1999) and Gravallese EM. et al., (2000) demonstrated that activated T lymphocytes and synovial fibroblasts in vitro were inhibited by administration of OPG. Furthermore, IL-17 concentrations were elevated in synovial fluid in human RA, and were found to be among the most potent stimulators of RANKL production (Kotake S. et al., 1999). The sequential action of IL-17 and RANKL is supported by the finding that osteoclastogenesis induced by IL-17 was completely inhibited by administration of OPG (Ferraccioli G. et al., 1996).

However, as previously described RA is an autoimmune disease characterized by a severe lymphocyte penetration into the synovial cavity resulting in the secretion of a multiplicity of cytokines, which eventually leads to destruction of joint tissues. Among the infiltrating cells are activated T cells, which produce specific cytokines capable of osteoclast progenitor cell expansion, fusion, and activation. Cultures of activated human T cells and human osteoblasts were used to study the possibility that lymphokines may act on osteoblasts to produce the osteoclastogenic factors IL-17, IL-6 and RANKL (Rifas L. and Avioli LV. 1999). IL-17, produced by activated T cells in the synovial tissues, enhances the production of PGE_2 synthesis in the osteoblast/stromal cells in adjacent bone. The presence of PGE_2 in these cells causes RANKL up-regulation which leads to RANK activation. M-CSF produced by osteoblast/stromal cells is also an essential soluble factor for osteoclast survival in mice cells. Elevation of IL-17 in RA synovial tissue may induce osteoclastogenesis, leading to joint damage Fig 1-9 (Kotake, et al., 1999).

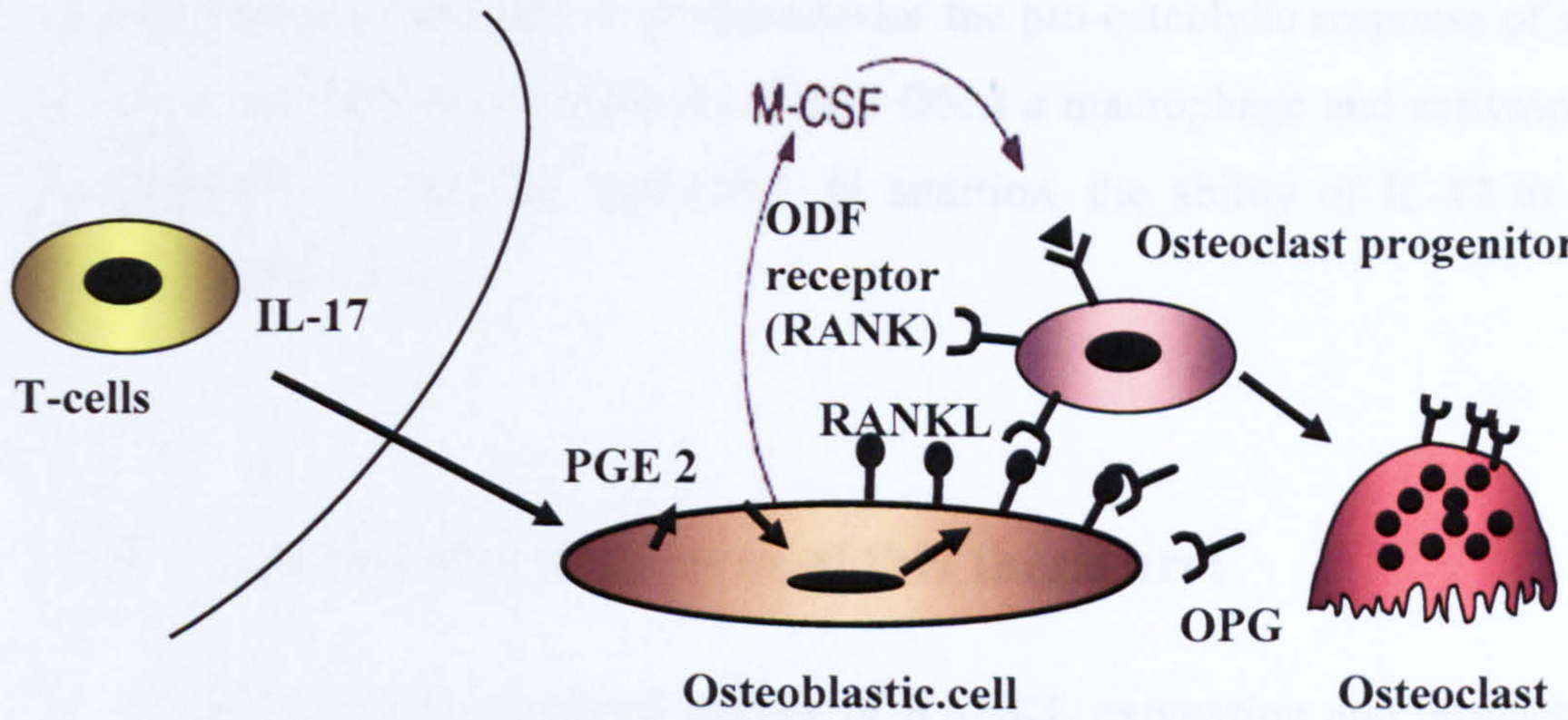


Fig-1.9 Osteoclastogenic mechanism mediated by activated T cells and osteoblasts. (taken from Kotake, et al., 1999).

1.7 Overall summary

RA is an autoimmune disease characterised by chronic inflammation of multiple synovial joints leading to progressive joint destruction and bone resorption. T cell recruitment and activation is believed to be a pivotal part of RA and leads to the accumulation of cytokines such as IL-17 and OSM in the joint space. The precise role of T cells in normal bone homeostasis is not fully understood but the T cell-derived cytokine IL-17 is known to support osteoclastogenesis. This is believed to be mediated through the up regulation of RANKL expression and production by marrow stromal and osteoblast cells. A recent study proposed that activated T cells regulate bone loss and joint destruction in adjuvant arthritis (Kong YY et al., 1999). Kotake, et al., (1999) demonstrated that activated T cells and synovial fibroblasts express RANKL and M-CSF which, may directly stimulate osteoclast differentiation, survival and activation. Activated T cells further influence local RANKL expression through the production of IL-17, which stimulates osteoblasts and synovial fibroblasts.

1.8 Aim of the study

The overall aim of this project is to characterise the pro-osteolytic response of synovial cells to the T cell-derived cytokine IL-17 and OSM a macrophage and activated T cell on the expression of RANKL and OPG. In addition, the ability of IL-17 to support osteoclastogenesis in vitro.

1.9 The Specific objectives of this thesis are:

- To determine the temporal pattern of RANKL expression and production by synovial cells treated with IL-17 and OSM and contrast this with the expression in osteoblasts.

- To determine the temporal pattern of OPG expression and production by synovial cells treated with IL-17 and OSM and contrast this with the expression in osteoblasts.

- To determine whether the temporal pattern of RANKL and OPG expression by synovial cells is altered when treated with IL-17 in combination with OSM, and to contrast this with the expression in osteoblasts.

- To investigate the formation multinucleated cells from PBMCs in response to IL-17.

- To investigate the effects of IL-17 on osteoclastogenesis.

- To investigate the possibility that IL-17 treated synovial cells are capable of mediating bone resorption independently of RANKL.

CHAPTER 2

Materials and Methods

2 General Materials and Methods

2.1 Materials

2.1.1. Cell, tissue culture reagents and plastic ware

Tissue culture solutions were Dulbecco's modification of Eagle's medium (DMEM) with 1000 mg/L glucose and pyridine, and 25mM HEPES, Alpha minimal essential medium (∝-MEM) with Glutamax-1 without ribonucleosides and deoxyribonucleosides, RPMI 1640 media, Fetal calf serum (FCS) (Life Technologies). Penicillin, streptomycin and L-glutamine were obtained from Life Technologies Ltd. Collagenase (type I from Clostridium histolyticum), and trypsin (type III, from bovine pancreas), Dulbecco's phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), gentamycin solution, nystatin suspension and Histopaqe 1077 were obtained from Sigma-Aldrich Company Ltd. Tissue culture 48-well plates and 162 cm^2, 75 cm^2 and 25 cm^2 vented cell culture flasks were obtained from Corning/Costar UK Ltd. (High Wycombe, UK). Cell scrapers were obtained from Greiner Labortechnik (Gloucester, UK). Universals (5 and 20 ml) were obtained from Bibby Sterilin Ltd (Stone, Staffs, UK). Syringe filters (0.2 μm) were from Pall Gelman Sciences (Northampton, UK). Sterile disposable scalpel blades were from Swann-Morton (Sheffield, UK). Flat-bottomed 96 well plates were from Dynatech Laboratories (Billington, UK).

2.1.2 Cytokines

Recombinant human IL-17 was obtained from R&D systems Europe Ltd UK. Human recombinant oncostatin M (OSM) was kindly donated by Prof. J. Heath, Department of Biochemistry, University of Birmingham, UK. OSM stock was at 10 μg/ml in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) stored at -80°C. IL-17 was at 20 μg/ml in PBS containing 0.1 % (w/v) BSA, stored at -20°C. Recombinant human RANKL, OPG and M-CSF were purchased from PeproTech EC Ltd (PeproTech House UK). RANKL, OPG and M-CSF stocks were at 20 μg/ml, 50 μg/ml and 20 μg/ml, respectively, in distilled water. Immediately prior to use the cytokines were diluted in culture medium and sterile filtered through a 0.2 μm filter.

2.1.3 Cell lines

The osteosarcoma cell lines used in this project were SaOS-2 cells and MG-63 cells both from European collection of cell cultures (ECACC) The European Tissue Culture Society (ETCS) UK Branch.

2.1.4 Molecular biology reagents

Formamide, 3-(N-morpholino) propanesulphonic acid (MOPS), diethyl pyrocarbonate (DEPC), 37% (V/V) formaldehyde and β-mercaptoethanol were obtained from Sigma-Aldrich Company Ltd. RNeasy Mini kits and Plasmid Maxi kits were purchased from Sigma. RNA ladder (1- 10 kD), agarose (electrophoretic grade) and salmon sperm DNA (10 mg/ml) were obtained from Life Technologise Ltd. [α-^{32}P]-dCTP, Ready-To-Go DNA labelling beads and ProbeQuant G50 micro-columns were purchased from Amersham Pharmacia Biotech UK Ltd. GeneScreen plus membrane was obtained from NEN life Science products, Inc. (Hounslow, UK). All RNA work was performed under RNase-free conditions using RNase-free reagents and materials. All reagents required for RNA work were molecular biology grade. DEPC water was prepared by the addition of DEPC to d.H_2O to a final concentration of 0.1% (v/v). This was left overnight, then autoclaved for at least 20 min to remove traces of the DEPC. Furthermore, all glassware was oven-baked at 200°C, for at least 3 hr and cooled to room temperature, prior to use

2.1.5 Complementary DNA probes

Complementary DNA (cDNA) clones for human RANKL and OPG were cloned and sequenced from the PCR products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was originally isolated by Fort et al. (1985). The size of OPG clones was (2.5 kb) human cDNAs and GAPDH clones was (2.2 kb) human cDNAs, in Northern blot analysis.

2.1.6 Other reagent

All other chemicals and biochemicals were commercially available analytical grade reagents obtained from Sigma-Aldrich Company Ltd UK., Gibco Life Technologies UK, R&D or Promega UK (Southampton).

2.2 Cells and cell culture

2.2.1 Synovial fibroblasts

Solutions

Culture medium

DMEM, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20µg/ml nystatin and 100 µg/ml gentamicin.

Dulbecco's phosphate buffered saline (DPBS)

DPBS containing 100 U/ml penicillin, 100 µg/ml streptomycin.

Method

Synovial tissue was obtained from patients undergoing joint replacement surgery at the Freeman Hospital Newcastle upon Tyne or the Queen Elizabeth Hospital, Gateshead, and stored at 4°C in DPBS containing 100U/ml penicillin and 100 µg/ml streptomycin. Tissue was used up to 5 days after removal from the patient. The culture conditions were based on the method of Dayer et al. (1976). Fat and fibrous connective tissue was carefully dissected away and the remaining synovium washed twice in DPBS and finely chopped. The synovium fragments were digested for 2 to 3 hours at 37°C in DMEM supplemented with 2 mg/ml of bacterial collagenase, which had been filtered through a 0.2 µm filter. After incubation in an orbital shaker (110 rpm), the tissue fragments were dispersed by gentle pipetteing with a Pasteur pipette and filtered with cell strainer 100 µm Naylon BD Falcon (Becton Dickinson Labware USA). The cell suspension was centrifuged for 5 min at a relative centrifugal force of (200g) at room temperature. The cell pellet was resuspended in DMEM containing 20% FCS. The cells were plated into T75 cm^2 tissue culture flasks (10 ml/flask). The following day, medium was removed from each of the flasks and replenished with fresh medium. The passage zero (p0) cells typically took 7-15 days to reach 90-95% confluence. Synovial fibroblasts were split 1:2, and grown up to passage 4 or 5 (p4 or p5) before use in

experiments. Before cytokine stimulation, cells were washed twice in DPBS and left for 24 hr in 10 ml of 0.5% FCS cultured medium/flask.

2.2.2 Cell lines

Solutions

Culture medium

DMEM, containing 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml nystatin and 100 µg/ml gentamicin. α-MEM containing 10% (v/v) FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml nystatin.

Dulbecco's phosphate buffered saline (DPBS)

DPBS containing 100 U/ml penicillin, 100 µg/ml streptomycin.

Method

Human osteosarcoma cell line SaOS-2 was cultured in ∝MEM, and MG-63 cells were cultured in DMEM. The cells were grown until they reached confluence at 37°C, 5% CO_2. Before experiments the cells were serum-reduced in medium containing 0.5% (v/v) FCS overnight. Then fresh medium with cytokines at different concentrations was added and the cells further incubated for different time periods.

2.3 RNA extraction

In experiments with osteosarcoma cell lines and human synovial fibroblasts, gene expression was assessed by analysis of mRNA levels. Total RNA was extracted from cells using Trizol reagent (Life Technologies) which is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987).

Method

Culture supernatants were decanted from the cells grown in a monolayer, and total RNA was extracted from the cells. Trizol reagent (2 ml) was added to each T75 cm^2 flask, worked over the cell monolayer and a scraper used to collect the lysate which

was then transferred to a 1.5 ml eppendorf tube. Chloroform (0.3 ml) was added; the tube inverted several times and placed at room temperature for 10 min. After centrifugation at 13000xg for 10 min at 4°C each tube has three distinct phases. An upper RNA containing layer which was carefully transferred to a new tube leaving < 0.1 ml of this layer to avoid DNA contamination from the interface. The lower phase, containing protein was stored at -20°C until required. Isopropanol (0.6 ml) was added to the aqueous phase containing RNA and placed at -20°C overnight (an alternative is to keep at -80°C for 2 hr). The RNA was spun at 13000xg for 15 min at 4°C, and the supernatant carefully discarded so as not to disturb the pellet. The pellet was washed with 0.7 ml of 70% ethanol and centrifuged at 13000xg for 5 min at 4°C. The ethanol was removed and the pellet air-dried until translucent (around 5 min). The pellet was reconstituted in 15 µl DEPC-H_2O. The absorbance of the RNA was measured using a GeneQuant II spectrophotometer (Amersham Pharmacia Biotech Ltd UK) at 260/280nm. The RNA was stored at -80°C until required.

2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Principles of RT-PCR sensitive methods for the detection and analysis of rare mRNA transcripts or other RNAs present in low abundance are an important aspect of most cell/molecular biology studies. RNA cannot serve as a template for PCR, so it must first be reverse transcribed into cDNA. Gattei V ET AL., (1997) described a combined technique (now commonly known as RT-PCR) in which reverse transcription (RT) is coupled with PCR amplification of the resulting cDNA. RT-PCR provides the possibility to assess gene transcription in cells or tissues. PCR and RT-PCR techniques have been instrumental in dental research. PCR and RT-PCR show many advantages including high specificity, sensitivity, and speed.

2.4.1 DNase treatment

All RNA was treated with DNase I to ensure there was no genomic DNA contamination. For 3 µg of RNA, 1 µl of 10x DNase I reaction buffer and 1 µl of DNase I (2.5 U/µl) (Amplification grade from Invitrogen (Gibco BRL) Life technologies UK) were added, and then DEPC-treated H_2O to a 10 µl final volume. After incubation for 15 min at room temp, 1 µl of 25 mM EDTA was added and then heated to 65°C for 10 min to inactivate the DNase. Each tube containing 11 µl was then split in half and 44.5 µl-distilled water was added to negative controls for RT-PCR reactions a further 5 µl DEPC-H_2O was added and mixed well.

2.4.2 cDNA synthesis

RNA was reverse transcribed into complementary DNA (cDNA) using the Superscript kit (Invitrogen (Gibco BRL) Life technologies UK). Between 3 - 5 µg of the total RNA was used as template from each sample for cDNA synthesis by SuperScript^{TM}II. $oligo_{dT12\text{-}18}$ (0.5 µg) was added; the volume was made up to 10.5 µl. The mixture was incubated at 70°C for 10 min then placed on ice. 5x first strand buffer 4 µl, 2 µl DTT, 1 µl of 10 mM dNTPS, 1 µl RNase inhibitor (40 U/µl) and 1 µl Superscript II reverse transcriptase (200 U/µl) (Sigma) were added. The final volume (20 µl) was incubated at 42°C for 1 hr. The cDNA was diluted to 50 µl and stored at -20°C.

2.4.3 Conventional PCR analysis

Complementary DNA was amplified by PCR using primer pairs to generate products corresponding to specific gene as listed in Table 2-1. Primers were designed against human sequences for RANKL, OPG, GAPDH, IL-17 and IL-17R in the EMBL/GenBank database.

The amplification mixture contained 1 U of Taq DNA polymerase (Life Technologies), 200 ng each of the 5` and 3` primers, 0.2 mM dNTPs (dATP, dCTP , dGTP and dTTP), 1.5 mM $MgCl_2$, 2 µl of 10x reaction buffer, and DEPC-H_2O to 25 µl and 2 µl of

template cDNA. A negative control reaction was run with each experiment and contained all the reagents except the cDNA template. PCR was performed for 35 cycles for GAPDH, RANKL, OPG, IL-17, and 38 cycles for IL-17R.

	Accession Number	5' (sense) primer 3' (Antisense) primer	Position (bp)	Product Size (bp)
GAPDH	AF019047	GGT GAA GGT CGG AGT CAA CGG GGT CAT GAG CCC TTC CAC GAT	542-1024	483
IL-17R	U94332	GGC TAA ACT GCA CGG TCA AG TAT TCC TGG TCA GGG TCA AC	287-722	436
RANKL	U32659	GCA CAT CAG AGC AGA GAA AG AGT AAG GAG GGG YYG GAG AC	124-682	386
OPG	U58917	TGC CCT GAC CAC TAC TAC AC CAA ACC TGA AGA ATG CCT CC	172-502	400
IL-17	M33197	GAA TCA CAA TCC CAC GAA ATC C GCC AAG TGT TAC CTC TGA AGC	69-589	520
Alkaline phosphatase	NM 000478	GGA CAT GCA GTA CGA GCT GA GGT GCA GAA GTG TAA ACC AC	1251-1551	281

Table 2.1 the Conventional PCR primers sequence for RANKL, OPG, GAPDH, IL-17 and IL-17R.

A PTC-100 Thermal Cycler (MJ Research (Genetic Research Instrumentation Gene House. UK) was programmed to perform the following PCR program. After an initial step at 95°C for 3 min to break down the complex tertiary structure of cDNA, each cycle consisted of 30 seconds denaturation at 94°C, annealing depends on the melting temperature of each primer pair (for RANKL; 60°C, OPG and GAPDH, 55°C IL-17R, 61°C) for 30 seconds then extension at 72°C for 30 seconds. This was followed by an additional extension step at 72°C for 5 min. In all cases, the primers were mRNA-

specific in that the recognition sites of the upstream and downstream primers resided in separate exons in the genomic sequence. Primer sequences and predicted PCR product sizes are shown in table 2-1. Amplification products were identified by electrophoresis on a 1% (w/v) agarose gel. Amplified products corresponding to human specific mRNAs were expressed as a ratio of the respective GAPDH product. To show that there were no false-positive results, PCR reactions were performed using non-reverse-transcribed RNA, and on reaction mixtures in which no RNA was added. Positive controls, including a number of human sample and cell line-derived cDNAs, were used to confirm the specificity of each primer pair under the conditions used. Products were analysed by agarose gel electrophorsesis and visualised by ethidium bromide staining under UV light. The identity of PCR products was confirmed by cloning into PGEM-T (Promega) and sequence analysis on an ABI DNA sequencer (Molecular Biology Unit, University of Newcastle).

2.5 Cloning of PCR products

2.5.1 Isolation of PCR fragment

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) as recommended by the manufacturer and the eluted products kept at -20°C. A small few aliquots ≤ 5 µl of recovered cDNA were run on 1% agarose gel to confirm the purification.

2.5.2 Ligation into pGEM-T

Purified PCR products were added to the ligation reaction containing T4 DNA ligase, as directed by the manufacturers with pGEM-T or pGEM-T Easy Vector (Promega). High efficiency competent cells (JM109) Promega UK (Southampton, UK) were used for the transformation as recommended, in order to obtain a reasonable number of colonies. Selection for transformants was on LB/ampicillin/Isopropyl-beta-D-thiogalactopyranoside (IPTG)/X-Gal plates. Two LB/ampicillin/IPTG/X-Gal plates were prepared (for each ligation reaction) and the plates were equilibrated to room temperature prior to plating. Ligation reaction 2 µl was added to a sterile 1.5 ml

microcentrifuge tube on ice. JM109 cells were removed from -70°C and placed in an ice bath until just thawed and then 50 µl of cells were transfered to each ligation. The tubes were gently flicked to mix and placed on ice for 20 min before heat-shock at 42°C for 45 to 50 seconds. The microcentrifuge tubes were immediately returned to the ice for 2 min, room temperature SOC medium 950 µl was then added to the tubes and incubated for 1.5 hr at 37°C with shaking (≅200 rpm). Transformed bacteria (100 µl) were spread onto the LB plates containing ampicillin. The plates were incubated upside-down overnight (16-18hr) at 37°C. Single white colonies from the plate were picked, and resuspended in 50 µl of distilled water, mixed well and kept at -70°C until required.

2.5.3 Harvest and lysis of bacteria

The resuspended colony 10 µl was used to inoculate 6.5ml of LB broth with 6.5 µl of ampicillin (50 µg/ml) in a 20 ml universal and incubated overnight at 37°C in a shaking incubator (200 rpm). From the cultured bacteria, a stock was first made; 450 µl of the culture was added to 50 µl of glycerol, mixed well and kept at -70°C until required. The rest of the culture was transferred to 1.5 ml microcentrifuge tubes and spun for 1 min at 13,000 xg. The supernatant was discarded and re-suspended in 200 µl of re-suspension solution as recommended in the Genelute plasmid mini-prep kit (Sigma). Plasmid was isolated using the Genelute kit as directed by the manufacturer. Elution buffer 80 µl was added to the column and spun for 1 min. The last step was repeated twice to make sure that most of the plasmid DNA was eluted and then the DNA was kept at -70°C until required.

2.5.4 Quantitation and digestion of plasmid DNA

The DNA was quantified by its absorbance at 260/280nm and then 5 µg/ml of plasmid DNA was digested. A typical digest was performed in a 10 µl volume. Thus, for 3 µl of plasmid DNA, 1 µl of buffer A was added, 1 µl of enzyme Sac I (10 U/µl) and enzyme Apa I (10 U/µl), followed by distilled water to a final volume of 10 µl. The plasmid DNA was incubated for 3 hr at 37°C and subsequently loaded onto a small

1.5% agarose gel and electrophoresed at 80 V for 1 hr. The relevant band was cut from the gel and placed in a microcentrifuge tube. The DNA was purified from the agarose gel using a QIAquick gel Extraction kit or kept at –70°C until purified.

2.6 Northern blot analysis

2.6.1 RNA electrophoresis

Principles: electrophoresis of RNA through an agarose gel allows separation of RNA based on size and electrostatic charge. Agarose is a linear polysaccharide. An agarose gel consists of inter-and intra-molecular hydrogen bonds within and between the long agarose chains. RNA is denatured to prevent hydrogen bond formation within or between polynucleotides. Formaldehyde is used as the denaturing reagent, and is included in the agarose gel. Moreover, samples are heat denatured in the presence of formaldehyde prior to electrophoresis, to disrupt the formation of secondary mRNA structures.

The gel tray, comb and electrophoresis tank were soaked in 0.5 M NaOH for 15 min to ensure they were free from RNase and then were washed with DEPC-treated H_2O.

1% agarose gel with 0.4 M formaldehyde

Agarose (0.6 g) was weighed; 54.75 ml DEPC distilled water and 7.5 ml of 10X MOPS were added and heated in a microwave until dissolved (approx. 45 sec on full power). Then the solution was left to cool in a fume hood (approx. 60°C) and then 12.75 ml 37% (v/v) of formaldehyde (Merck) was added, the solution swirled and poured immediately into the sealed gel tray.

10x running buffer

200 mM MOPS, 50 mM sodium acetate and 10 mM EDTA (pH 7.0).

5X RNA loading buffer

0.25% (v/v) Bromophenol blue, 4 mM EDTA, 7.2% (v/v) formaldehyde (37% solution), 20% (v/v) glycerol, 31% (v/v) formamide, 40% 10x running buffer and 0.1 mg/ml ethidium bromide.

Methods

RNA was electrophoresed using 1% agarose /0.4 M formaldehyde gels. Equal amounts of RNA (typically: 10 - 20 µg for human osteosarcoma cells SaOS2, MG-63 and 8 - 15 µg for human synovial fibroblast) were made up to a volume of 20 µl with DEPC d.H_2O. A RNA ladder (1 kDa) was prepared in the same manner. To each sample, 10 µl of 5x RNA loading buffer was added. Samples were then incubated at 55°C for 15 min and placed on ice. The gel was run at 60 V for 1 – 2 hr in 1x running buffer, viewed under UV light to check the integrity of the RNA and photographed.

2.6.2 RNA transfer

Principle: Through the process of capillary action, RNA can be transferred from the agarose gel onto a nylon membrane. The RNA can then be "cross-linked" permanently onto the membrane through baking or exposed to UV irradiation.

Solutions

20x saline-sodium citrate (SSC)

This reagent is supplied as a 20x concentrate: 0.3 M sodium citrate, with 3 M NaCl, pH 7.0.

Method

RNA was transferred to Hybond-N (Amersham Pharmacia Biotech) with 200 ml 15x SSC overnight as follows. Formaldehyde was removed from the gel by washing in DEPC d.H_2O (3 x 5 min washes). The Hybond-N membrane and four pieces of filter paper were cut to the size of the gel. One piece of filter paper was cut longer than the size of the gel to act as a wick. The membrane, filter paper and wick were then pre-soaked in 15x SSC. The transfer stack was prepared on a sponge in the following order: one piece of filter paper, the wick, and the gel (upside down), Hybond-N membrane, three sheets of filter paper, approximately (5 – 10 cm) of paper towel, a glass plate and a weight. Cling filming to prevent evaporation and ensure there was no contact between the wick and the layers above the gel was used to surround the stack. The blot was removed the following day, checked under UV light to ensure transfer of

RNA and washed briefly in 2x SSC to remove traces of agarose. The membrane was blotted dry and then baked at 80°C for at least 2 hr, in order to cross-link the RNA onto the membrane. The blot was stored between two pieces of filter paper at room temperature until required.

2.6.3 Labelling of cDNA probes and membrane hybridisation

2.6.3.1 Labelling cDNA probes

Principles: the random primed labelling method was used to label the cDNA probe. The cDNA was denatured and then renatured in the presence of a mixture of different random sequences of hexamers. The hexamers bind to the cDNA where they encounter the complementary sequence and the DNA therefore rapidly acquires an approximately random distribution of hexamers. The hexamers have an exposed 3'-hydroxyl group and act as primers for the synthesis of a fresh cDNA catalysed by the Klenow fragment of DNA polymerase with the incorporation of $[\propto^{32}P]$-dCTP. Ready-to-Go DNA labelling beads are used. Each bead consists of dATP, dGTP, dTTP, klenow fragment and random oligodeoxyribonucleotides.

Materials

cDNA probe.

Ready to Go tube containing DNA labelling beads (Amersham Pharmacian Biotech).

$\propto^{32}P$-dCTP.

Column chromatography (G50 micro columns).

Methods

The appropriate required volume of cDNA insert (25 ng cDNA) was adjusted to 45 µl with $d.H_2O$. The probe was boiled for 5 min (then immediately put on ice to prevent renaturation). This was then added to a Ready to Go tube containing 'DNA labelling

beads'. $\propto^{32}$P-dCTP (5 µl) (3000 Ci/mmol) was added and the mixture incubated at 37°C for 30 min. The labelled probe was purified from unmincorporated label by column chromatography (ProbeQuant G50 micro columns) according to the manfacturer's instructions. The probe was assessed for incorporation of label in ß-counter. The probe was labelled to $5 - 7 \times 10^6$ Bq/µg DNA.

2.6.3.2 Hybridisation

Principle: Salmon sperm DNA was used to block non-specific sites on the blot and labelled probe was added that allowed hybridising with its specific mRNA on the blot. After hypridisation, blot were washed to remove unbound label and labelled mRNA visualised by phosphorimaging.

Materials

QuikHyb® Hybridization solution (Stratagene).

Salmon sperm DNA.

Methods

The blot was pre-soaked in 100 ml of 2x SSC and placed into a hybridisation bottle. The membrane was then incubated with 10 ml of Hybridization solution following the manufacturer's protocol. The bottle was placed (with a balance) into the hybridisation oven and incubated at 68°C for at least 30 min. The labelled probe was added to 100 µl of 10 mg/ml salmon sperm DNA and boiled for 5 min and immediately placed on ice for 5 min to prevent renaturation. The labelled probe was directly added to the hybridisation bottle and incubated at 68°C for 2 hr. The membrane was washed twice with 100-200 ml 2x SSC buffer contained 0.1% SDS for 15 min at room temperature. Then the membrane was washed once with 100-200 ml 0.1x SSC buffer + 0.1% (w/v) SDS washing solution for 30 min at 60°C. The blot was blotted semi-dry on filter paper, wrapped in cling film and exposed to a Storage phosphor screen (Molecular Dynamics, Chesham, UK) overnight at room temperature. Radioactive bands were visualised by using a Phosphor Imager (Storm 860, Molecular Dynamics).

2.6.4 Stripping RNA blots

solutions

0.1% SSC buffer and 0.1% SDS

Methods

The probe then stripped from the blot by washing (3 times) for 5 min with 200 ml of boiling 0.1% SSC buffer with 0.1% SDS. Blots were then re-exposed to the phosphor screen to check if they were adequately stripped. If the blot was not stripped very well, the blot was washed again in boiling 0.1% SSC buffer and 0.1% SDS for 15 minutes. In order to prevent the blot drying out between each probing, the blot was wrapped in cling film, followed by aluminium foil and stored at –20°C.

2.7 Protein electrophoresis and Western Blotting

2.7.1 Protein assay

Principle: Protein concentrations can be evaluated based upon the formation of a colorimetric complex. The bicinchoninic acid reagent supplied forms a complex with the copper I produced by the interaction of protein with alkaline copper II. This complex is water-soluble allowing spectrophotometric measurement in aqueous solution at 560 nm.

Methods:

A standard curve was constructed using BSA (2 mg/ml stock) diluted in H_2O; a range of standards (0.625 – 2 mg/ml BSA) were prepared in a 1 ml total volume. Sample or standard 10 µl was added in triplicates to 96 well microtitre plates, and the reaction initiated by addition of 200 µL of assay reagent made up according to the manufacturer's instructions. The plates were incubated for 30 min at room temperature prior to the absorbance being read at 560 nm. Protein concentrations were estimated from the standard curve. The dilution factor was taken into account to determine the protein concentration of the undiluted samples.

2.7.2 Western Blot

2.7.2.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Principle: Proteins are separated based on size by SDS-PAGE performed under reducing conditions (Laemmli UK, 1970). The polymerisation of acrylamide is initiated by the addition of ammonium persulphate (APS) and the base TEMED. TEMED catalyses the decomposition of persulphate to give a free radical which initiates the polymerisation reaction. Samples are prepared by boiling in β-mercaptoethanol and SDS. β-mercaptoethanol reduces any disulphide bridges and the SDS, an anionic detergent denatures proteins by wrapping around the polypeptide backbone in a ratio of approximately 1.4 grams SDS per gram of protein. The bound SDS masks the charge of the proteins, forming anionic complexes with constant net negative charge per unit mass. The purpose of the stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. The protein-SDS complexes concentrate in a very tight band between glycinate and chloride boundaries. Once the glycinate ions reach the separating gel, it becomes more ionised due to the higher pH and its mobility increases. The interface between the chloride and glycinate ions leaves behind the protein-SDS complexes, which are left to electrophorese at their own rates. The protein-SDS complexes continue to move towards the anode. Proteins are separated by their size. Smaller proteins pass more easily through the pores of the gel. Larger proteins are retarded by frictional resistance due to the sieving effects of the gels. After the tracking dye (bromophenol blue) reaches the bottom of the gel, electrophoresis is stopped.

Materials:

N,N,N'N'-tetramethylenediamine (TEMED), Acrylamide/bis-acrylamide (37.5:1) solution [40% (w/v)] was obtained from Anachem (Luton, UK). Pre-stained protein SDS-PAGE molecular weight standards (Broad Range) were obtained from BIO-RAD Laboratories UK.

Solutions:

10% Resolving gel buffer containing 0.51M Tris base, pH 8.8.

Stacking gel buffer containing 142 mM Tris base, pH 6.8.

5x running buffer consisted of 200 mM Tris base, 192 mM glycine and 0.1 % (w/v) SDS.

For monitoring the progress of the gel run and the efficiency of the Western transfer 10 µl of pre-stained standard were used.

Methods:

Electrophoresis was carried out in a Bio-Rad Mini-Protean II apparatus with 1.5 mm spacers and combs. For a 10% gel, 3.75 ml of a 40% stock acrylamide-bis-acrylamide solution was mixed with 10% of gel buffer, pH 8.8 (11 ml). All solutions were allowed to reach room temperature before mixing. Gel mixture (14.2 ml) was polymerised by the addition of 20 µL of TEMED and 75 µl of a 10% (w/v) solution of APS, and the solution poured into a gel cassette and left to polymerise. Following polymerisation, a 4% stocking gel was formed by mixing 1 ml of the 40% stock acrylamide/bisacrylamide solution with 8.8 ml of stacking gel buffer, 50 µL of 20% SDS, 100 µl of 10% APS and 20 µl of TEMED.

Reduced samples were prepared for electrophoresis by addition of SDS loading buffer containing 142 mM Tris base pH 6.8, 0.8g SDS, 50% of glycerol (v/v), 0.5% bromophenol blue (w/v) and 50 mM DTT (di-thio-threitol) and a nonreduced samples which were the same reagent but with out DTT. The samples were boiled for 5 min in a water bath. Following a brief centrifugation, samples were applied to the gel along with molecular weight markers and electrophoresed at 150V for approximately 1.5 hr, or until the dye front passed the end of the gel. Following electrophoresis gels were transferred to a nitrocellulose membrane.

2.7.2.2 Protein Transfer

Principle: Proteins are electro-transferred from a SDS-PAGE gel onto nitro-cellulose membrane. Proteins bind irreversibly by hydrophobic interactions to the membrane. The protein is transferred by applying a current. This is the actual blotting process and is necessary to expose the proteins. The membrane is "sticky" and binds proteins non-specifically (i.e., binds all proteins equally well).

Materials

1,10-phenanthroline and polyoxyethylenesorbitan monolaurate (Tween 20) were obtained from Sigma-Aldrich Company Ltd. Precision protein standards and

Streptavidin-horseradish peroxidase (HRP) were obtained from Bio-Rad Laboratories. Enhanced chemiluminescence (ECL) Western blotting detection reagents and Hyper Film were obtained from Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK). Streptavidin-HRP and HRP-conjugated antibodies were obtained from DAKO Ltd. (Cambridgeshire, UK).

Solutions

Western Transfer Buffers: Transfer buffer for blotting contained 190 mM glycine, 25 mM Tris base and 20% (v/v) methanol.

Blocking Buffer: 5% (w/v) Marvel fat-free dried milk in PBS/1%Tween.

Method

Western transfer of proteins for immunoblotting was carried out using a Semi-dry blotter. All materials were soaked in the transfer buffer prior to use. A sandwich was formed on the anode of the blotter consisting of 9 sheets of filter paper, 1 sheet of nitrocellulose, the SDS gel and finally a further 9 sheets of filter paper. A current of 80 mA for each gel was then passed through the sandwich for 1.5 hr. Following transfer the blot was reversibly stained with Ponceau Red solution to check whether the transfer was completed. Then the membrane was washed with PBS, 0.5%Tween and blocked over night in blocking solution (5% milk in PBS/0.5%Tween). The membrane was screened with the Anti h-OPG antibody (R&D) at 1:1000 for one hour. The membrane was then washed once with PBS/Tween for 5 min and the secondary antibody (Rabbit anti-goat HRP; Dako po449) was added at 1:10,000 for 30 min. The membrane was then washed with PBS/Tween three times, for 10 minutes. Using ECL reagents, antibody-protein complexes were detected as directed by the manufacturer. The light emitted is detected by exposing the blot to blue-light sensitive autoradiography film.

2.8 Real-Time PCR

Principles: Real-time quantitative PCR is a powerful tool that can be used for gene expression analysis. Two types of real time PCR were used (Taq-Man and LightCycler). Taq-Man is based on fluorescence labelled sequence-specific hybridization probes (resonance energy transfer). The LightCycler uses the DNA binding dye, SYBR green I. This dye binds in the minor groove of double-stranded

DNA in a sequence independent way. When it binds, its fluorescence increases over 100-fold.

With real-time PCR it is possible to detect a signal (threshold cycle) and use this to determine the starting level of a certain mRNA preparation. Fluorescent signal from each PCR reaction is collected as peak-normalized values plotted versus the cycle number. Reactions are characterized by comparing threshold cycle (C_T) values. The C_T is a unit-less value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold above baseline. Samples with high starting copy number show an increase in fluorescence early in the PCR process, resulting in a low C_T number, whereas lower starting copy number results in a higher C_T number. Quantitated transcript levels of the GAPDH gene are used as the endogenous control; with each unknown sample normalized to GAPDH content. The aim of real time PCR is to improve the sensitivity and accuracy of the quantification, especially in the case of low abundance mRNA (Dhar AK et al., 2001; Mackay M. 2004).

2.8.1 LightCycler

Principles: The Quantitative PCR instrument detects accumulation of PCR products, allowing easy and accurate quantitation in the exponential phase of PCR reactions. For the monitoring of PCR with SYBR Green, conventional PCR primers are needed. The primers used for quantitative PCR can be used for LightCycler PCR experiments by using SYBR Green I.

A successful, highly specific and sensitive quantitative PCR experiment primers must fulfil some general properties, such as firstly the target sequence should contain only a single annealing site. Secondly the primers should not contain any intra-molecular sequence complementarities, which could form secondary structures. No inter-molecular complementarities should exist between the primers, which could potentially lead to the formation of primer-dimers. Finally target product size should ideally be 50-150 bp in length and should not exceed 500 bp.

2.8.2 Oligonucleotide Primers and TaqMan Probe Design

RANKL and OPG complementary DNA (cDNA) sequences (GenBank Accession No. AF019047 and U94332, respectively) were evaluated using the Primer Express software (Perkin-Elmer). The forward and reverse primers were designed to lie in adjacent exons (separated by a long intron) to prevent an amplification of genomic DNA that may be contained in the samples. The primers for RANKL and OPG had a calculated annealing temperature of 65°C. Primers were ordered from (Sigma-Genosys Ltd). For RANKL, forward and reverse primers were 5'AGAGAAAGCGATGGTGGATG and 5'TATGGGAACCAGATGGGATG, respectively, creating an amplicon of 116 bp. For OPG, forward and reverse primers were 5'AAGAAGATCATCCAAGATATTGACC and 5'CGGTAAGCTTTCCATCAAGC, respectively, creating an amplicon of 108 bp. The primers for GAPDH had a calculated annealing temperature of 60°C. The Taqman GAPDH primers and probe were used from R&D (part number 402869) (Rubin j. et al 2002).

2.8.3 Quantitation of RANKL and OPG mRNA levels by QT-PCR

To quantify RANKL and OPG cDNA levels: the relative standard curve method and the comparative C_T method were used. In the relative standard curve method, standard curves with known amounts of total cDNA (relative) are analysed for the gene of interest (RANKL and OPG). For each unknown sample, the relative amount is calculated using linear regression analysis from respective RANKL and OPG standards curves. Expression values are then obtained by division of the RANKL and OPG value by the GAPDH value.

2.8.4 Total RNA quantification for Real Time PCR.

Principles: RiboGreen RNA to quantitate as little as 1 ng/ml RNA with a fluorescence microplate reader, standard spectrofluorometer or filter fluorometer, using fluorescein

excitation and emission wavelengths. The excitation maximum for RiboGreen reagent is ~500 nm and the emission maximum is ~525 nm using a fluorescence microplate reader. Total RNA (2-3 μg) was reverse transcription as reported in section (2.4.2). Then the cDNA was diluted 5 times for real time PCR and 2μl of template used for each reaction.

2.8.5 Polymerase chain reaction

PCR amplification was carried out in a 20 μl volume with 2 μl of LightCycler DNA Mastermix (Roche Molecular Biochemicals, Mannheim, Germany). Quantitative (QT) PCR amplification and analysis were achieved using a LightCycler instrument. For each primer combination optimal $MgCl_2$ (2 mM) concentration and annealing temperature were experimentally determined for OPG and RANKL primer pairs (65°C) and for GAPDH primer pairs were 60°C. The reaction mixture consisted of cDNA (2 μl), 0.5 μl (which is 0.3 mM) of each primer, 2 μl LightCycler FastStart DNA Master SYBR Green I mix (Roche, 2239264), and $MgCl_2$ (2 mM), in a total volume of 20 μl. cDNA conditions to be compared were made with the same PCR master mixes and within a single LightCycler run. All templates were amplified using the following LightCycler protocol. The FastStart polymerase was activated and cDNA denatured by a preincubation for 10 sec at 95°C, the template was amplified for 45 cycles with denaturation for 20 sec at 95°C, annealing of primers at 65°C programmed for 5 sec, and extension at 72°C for 10 sec. Fluorescent data were acquired during each extension phase. After 45 cycles a melting curve was generated by heating the sample to 95°C programmed for 0 sec followed by cooling down to 60°C for 15 sec and slowly heating the samples at 0.1°C to 95°C while the fluorescence was measured continuously. Fast loss of fluorescence is observed at the denaturing/melting temperature of a DNA fragment, which is a unique feature of that individual fragment. The melting peak can be obtained by plotting the negative first derivative of fluorescence against temperature. Finally, the samples were cooled down to 40°C for 30 sec.

The fluorescence emitted by the reporter dye was detected online in real-time, and the threshold cycle (C_T) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample (Zijlstra, A. et al. 2002).

The fluorescence emitted by the reporter dye was detected online in real-time, and the threshold cycle (C_T) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample (Zijlstra, A. et al. 2002).

2.8.6 Interpretation of QT-PCR data-Standard curve

Standard curves were generated from cDNAs made from good quality total RNA. The cDNA was serially diluted 12 times starting with the equivalent of 250 ng/ml of total RNA down to 0.061 ng/ml as shown in (Fig 2-1a) and (Fig 2-1b). The C_T values were subsequently used to calculate and plot a linear regression line by plotting the logarithm of template concentration (X-axis) against the corresponding threshold cycle (Y-axis) as shown in (Fig 2-2).

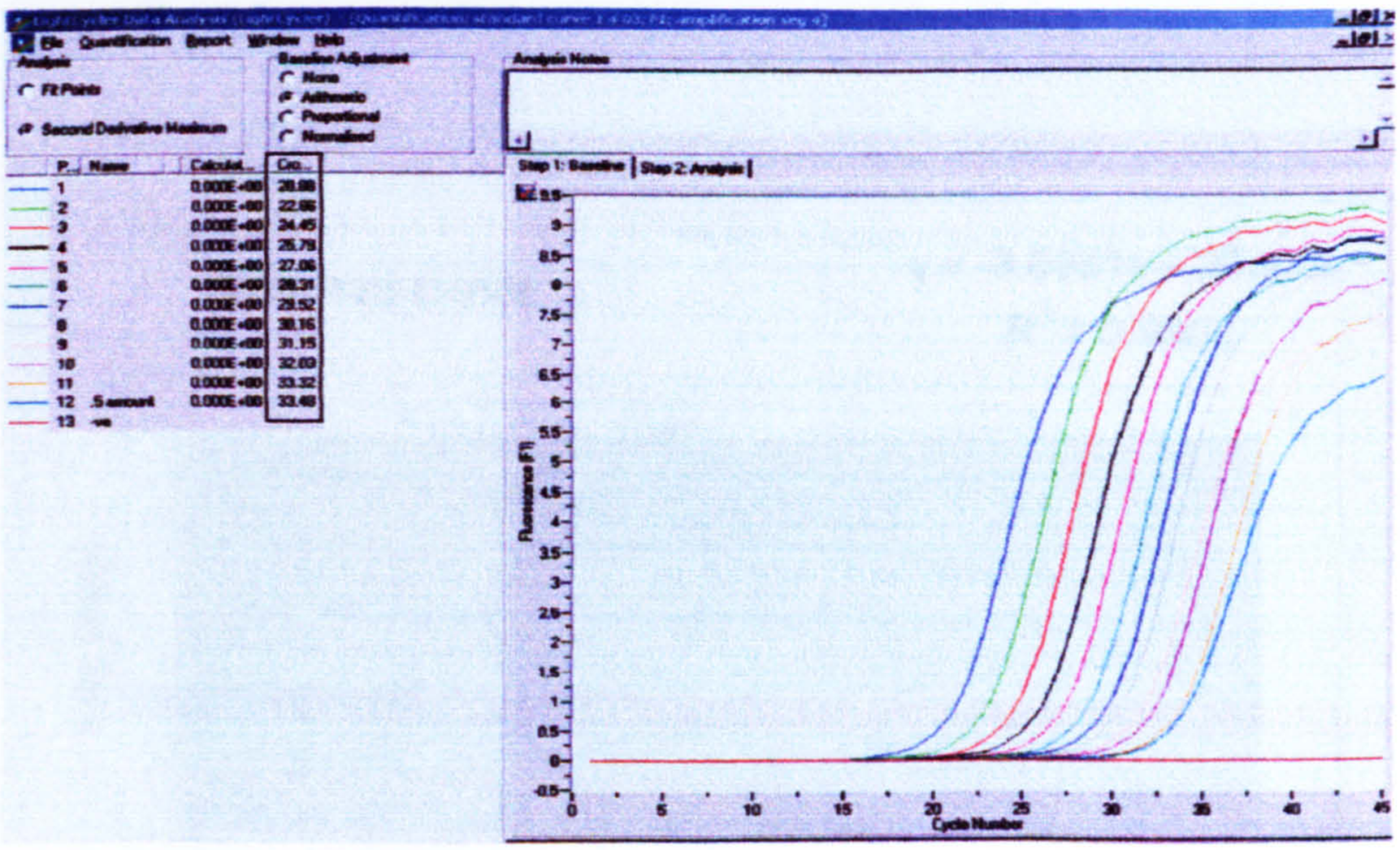


Fig 2.1a shows the CT value from each sample from standard curve. An example of the CT report observed from Taq-Man CT values are in the square.

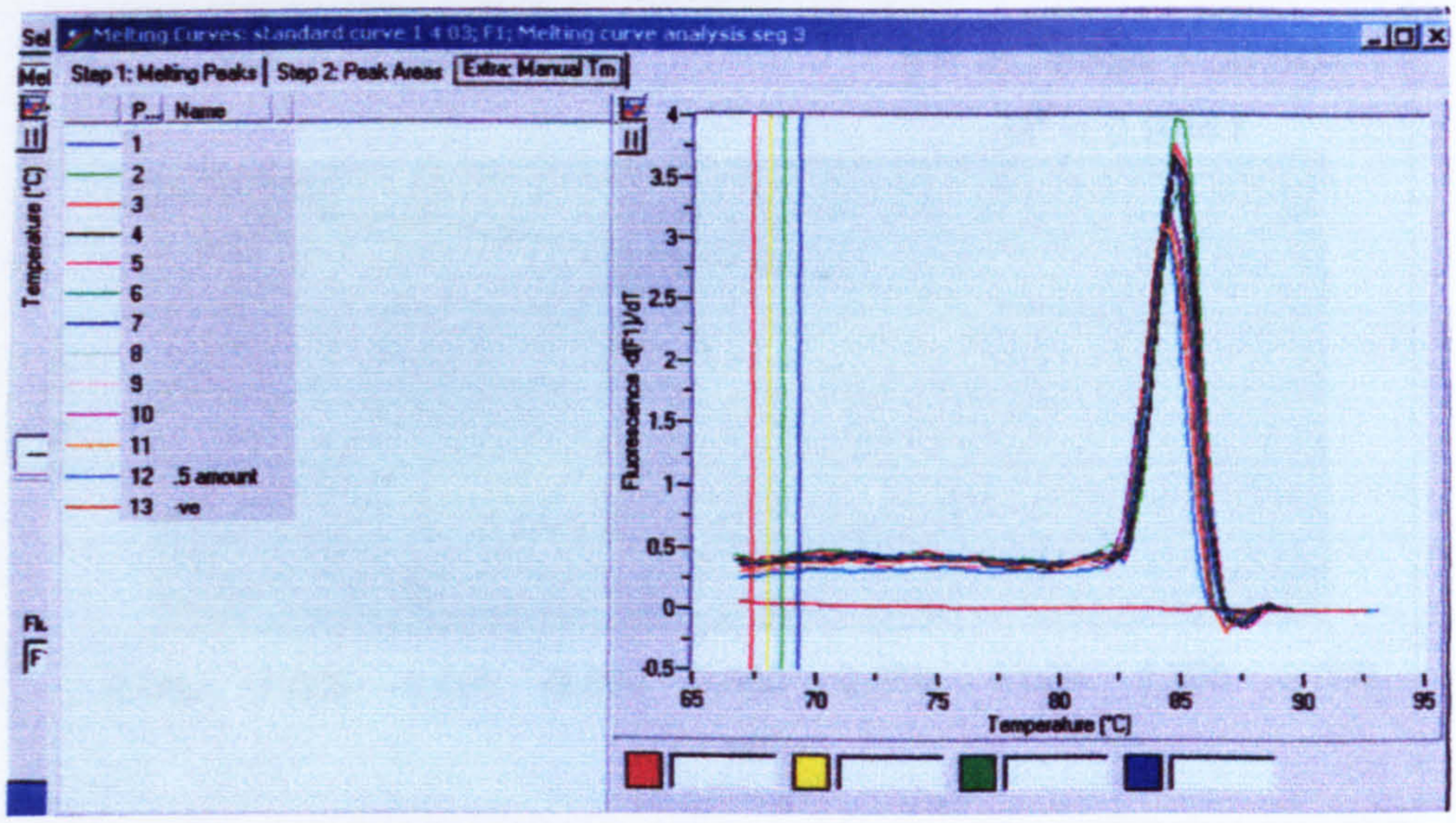


Fig 2.1b The melt curve of the standard samples, showing only one peak at equivalent temperature for all samples as observed from Taq-Man.

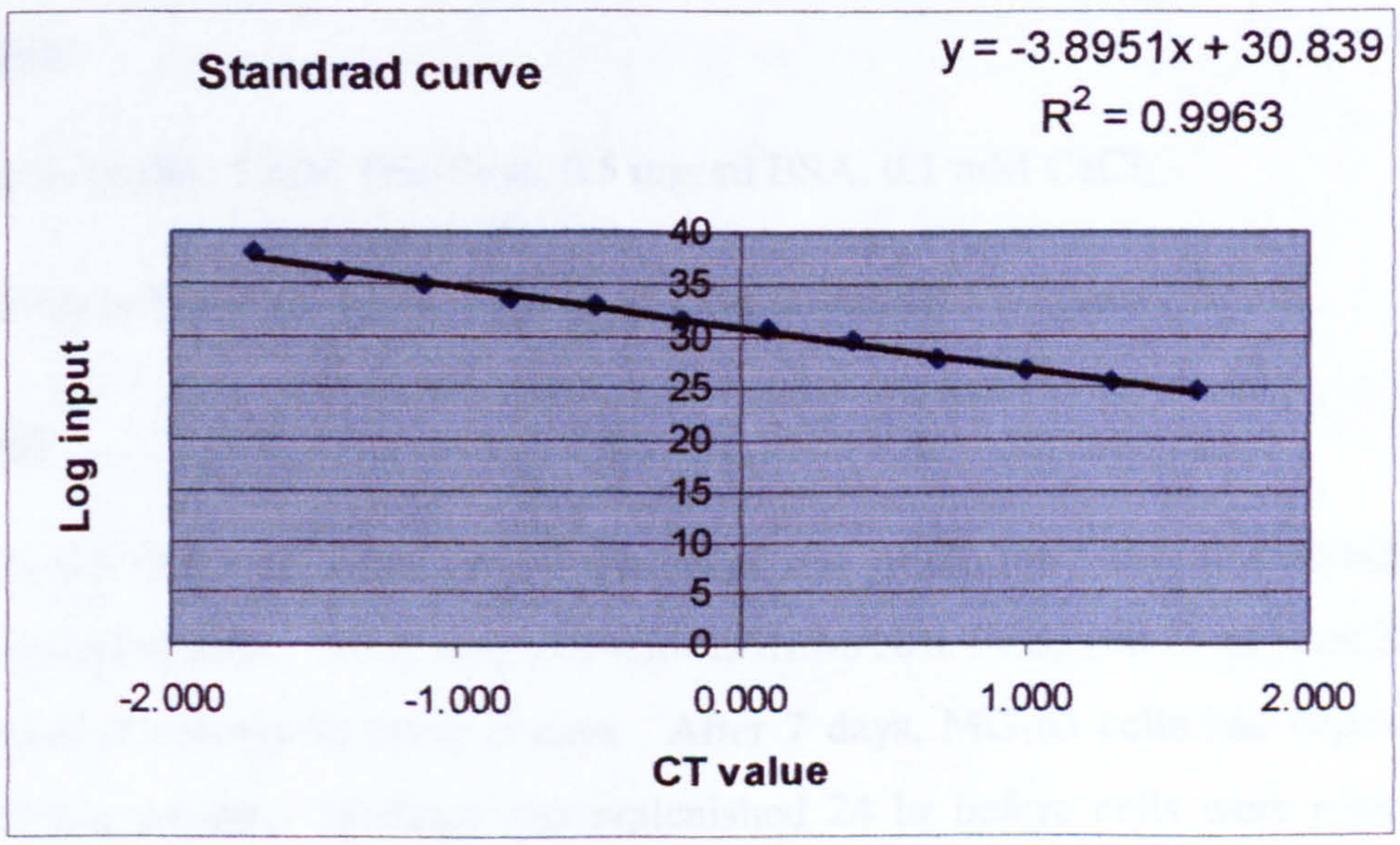


Fig 2.2a An example of the calculated standard curve for the expression of OPG mRNA.

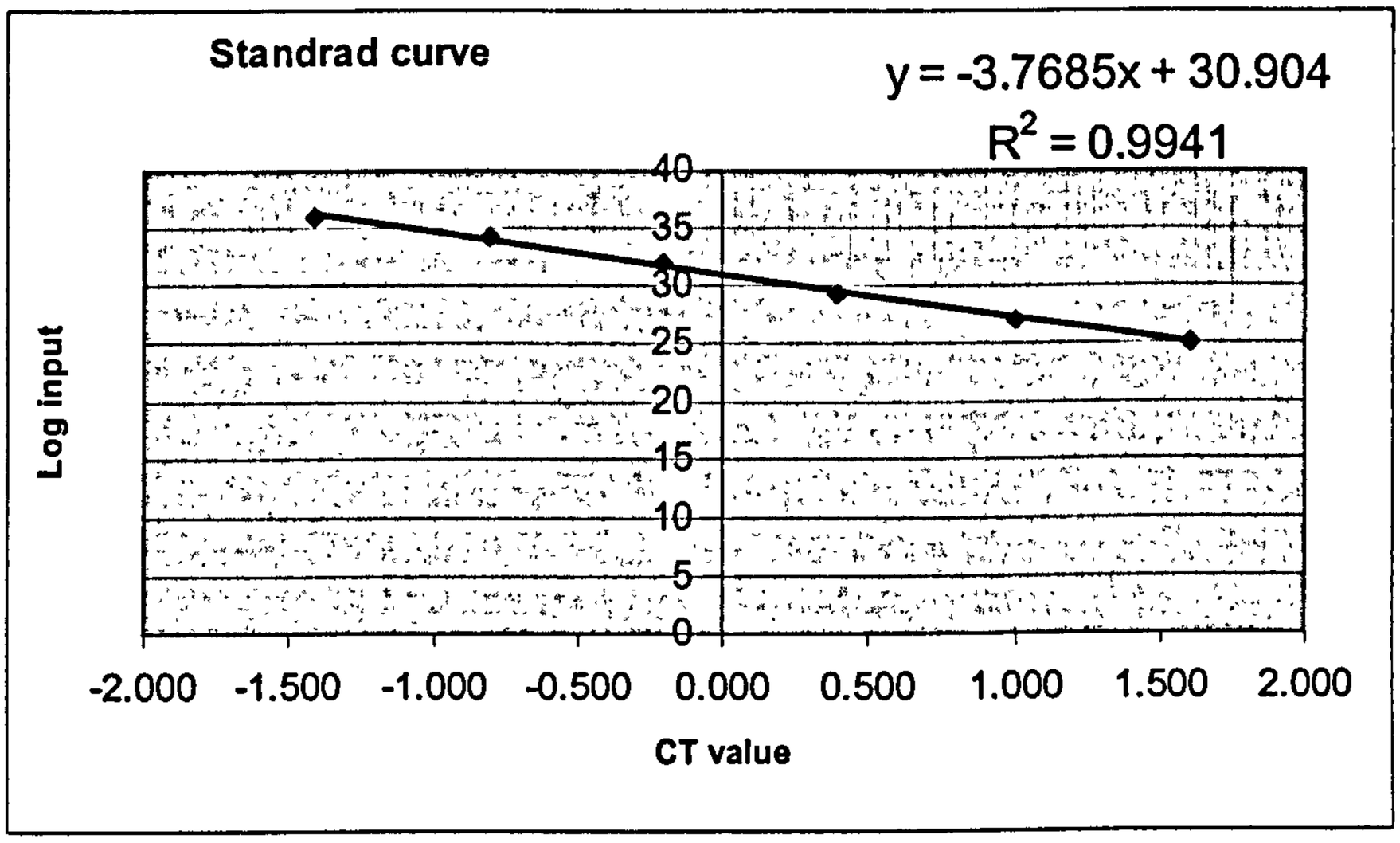


Fig 2.2b Established general standard for OPG, RANKL and/or GAPDH primers. Six dilutions for each individual experiment were used.

2.9 Osteoclast-like cell formation in vitro

2.9.1 Extracellular matrix (ECM) isolation

Solutions:

Hypotonic buffer: 5 mM Tris-Base, 0.5 mg/ml BSA, 0.1 mM $CaCl_2$.

Hypotonic buffer with Igepal: hypotonic solution with 0.5% Igepal (Sigma).

Method:

MG-63 cells (2.8 x 10^4 cells/well) were seeded and grown for 7 days in each well of an 8 well chamber slide. Cells were fed with DMEM/10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin every 3 days. After 7 days, MG-63 cells had deposited an extracellular matrix. Medium was replenished 24 hr before cells were removed to leave the ECM in the culture wells. The cell layer was washed twice with DPBS and cells were removed by incubation at 37°C with hypotonic buffer for 8 min followed by

hypotonic buffer with Igepal for 3 min at 37°C. The remaining ECM was washed once in DPBS containing 10% FCS followed by two washes in DPBS alone. The chamber slides were stored at –20°C until required. Before use, slides were washed twice in DPBS and once in DMEM medium.

2.9.2 Isolation Peripheral Blood Mononuclear cells

Preparation:

Human peripheral blood was collected from healthy normal donors in syringes and immediately transfered to a universal tube containing 1,000 U/ml of heparin solution and inverted 3-4 times to mix. The Histopaqe gradient 1077 (Sigma) was removed from the fridge and allowed to warm to room temperature. Histopaque (15 ml) was pipetted on to a filter disc in a 50 ml leucosep tube. The leucosep tube (s) were centrifuged at 1100 rpm (200xg) for 5 min to allow the histopaque to travel through the filter. Heparinised blood was then diluted (1:1) with DPBS and gently pipetted in to the filter tube; only 30 ml of blood was used in each leucosep tube. Immediately, the gradient was then centrifuged at 1500 rpm (272xg) for 30 min. After centrifugation, the mononuclear cell layer is visible as a white fluffy layer sandwiched between a clear histopaque and yellow plasma layer interface. The cells were harvested from the leucosep tube using a 3 ml plastic Pasteur pipette, using a gentle swirling motion to remove the cell layer. Then the cells were pipette into a 30 ml universal and centrifuged at (327g) 1800 rpm for 5 min. The cells were washed by resuspended the pellet in 20 ml of Dulbecco's PBS and centrifuged at (200g) 1100 rpm for 1-2 min, the cells were resuspended in 10 ml of media RPMI 1640 with 20% FCS. The numbers of cells in the resulting suspension of peripheral blood mononuclear cells (PBMCs) were counted using a haemocytometer.

2.9.2.1 Cultured of peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were cultured in the prepared 8-well chamber slide. Cells were seeded at 5 x 10^5 cells/well for 24 hr in medium/FCS (20% vol/vol). Then fresh untreated medium was added as –ve control, and to the treated wells; medium/FCS with IL-17 at different

concentrations and RANKL and M-CSF were added as a positive control. The cultures were maintained for 21 days; culture medium was replenished every three days with fresh medium supplemented with the agents as described above.

2.9.2.2 Preparation of Peripheral Blood/Synovial Fibroblast Cell Cocultures

PBMCs cells (5×10^5) were cultured in the prepared 8-well chambered slide for 24 hr in medium/FCS (20% vol/vol). Then 2×10^4 SFB cells were added to each well and incubated overnight with untreated medium/FCS. Then the cultures were replenished with fresh untreated medium as -ve control, medium/FCS with a low and a high concentration of IL-17, RANKL, M-CSF, and OPG were added. The cultures were maintained for 21 days, during which time the culture medium and added factors (including cytokines/growth factors) were replenished every 3 days.

2.9.2.3 Culture of PBMCs on ivory slices

Elephant ivory (a kind gift of HM customs and Excise, Heathrow Airport, London, UK) was cut into small pieces, then into a thin slices (0.6 mm thick) of (0.8 cm) diameter is with an Isomet 2000 (BUEHLER DIAL Engineering UK) using a slow speed diamond-edged wafering blade. These slices were washed and sterilized with 70% ethanol then placed in 96 or 48 well tissue-culture plates. PBMCs (5×10^5 in 96 wells) plates and (1×10^6 in 48 wells) plates were seeded in each well and cultured on ivory slices for (2 hr - 4hr). The ivory slices were then washed in culture medium to remove the nonadherent cells and debris. Then the culture media/FCS 20% and medium containing OPG were used as a negative control, medium/FCS with RANKL + M-CSF as a positive control, a low and a high concentration of IL-17, IL-17 + M-CSF at different concentrations and M-CSF were added. The experiments were performed with five replicates. The cultures were maintained for 21 days, during which time the medium and added factors (including cytokines/growth factors) were replenished every 3 days.

2.9.2.4 PBMCs/Synovial Fibroblast co-cultures on ivory slices

Ivory slices were placed in (96 or 48) well tissue-culture plates. Then PBMCs (5×10^5 – 1×10^6) were added to each well and cultured on ivory slices for (2 hr – 4 hr). The wells were then washed in medium/FCS 20% to remove nonadherent cells and debris. SFB cells (2×10^4) were then added and incubated overnight, the fresh medium/FCS with OPG was added as a negative control, medium/FCS with RANKL + M-CSF as a positive control, IL-17, IL-17 + M-CSF and M-CSF were added in 5 wells to each treatment. The cultures were maintained for 21 days, during that time the medium and added factors (including cytokines/growth factors) were replaced every 3 days.

2.9.3 Cytochemical and Functionl Assessment of osteoclast Formation

Solutions:

4% paraformaldehyde in DBPS.

For TRAP, a commercially available kit (Sigma, UK) was used. 4'-6-Diamidino-2-phenylindole (DAPI) (10 µg/ml) stock solution in dH_2O stored in dark at -20°C, and Triton X-100 (T-ocytlphenoxypoly-ethoxyethanol) were obtained from Sigma.

Methods:

After culture for an indicated period, cells were fixed in 4% paraformaldehyde solution for 10 min then washed twice in DBPS. After staining for acid phosphatase, naphthol AS-BI phosphate is used as a substrate, in the presence of 1.0 mol/l tartrate. The product then reacts with Fast Garnet GBC salt. TRAP-positive cells containing more than three nuclei were counted as OCLs as shown in Fig 2.3.

DAPI was used to visualize the DNA in our experiment and therefore to determine the number of nuclei in each OCL (Fig 2.3). For DAPI binding the slides were washed two times with PBS for 5 min each time then DAPI (10 µg/ml) with 0.1% Triton x-100 in PBS was added for 30 min. After incubation the slides were washed three times in PBS. The images were visualized with an incidence fluorescent microscope.

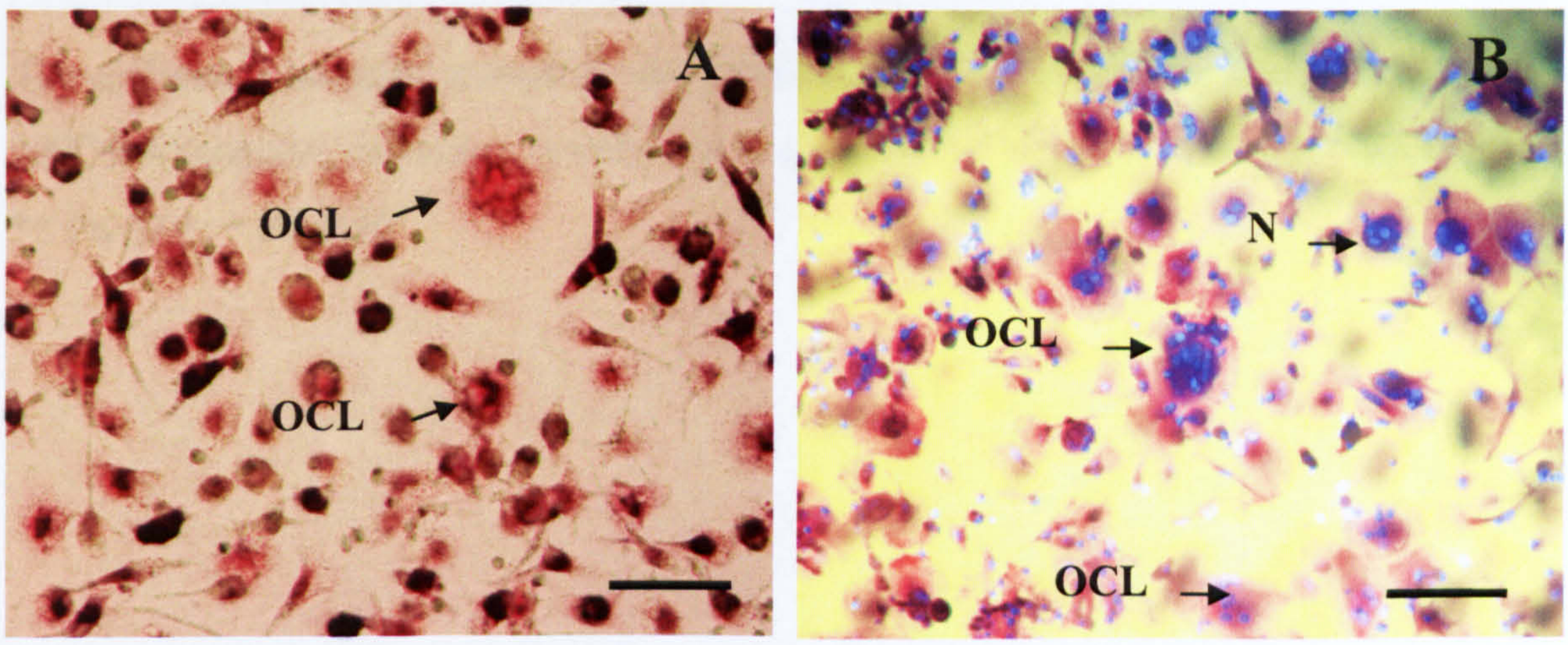


Fig 2.3 shows the multinucleated cells stained for TRAP with or without DAPI. **A** is the PBMC treated with RANKL and M-CSF (30 + 25 ng/ml) as a positive control stained only for TRAP-activity. **B** The positive control is stained for TRAP and DAPI to make clear that the cells which were counted have three or more nuclei. The bar is 50 μm. Osteoclast-like (OCL) and Nuclei (N).

Functional assessment of osteoclast formation was determined at the end of the co-culture period using a resorption assay. Cells were stained for TRAP activity. The cells were then removed from the ivory slices by immersing them in 10% (vol/vol) sodium hypochlorite in distilled water for 30 min at room temperature. Ivory slices were washed in distilled water, and briefly sonicated, to completely remove all cells. This permitted examination of the ivory surface for evidence of lacunar resorption (Louise, E. et al. 1996). The slices were stained with 0.5% (w/v) toluidine blue for 3 min, air dried, and examined by reflected light microscopy. The surface of each slice was examined for evidence of lacunar resorption using a Metallurgical microscope (Axiovert 100A ZEISS Germany). The extent of resorption was determined by measuring the area of discrete pits counted on each slice. Resorption pits were observed as either individual small pits or large multilocular areas.

2.10 Statistical analysis

The difference between sample group means was tested for statistical significance by ANOVA (analysis of variance) with Tukey and Duncan post test, where *** P≤ 0.001; ** P≤ 0.01; * P≤ 0.05

CHAPTER-3

RESULTS

3 The effect of IL-17 on the expression and production of OPG by synovial fibroblasts and osteoblast-like cells

3.1 Introduction

RA is a chronic inflammatory joint disease, and bone erosion is a major complication (Firestein GS. et al., 1990; Chen E. et al., 1993). In areas of pannus infiltration, erosion of cartilage and subchondral bone is common, leading to characteristic marginal erosions seen radiographically in this disease. The area of contact between pannus and cartilage/bone represents an erosive front, and the role of osteoclasts in the erosion of bone in arthritis has been recognized (Bromley M. and Wooley DE. 1984; Suzuki Y. et al., 1998). IL-1, IL-6 and TNF-α are proinflammatory cytokines that mediate the continuation of synovitis and cartilage/bone destruction and are produced in increased quantities by RA synovium and detected in synovial fluid (Arend WP et al., 1998; Feldmann M. et al., 1997). The important role of these cytokines on osteoclastic recruitment, proliferation and differentiation has been described (Pfeilschiffer J. et al., 1989; De la Mata J. et al. 1995). Furthermore, the potency of IL-17 as a stimulator of osteoclastogenesis has been shown in vitro (Kotake S. et al. 1999), and this T-cell–derived cytokine was found in the synovium of patients with RA (Kotake S. et al. 1999; Chabaud M. et al. 1999). OPG inhibits osteoclast formation in a co-culture system of osteoblasts and bone marrow cells, pit formation by osteoclasts in vitro, and bone resorption in organ culture of fetal mouse long bones (Kwon BS. et al., 1998; Simonet, WS. et al., 1997; Okada T. et al., 2003).

The importance of OPG in normal bone physiology has been shown in OPG deficient mice produced by targeted disruption of the gene (Mizuno A. et al., 1998, Bucay N. et al., 1998). OPG knockout (OPG -/-) mice were viable and fertile; they exhibited severe osteoporosis marked by excessive bone resorption caused by enhanced osteoclast formation and function. The strength and mineral density of their bones were decreased dramatically (Mizuno A. et al., 1998, Bucay N. et al., 1998). Bone histomorphometric analysis showed that the osteoblast surface as well as the osteoclast surface was increased in OPG-deficient mice (Bucay N. et al., 1998). OPG (-/-) mice also developed medial calcification of the aorta and renal arteries (Bucay N. et al., 1998). These results indicate that OPG is a physiological regulator of osteoclast-mediated bone

resorption during postnatal bone growth. In vivo administration of OPG results in an increase in bone mineral density and bone volume associated with a decrease of active osteoclast number in normal and ovariectomized rats. Serum Ca_{+2} concentrations were also decreased by injecting OPG into rats (Morony S et al., 1999; Tomoyasu A. et al., 1998; Yamamoto M. et al., 1998). In addition OPG administration has been shown to be anti-resorptive in postmenopausal women and in patients with lytic bone metastases (Kong YY and J. M. Penninger. 2000).

Surface plasma resonance experiments revealed that RANK, RANKL and OPG are able to form a tertiary complex; suggesting that OPG is not only a soluble decoy receptor for RANKL but also may have direct effects on osteoclast functions (Theoleyre S. et al., 2004). In addition, RANK and RANKL expression are inhibited from the murine monocyte cells RAW-264.7 stimulated with OPG while MMP-9 and Cathepsin K mRNA expression were not modulated. This suggests that some of the OPG effects are RANKL-dependent (Wittrant Y. et al., 2004).

For instance, a recent study reported that estrogen appears to inhibit the effects of RANKL by enhancement of the production of OPG (Aubin JE. and Bonnelye E. 2000). In addition, RANKL secreted by T cells can induce osteoclastogenesis and this mechanism is enhanced by cytokines such as TNFα, IL-1 and IL-17. Conversely, this system is blocked by OPG, IL-4 and IL-10, which inhibit both inflammation and osteoclastogenesis (Saidenberg Kermanac'h N et al., 2002). This information may explain part of the abnormal phenomena in diseases such as RA characterized by both inflammation and destruction. Activated T cells within the rheumatoid synovium express RANKL. Cells within the synovium are capable of differentiating to osteoclast-like cells under some conditions, particularly when supported by M-CSF and RANKL. This suggests that bone erosion seen in RA might result from the RANKL/RANK system through activated T cells. This opens up the possibility that OPG may have therapeutic effects in RA (Saidenberg et al., 2002). The new understanding provided by the RANK/RANKL/OPG paradigm for both differentiation and activation of osteoclasts has had tremendous impact on the field of bone biology and has opened new avenues for development of possible treatments of diseases characterized by excessive bone resorption.

3.1.1 The aims of this chapter are to:

- Determine the effects of IL-17 and OSM on the expression of OPG by SFB derived from patients with RA.

- Establish the effects of IL-17 and OSM on the expression and production of OPG by SFB derived from patients with OA.

- Establish the effects of IL-17 and OSM on the expression and the production of OPG by osteoblast-like (human osteosarcoma) MG-63 cells.

- Demonstrate the effects of IL-17 and OSM on the expression and the production of OPG by osteoblast-like (human osteosarcoma) SaOS2 cells.

3.2 Materials and Methods

SaOS-2, MG-63 cells and human fibroblasts were cultured and stimulated as outlined in (2.2.1 and 2.2.2). After cytokine treatment, gene expression was assessed by analysis of mRNA levels as mentioned in section (2.3) and reverse transcribed into complementary DNA (cDNA) section (2.4.1 and 2.4.2).

Complementary DNA was amplified by PCR using primer pairs to generate products corresponding to specific gene products as listed in Table 2-1 section (2.4.3). Primers were designed against human sequences for OPG, GAPDH. PCR was performed for 35 cycles for OPG and GAPDH. Amplification products were identified by electrophresis on a 1% (w/v) agarose gel as mentioned defore in chapter-2 section (2.3.4)

Furthermore gene expression was assessed by Northern blot analysis. Agarose gel were prepared as mentioned in (2.6.1). RNA was electrophoresed in equal amounts. OPG and GAPDH probes were labelled as reported in section (2.6.3). In addition OPG production was assessed by Western Blot analysis; equal amounts of proteins were used for Western blot and screened for OPG as in section (2.7.2.2).

For real time PCR, the OPG cDNA sequence was used to design the forward and reverse primers, spanning intron/exon boundaries to prevent the amplification of genomic DNA that may be contained in the samples. The primers for OPG had a calculated annealing temperature of 65°C. For OPG, forward and reverse primers created an amplicon of 108bp. The primers for GAPDH had a calculated annealing

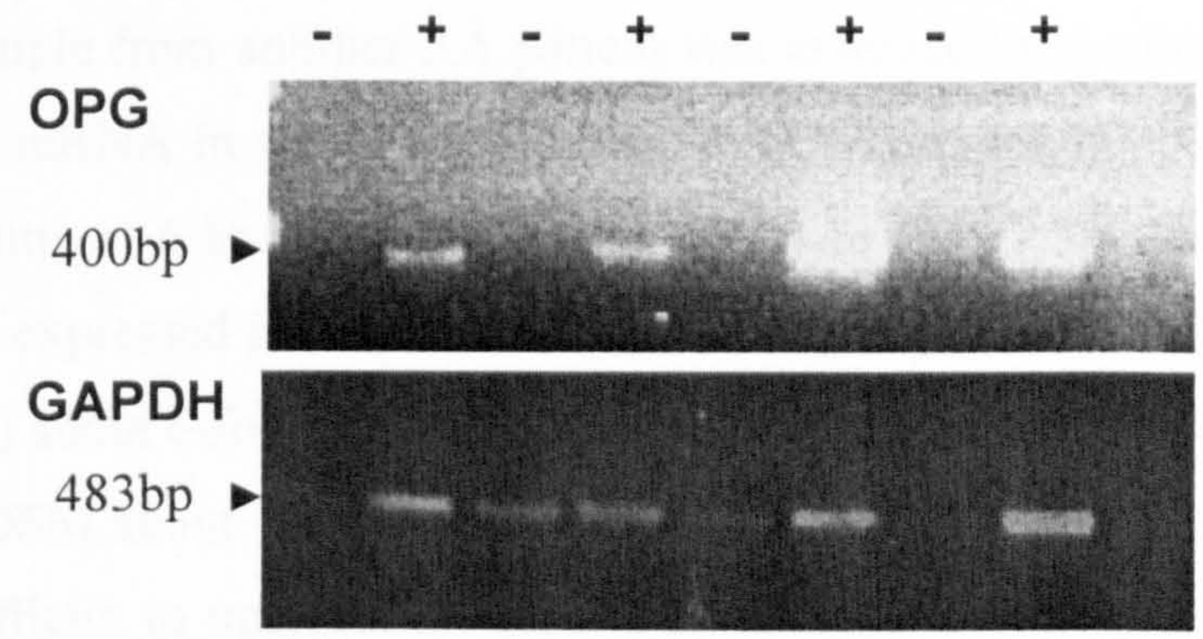
temperature of 60°C. The Taq-man GAPDH control reagents primers and the probe were used from R&D (part number 402869) as in section (2.8.2). In the elative standard curve method, standard curves with known amounts of total cDNA (relatively) are analysed for the gene of interest OPG. For each unknown experiment, the relative amount is calculated by division of the OPG value with GAPDH value as in section (2.8.3). The reverse transcription was performed in 20 µl reactions as mentioned in section (2.8.5). Then the cDNA was diluted 5 times for real time and 2 µl of template was used as for each reaction.

PCR amplification was carried out in a 20 µl volume with 2 µl of LightCycler DNA Mastermix as in section (2.8.5). All templates were amplified following LightCycler protocol. The fluorescence emitted by the reporter dye was detected online in real-time, and the threshold cycles (C_T) of each sample together with the standard curve samples, were recorded as a quantitative measure of the amount of mRNA product in the original sample.

3.3 Results

3.3.1 The effects of IL-17 and OSM on the expression of OPG by SFB derived from patients with RA.

To study the effects of IL-17 and OSM on the expression of OPG by synovial cells in vitro. SFB cells were examined using tissue obtained at joint replacement surgery to assess the ability of SFB from RA to express and produce OPG mRNA. In this experiment no DNase treatment was used as a result the cDNA in some samples was contaminated with the genomic DNA. However, The results from this experiment showed that there was no increase in the expression of OPG in response to IL-17 (5 and 50 ng/ml) at 6 hr but there was increase in OPG mRNA expression following OSM (10 ng/ml) treatment as shown in (Fig 3.1). These results were later confirmed by Northern blot analysis using the same mRNA (Fig 3.2).



IL-17	**5**	**50**	**-**	**-**
OSM	**-**	**-**	**10**	**-**

6 hr

Fig 3.1 The expression of OPG mRNA by human RA SFB. SFB (RA) were cultured, serum reduced to 0.5% FCS overnight and stimulated for 6 hr with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). RNA was extracted using trizol reagent. Total RNA was analyzed by RT-PCR with specific primers using the conditions described in Materials and Methods. PCR was performed for 35 cycles. The PCR products were run on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. (+) is cDNA and (-) is negative control containing untranscribted total RNA.

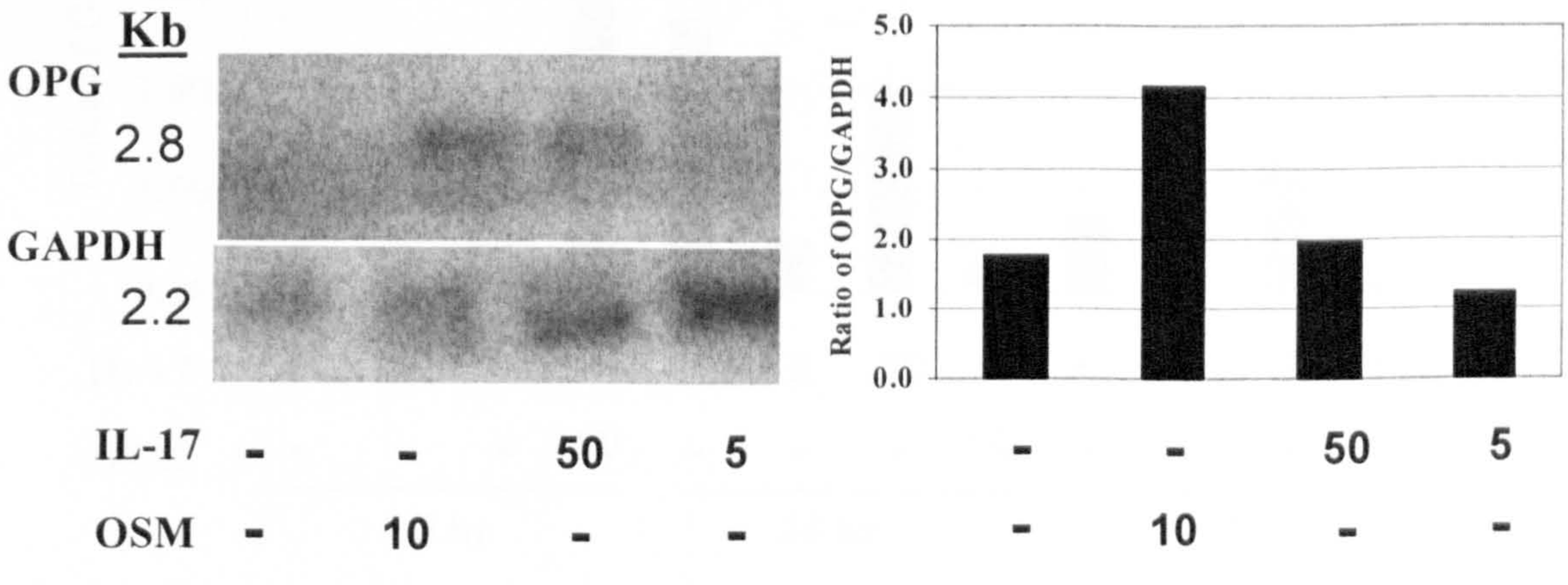


Fig 3.2 The expression of OPG mRNA by human RA SFB. SFB (RA) cells were cultured, serum reduced to 0.5% FCS overnight and stimulated for 6 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). RNA was extracted using trizol reagent. Equal amounts of RNA were electrophoresed using 1% agarose/0.4M Formaldehyde and Northern analysis performed. GAPDH was used to normalise for RNA loading.

A second sample from another RA patient was assessed by quantitative PCR (real time PCR). OPG mRNA in these cells appears to be expressed in a biphasic way with the cells responding at 6 hr and 48 hr but not at 24 hr (Fig 3.3). However, there was no OPG mRNA expressed in response to IL-17 (5 ng/ml) but levels were enhanced by IL-17 (50 ng/ml) about 6-fold at 6 hr while the expression of OPG mRNA was increased in response to OSM about 19-fold compared to the control. The control is very high at 24 hr so it is difficult to interpret the results at this time point. In addition, there was no increased expression of OPG mRNA by IL-17 (5 ng/ml) while the expression was increased by IL-17 (50 ng/ml) about 1.5-fold at 48 hr. There was a decrease in expression of OPG mRNA level in response to OSM at 24 hr and 48 hr compared to the controls.

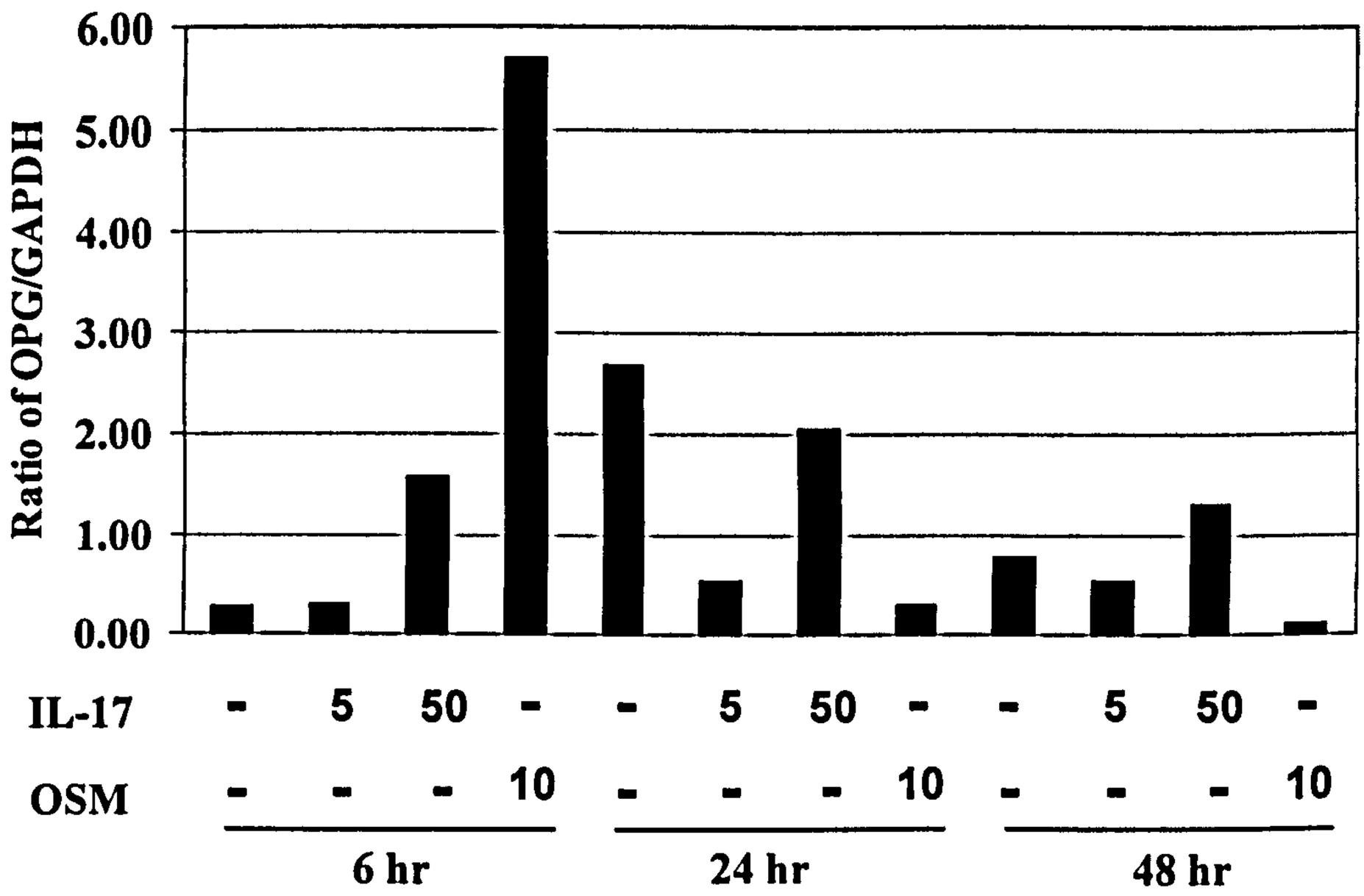


Fig 3.3 The expression of OPG mRNA by human RA synovial fibroblast. SFB (RA) cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). Total RNA was analyzed by RT and Quantitative PCR analysis with OPG primers using the conditions described in Materials and Methods.

3.3.2 The effects of IL-17 and OSM on the expression and production of OPG mRNA from SFB cultured from patient with OA.

3.3.2.1 OPG mRNA expression by SFB (OA).

A sample from a patient with OA was assessed by conventional PCR. The results show that there was no expression of OPG mRNA from cells stimulated with IL-17 (5 and 50 ng/ml) but there was a very low level of OPG mRNA expressed by cells treated with OSM (10 ng/ml) at 6 hr compared with the control. OPG mRNA expression was dose dependently enhanced in the cells stimulated with IL-17 and the expression of OPG mRNA was increased in cells treated with OSM compared to untreated cells at 24 hr. At 48 hr OPG mRNA expression was still increased by IL-17 treatment, but apparent levels were higher in cells treated with IL-17 (5 ng/ml) than cells treated with IL-17 (50 ng/ml). The expression of OPG mRNA from cells stimulated with OSM was enhanced at 48 hr but less than the cells treated with OSM at the 24 hr time point compared to the control as shown in Fig 3.4.

However, another two samples from patients with OA were examined by Northern blot analysis and the pattern of both samples shows that OPG mRNA is expressed in response to IL-17 (5 and 50 ng/ml) at a very low level at 6 hr and the expression is slightly more elevated by IL-17 (5 ng/ml) than by IL-17 (50 ng/ml) at 6 hr. The expression of OPG mRNA was low but slightly elevated by IL-17, dose dependently at 24 hr. In general, the expression of this gene was higher at 6 hr compared to 24 hr. There was expression of the gene following OSM treatment at 6 hr and 24 hr compared to the control. OPG mRNA was expressed in response to IL-17 dose dependently and also upon treatment with OSM at 48 hr (Fig 3.5). In addition, the second sample showed that the gene was expressed in response to IL-17 (5ng/ml) at 6 hr and to IL-17 (50 ng/ml) at 24 hr at low levels while the gene was expressed at high levels in response to IL-17 dose dependently at 48 hr (Fig 3.6). However, the general pattern of these results from conventional PCR and Northern blot analysis showed that expression of this gene was elevated in response to IL-17 at 48 hr.

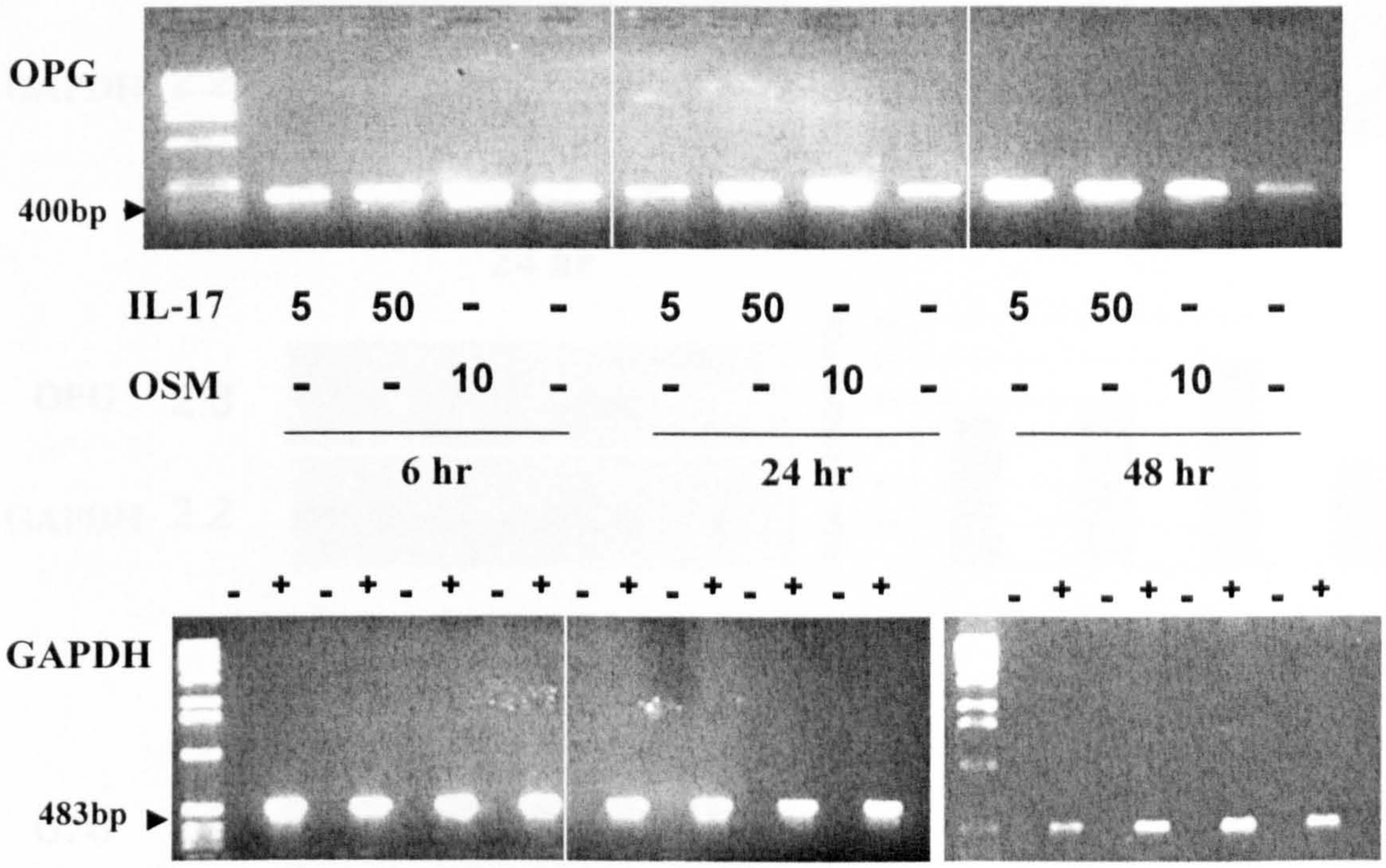
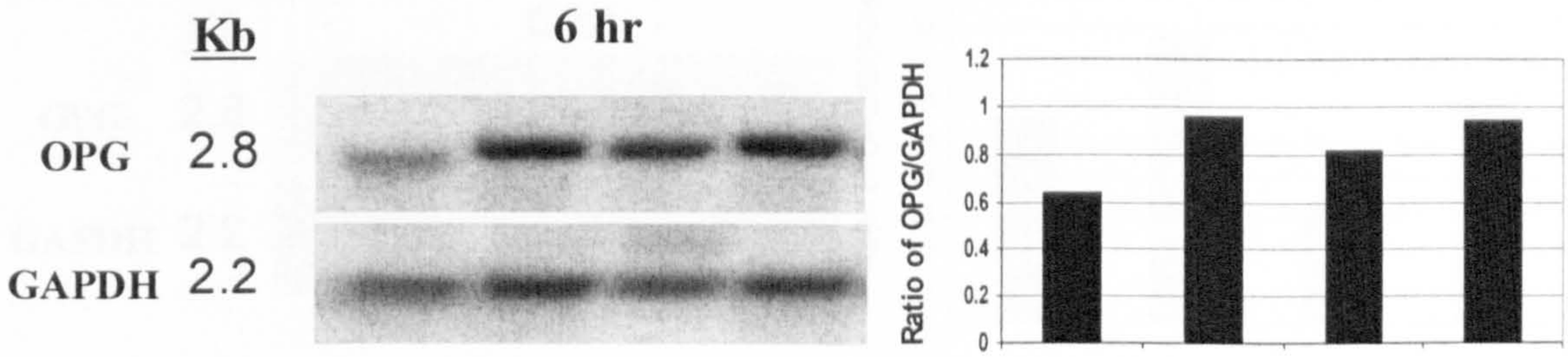


Fig 3.4 The expression of OPG mRNA by synovial fibroblasts cells. SFB from an osteoarthritis patient were cultured and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). RNA was extracted by using trizol reagent. Total RNA was analyzed by RT-PCR with OPG primers using the conditions described in Materials and Methods. PCR was performed for 35 cycles. The PCR products were run on 1% agarose gel, stained with ethidium bromide and visualized under UV light. (+) is cDNA and (-) is negative control containing untranscribted total RNA.



IL-17	-	5	50	-		-	5	50	-
OSM	-	-	-	10		-	-	-	10

Fig 3.5 The expression of OPG mRNA by human OA SFB. SFBs cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). RNA was extracted using trizol reagent. Equal amounts of RNA were electrophoresed using 1% agarose/0.4M Formalehyde and Northern analysis performed. GAPDH was used to normalise for RNA loading.

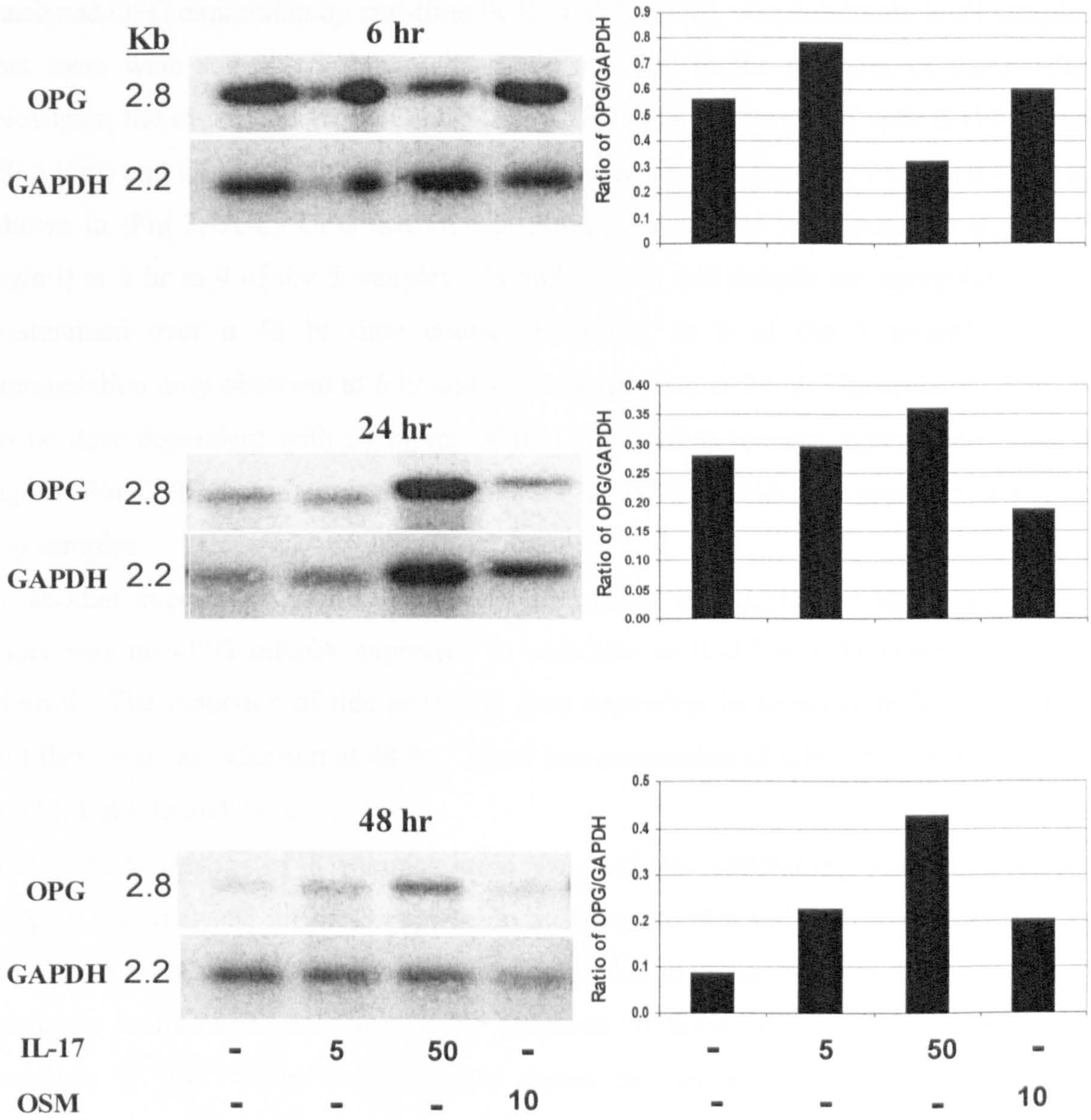


Fig 3.6 The expression of OPG mRNA by synovial fibroblasts from an OA patient. SFBs cells were cultured, serum starved overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). Total RNA was harvested Equal amounts of RNA were electrophoresed using 1% agarose/0.4M Formalehyde and Northern analysis performed. GAPDH was used to normalise for RNA loading.

I have used four samples from OA patients undergoing joint replacement surgery and analysed OPG expression by real-time PCR. OPG mRNA was detectable in all samples but there were some variation in its expression and in the response of these cells. However, the expression of OPG mRNA by SFB cells is stimulated with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) compared to untreated cells for 6 hr, 24 hr and 48 hr as shown in (Fig 3.7A-C) OPG mRNA was strongly expressed in response to IL-17 (50 ng/ml) at 6 hr in 4 of the 5 samples. In addition, in one sample the upregulation was maintained over a 48 hr time course. However, in 3 of the 5 patient samples upregulation only observed at 6 hr and was not apparent at 24 hr. Upregulation appears to be dose dependent with 50 ng/ml of IL-17 providing stronger upregulation than 5 ng/ml. For OSM stimulation, no upregulation of OPG mRNA was apparent in any of the samples.

In another experiment with OA synovial fibroblasts (Fig 3.7D) the results show that there was no OPG mRNA expressed in response to IL-17 at 6 hr compared to the control. The induction of this gene was dose dependent in response to IL-17 at 24 hr but there was no induction at 48 hr. There was expression of OPG mRNA in response to OSM at 6 hr and 24 hr.

The general pattern of 8 samples from OA patients undergoing joint replacement surgery and analysed for OPG expression by Northern blot and real-time PCR analysis are shown in Table 3.1. OPG mRNA was detectable in all samples but there were some variation in its expression and in the response of these cells. OPG expression in response to IL-17 from these samples shows that in some of them OPG mRNA expression was up-regulated at 6 hr and in the other samples the expression enhanced at 48 hr.

3.3.2.2 OPG production from SFB (OA)

The production of OPG from (OA) synovial fibroblasts was studied by Western blot analysis. These experiments showed that the pattern of OPG production was increased by IL-17 (50ng/ml) and OSM in the conditioned medium of both samples at 6 hr. OPG production was increased in the first sample by IL-17 (5 ng/ml) and in the second sample by IL-17 (50 ng/ml) and OSM at 24 hr compared with the control. While OPG production was increased by IL-17 (5 and 50 ng/ml) in the first sample and by only IL-

17 (50 ng/ml) at 48 hr and the production of OPG was decreased in response to OSM with increase in time (Fig 3.8).

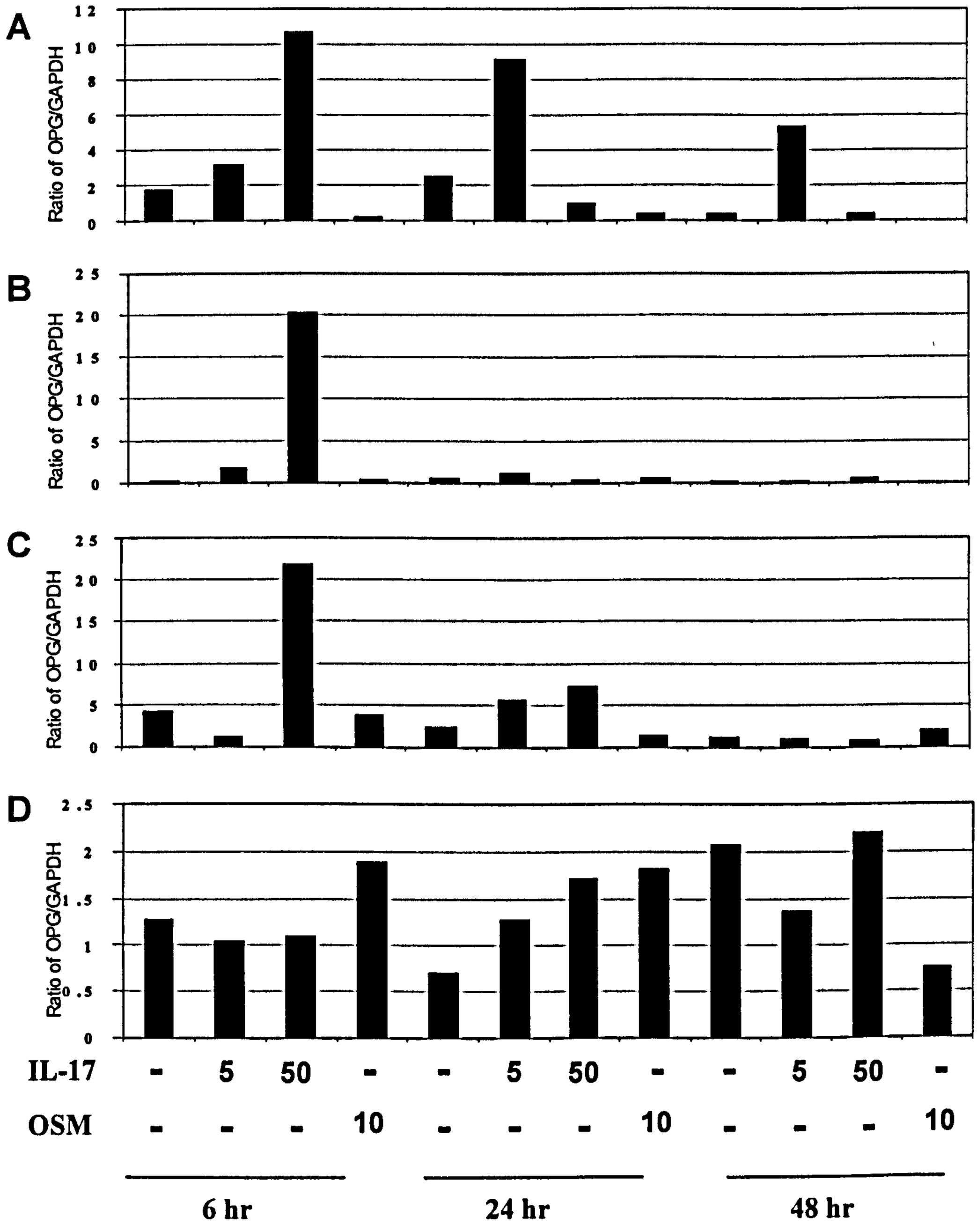


Fig 3.7 The expression of OPG mRNA by human synovial fibroblast from OA patient. SFB cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and compared to untreated cells. Total RNA was analyzed by Quantitative PCR analysis with OPG primers using the conditions described in Materials and Methods for four independent samples (**A, B, C** and **D**).

SAMPLE NO	6 HR				24 HR				48 HR				ANALYSIS
	Cont	IL-17 5ng/ml	IL-17 50ng/ml	OSM 10ng/ml	Cont	IL-17 5ng/ml	IL-17 50ng/ml	OSM 10ng/ml	Cont	IL-17 5ng/ml	IL-17 50ng/ml	OSM 10ng/ml	
OA1	1	1.5	1.33	1.50	0.60	0.68	0.83	0.58	0.42	1.67	1.92	1.83	N
OA2	1	1.38	0.86	0.52	0.48	0.48	0.66	0.33	0.10	0.36	0.69	0.35	N
OA3	–	–	–	–	1	1.4	1.2	0.8	–	–	–	–	N
OA4	1	5.2	3.2	4.8	–	–	–	–	1.8	3	3.52	6	N
OA5	1	1.6	5.5	0.25	1.4	5	0.5	0.25	0.25	2.75	0.25	0.25	QRT
OA6	1	8	82	2	2	4	2	2	1	1	2	1	QRT
OA7	1	0.5	5.63	0.75	0.5	1.5	1.88	0.25	0.23	0.23	0.2	0.5	QRT
OA8	1	0.85	0.88	1.38	0.58	0.96	1.35	1.38	1.62	1.38	1.73	1.35	QRT

Table 3-1 The effect of IL-17 (5 and 50 ng/ml) and (OSM 10 ng/ml) on the expression of OPG mRNA by synovial tissue. Cells from osteoarthritis patients using Northern blot analysis (N) and Quantitative real time PCR analysis (QRT). The results were normalised to control at 6 hr to allow for comparison, and the inductions are shown in fold compared to the control.

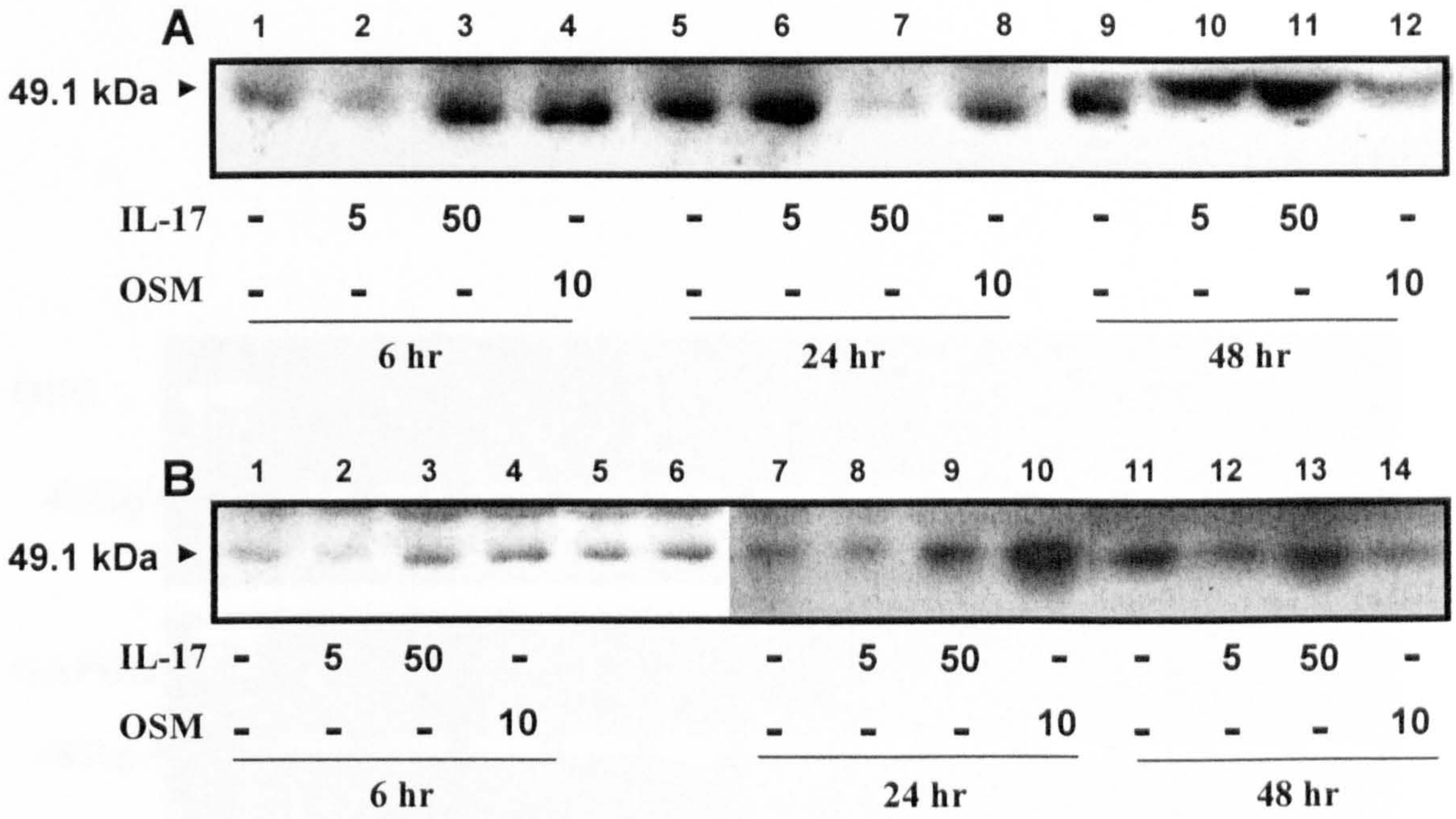


Fig 3.8 The OPG production by huma synovial fibroblasts (SFB). SFB cells were cultured, serum-reduced to 0.5% overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). The conditioned media were collected for Western blot analysis. (**A**) Lanes 1-8 were run on a separate gel. (**B**) Lanes 1-6 were run on a separate gel and samples 5 and 7, and 6 and 8 are duplicate samples. These data were representative of two independent experiments.

3.3.3 The effects of IL-17 on the expression and production of OPG by human osteosarcoma cell line, MG-63

3.3.3.1 OPG mRNA expression by MG-63

No increase in OPG mRNA expression was seen in MG-63 cells stimulated with IL-17 (5 and 50 ng/ml) and with OSM (10 ng/ml) at 6 hr compared to the untreated cells. While the expression of OPG mRNA was increased by IL-17 and OSM at 24 hr and 48 hr compared to the control, there was no clear difference between the different doses of IL-17 (Fig 3.9). Northern analysis was performed and representative results are shown in Fig 3.10. OPG mRNA levels are not significantly different between treatments at 6 hr and 24 hr. There was some evidence of elevated OPG mRNA levels at 48 hr when treated with IL-17 or OSM.

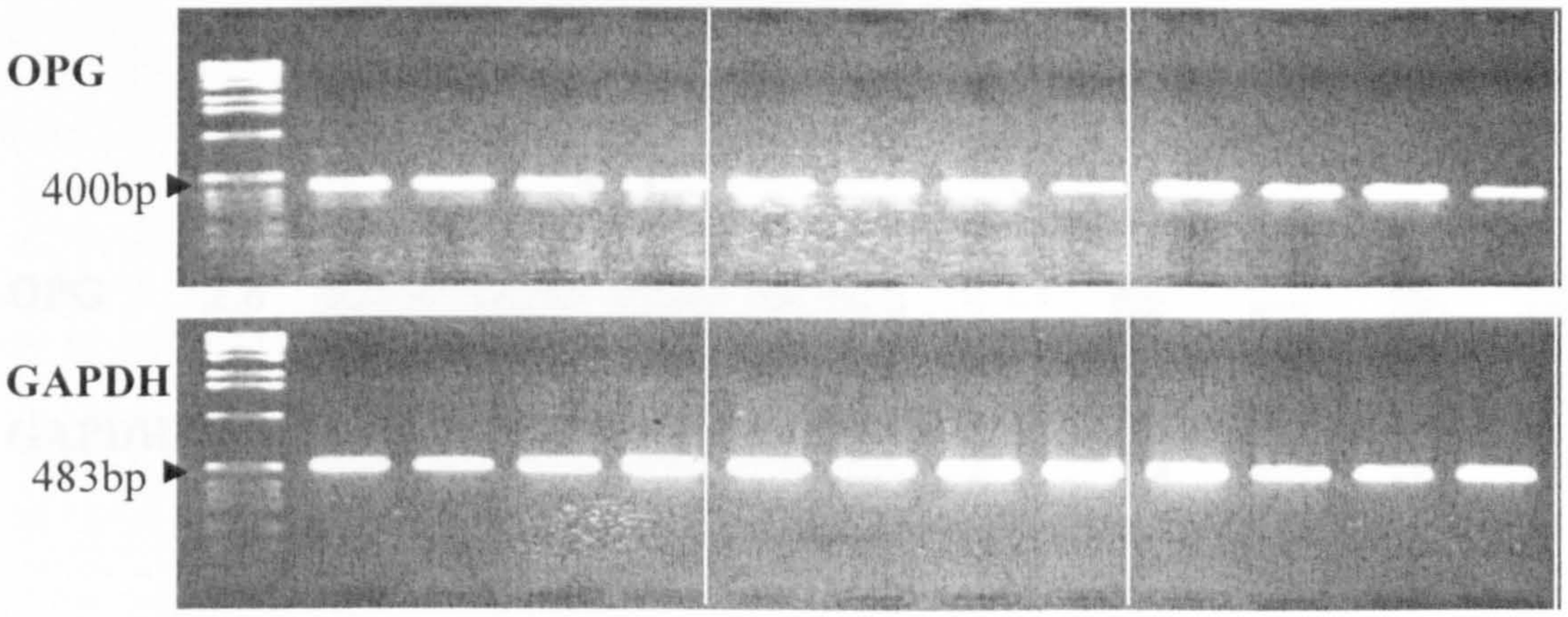


Fig 3.9 The expression of OPG mRNA by conventional PCR by MG-63. MG-63 cells were stimulated with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). The cells were cultured for 6 hr, 24 hr and 48 hr. RNA was extracted by using trizol reagent. Total RNA was analyzed by RT-PCR with OPG primers using the conditions described in Materials and Methods. PCR was performed for 35 cycles. The PCR products were run on 1% agarose gel, stained with ethidium bromide and visualized under UV light. This result is representative of three independent experiments.

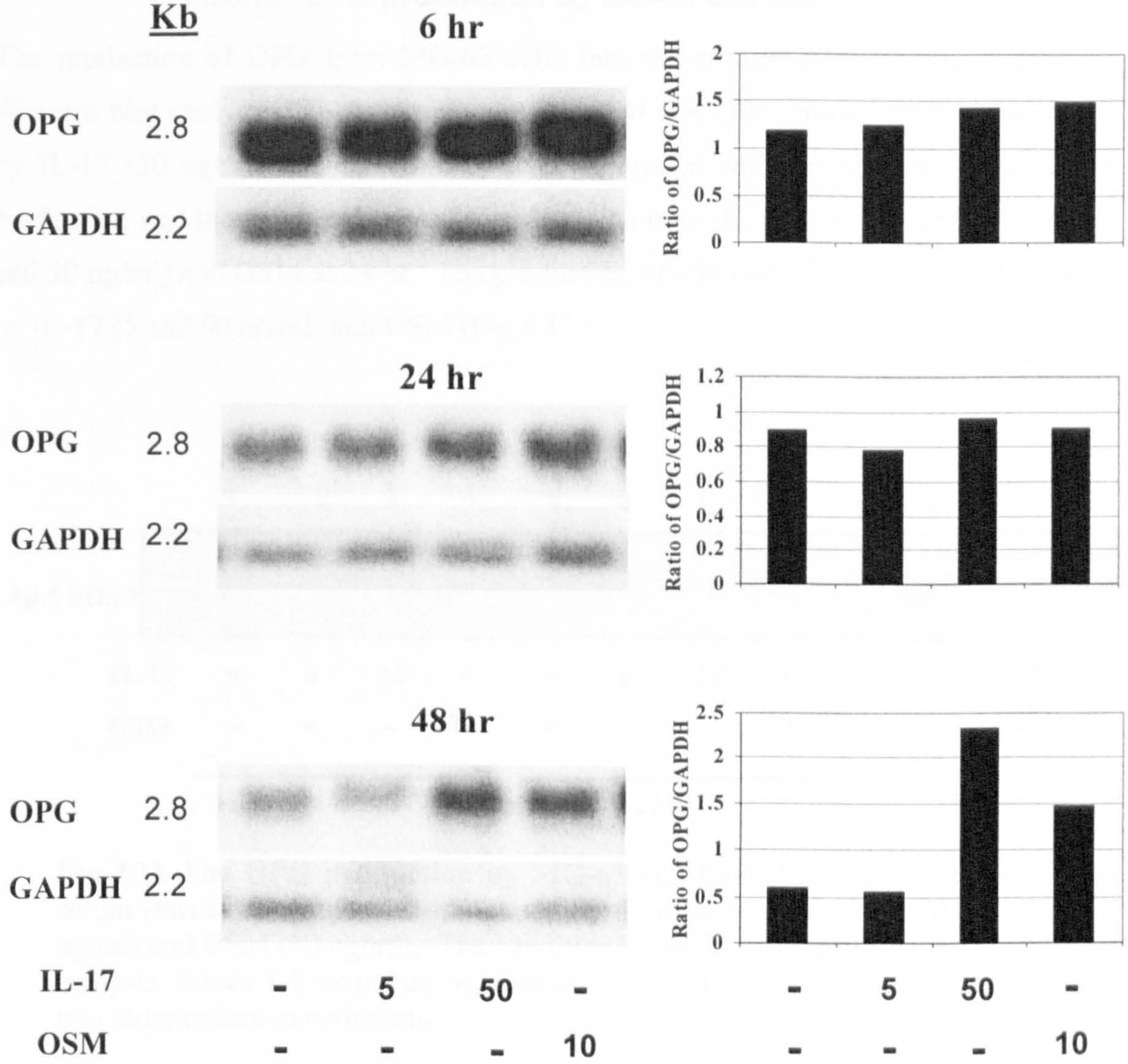


Fig 3.10 The expression of OPG mRNA by Northern blot analysis by MG-63. MG-63 cells were cultured, serum starved overnight and stimulated with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml), the cells were cultured for 6 hr, 24 hr and 48 hr. Total RNA was harvested. Equal amounts of RNA were electrophoresed using 1% agarose/0.4M Formalehyde and Northern analysis performed. GAPDH was used to normalise for RNA loading. This result is representative of three independent experiments.

3.3.3.2 OPG production by MG-63 cell line

The production of OPG from MG-63 cells into the culture medium was studied by Western blot analysis. These experiments showed that OPG production was increased by IL-17 (50 ng/ml) and by OSM at 6 hr compared with the control. Also, OPG production was increased in the conditioned media from the cells stimulated by IL-17 (5 and 50 ng/ml) and OSM at 24 hr. The production of OPG was further detected at 48 hr by IL-17 (5 and 50 ng/ml) and OSM (Fig 3.11).

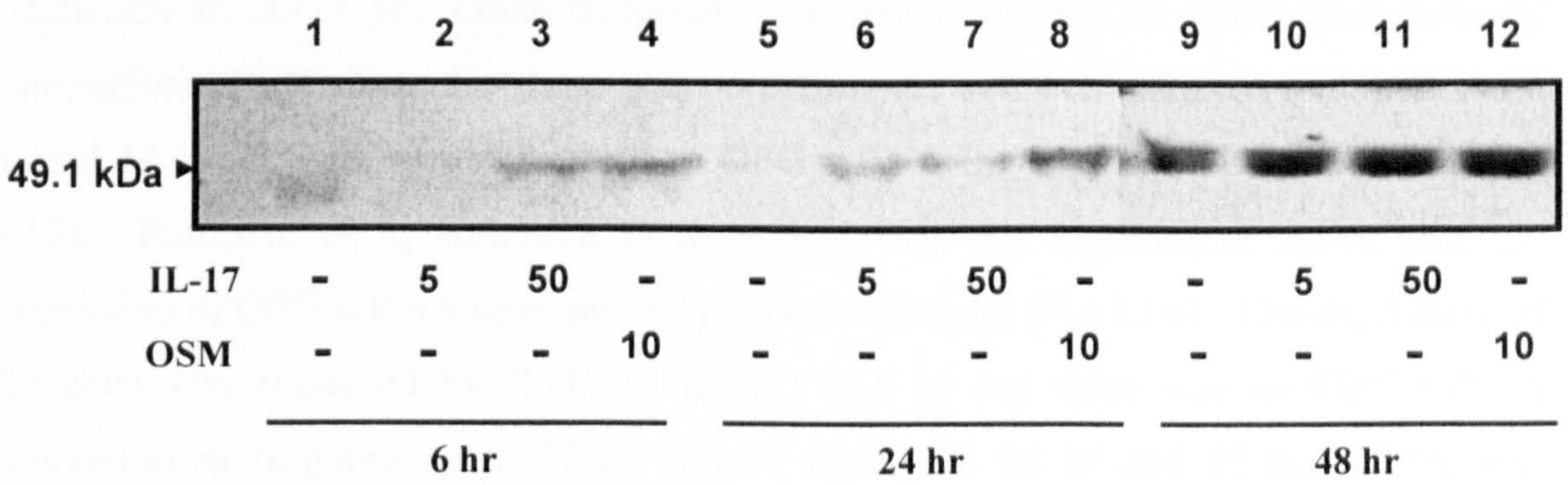


Fig 3.11 The OPG production by MG-63 cell line. MG-63 cells were cultured, serum starved overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). The conditioned media have collected for Western blot analysis. Lanes 1-8 were run on a separate gel. These data were representative of two independent experiments.

3.3.4 The effects of IL-17 on the expression of OPG by SaOS-2 cells.

3.3.4.1 OPG mRNA Expression by SaOS-2

In this study I examined the effects of IL-17 and OSM on the expression of OPG mRNA by human osteosarcoma cells, SaOS-2. The results showed that OPG mRNA was apparently expressed in all the treatments (Fig 3.12). There was no clear difference between the different treatments and the control at each of the different time points. Northern blot analysis was also performed for these experiments (Fig 3.13). These studies show that there was no increase in OPG mRNA expression in cells stimulated with IL-17 (5 and 50 ng/ml) at 6 hr and 24 hr compared to the untreated cells. There

was enhancement in the level of OPG mRNA in an apparent dose dependent response to IL-17 at 48 hr. OPG mRNA was elevated in response to OSM at 6 hr and there was no further regulation of OPG mRNA levels by OSM at 24 hr and 48 hr.

Quantitative RT-PCR was also performed on the same total RNA used in the Northern blot analysis. The results for 24 hr and 48 hr showed that there was no OPG mRNA expressed in response to IL-17 (5 and 50 ng/ml) at 24 hr compared to the control and OPG was enhanced in response to IL-17 (5 and 50 ng/ml) dose dependently at 48 hr compared to control. There was no expression of OPG mRNA in response to OSM at 24 hr and 48 hr (Fig 3.14). SaOS-2 cells, after several passages old, responded differently to IL-17 and OSM treatment. However, the results from these cells by conventional PCR show that there was no difference between different treatments at 6 hr and 48 hr but there was expression of OPG mRNA in response to OSM at 24 hr (Fig 3.15). Furthermore, quantitative PCR analysis for this experiment shows that the expression of OPG mRNA after several passages changed (Fig 3.16). The expression of the gene was enhanced by IL-17 (5 ng/ml) at 6 hr but there was no OPG mRNA expression in response to IL-17 (5 and 50 ng/ml) at 24 hr and 48 hr. There was expression of OPG in response to OSM at 6 hr and 24 hr, and this was increased at 48 hr. In general, the expression of OPG mRNA was at a very low level compared to the previous experiments.

3.3.4.2 OPG production by the SaOS-2 cell line

The production of OPG from the human osteosarcoma SaOS-2 cell line was studied by Western blot analysis. The pattern of results showed that OPG production was increased by IL-17 compared with the control. The production of OPG was increased dose dependently by treatment with IL-17 (5 and 50 ng/ml) and increased by OSM at 24 hr compared to the control. The production of OPG was induced by IL-17, but not dose dependently, and the production of OPG by OSM was decreased at 48 hr (Fig 3-17).

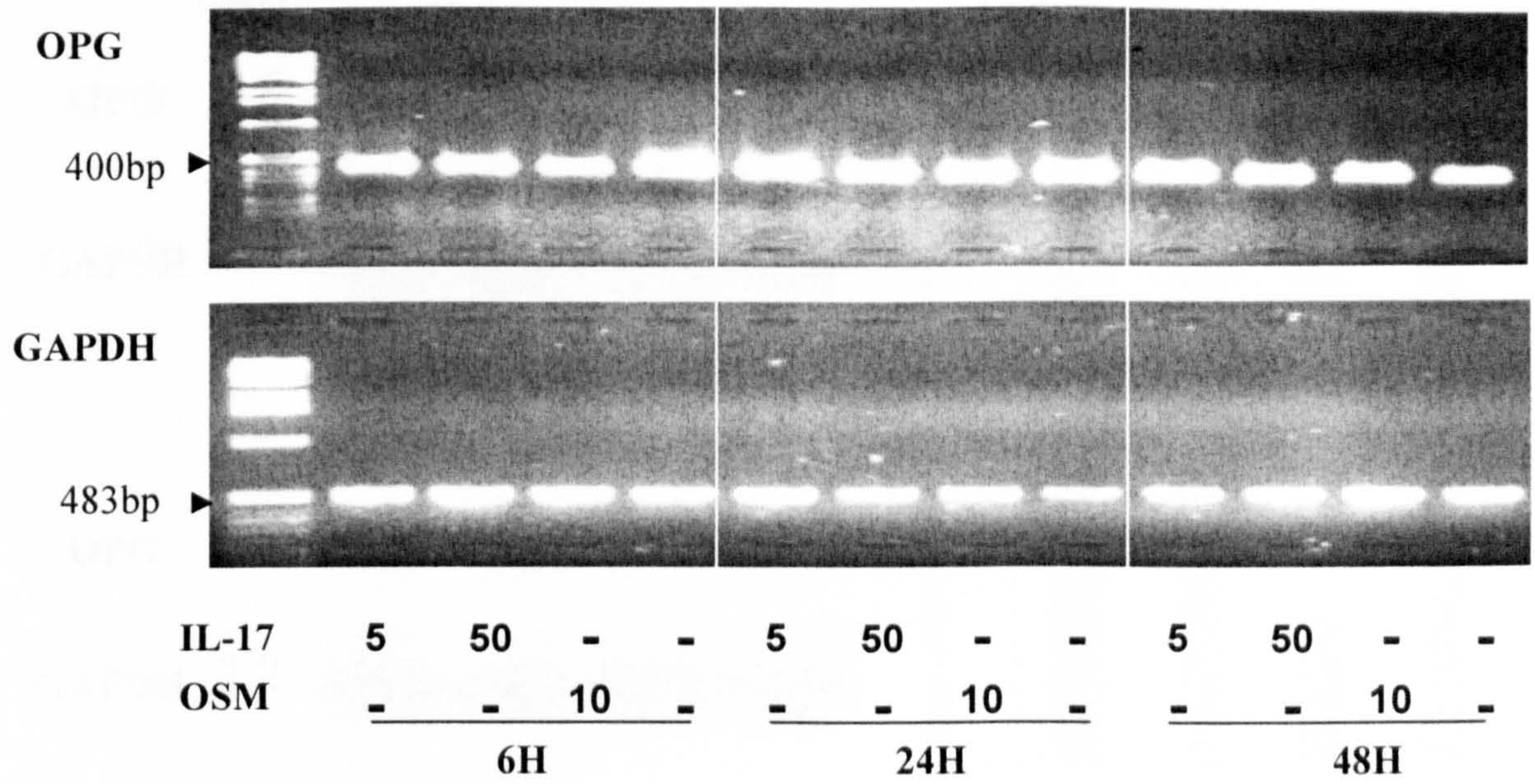


Fig 3.12 The expression of OPG mRNA by conventional PCR. SaOS-2 cells were stimulated with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) for 6hrs, 24 hr and 48 hr. RNA was extracted by using trizol reagent. Total RNA was analyzed by RT-PCR with OPG primers using the conditions described in Materials and Methods. PCR was performed for 35 cycles. The PCR products were run on 1% agarose gel, stained with ethidium bromide and visualized under UV light. This result is representative of three independent experiments.

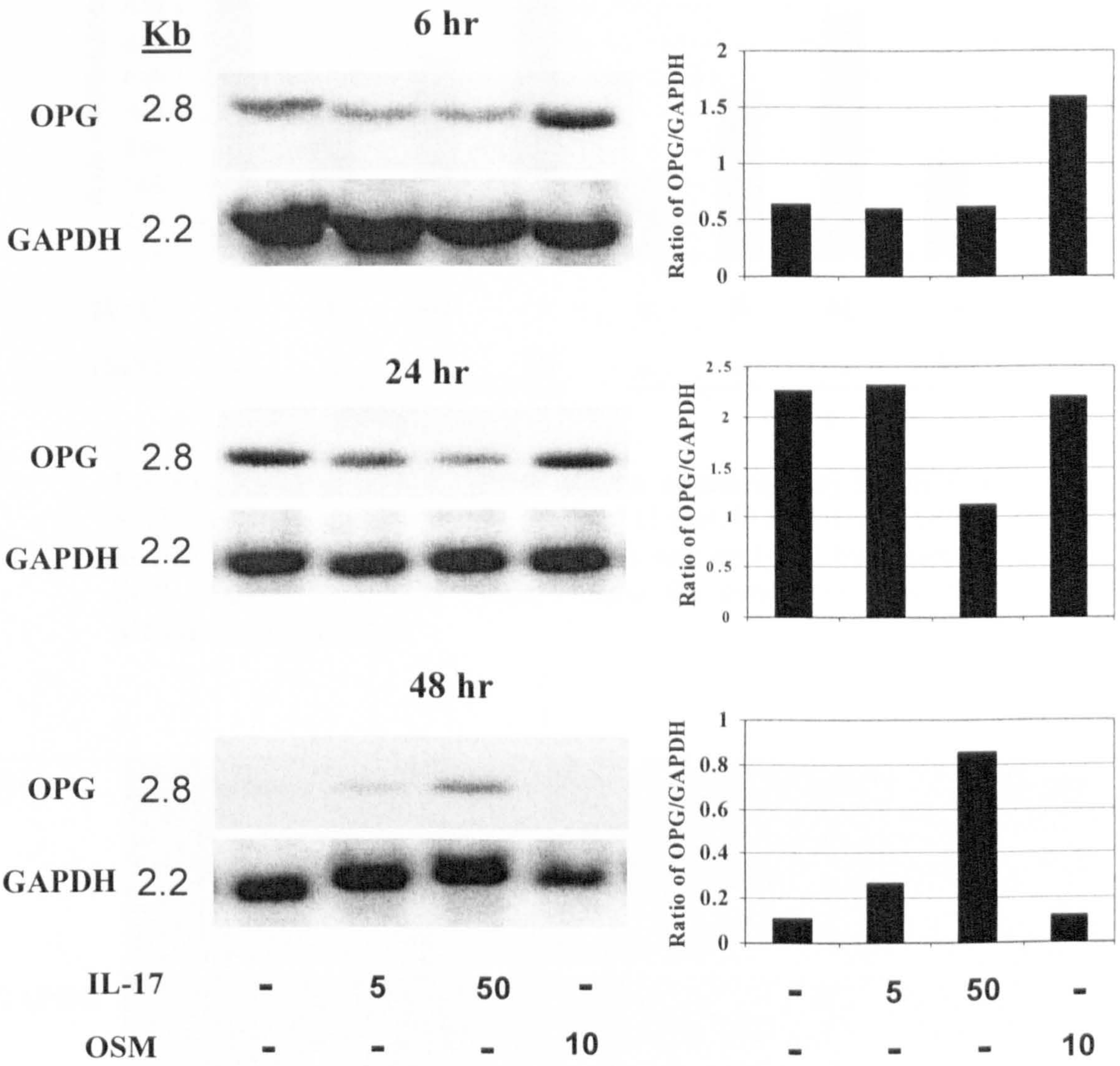


Fig 3.13 The expression of OPG mRNA by SaOS-2 cells. SaOS-2 cells were stimulated with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). The cells were cultured for 6 hr, 24 hr and 48 hr. Total RNA harvested and analysed with Northern blot. GAPDH was used to normalise for RNA loading. This result is representative of three independent experiments

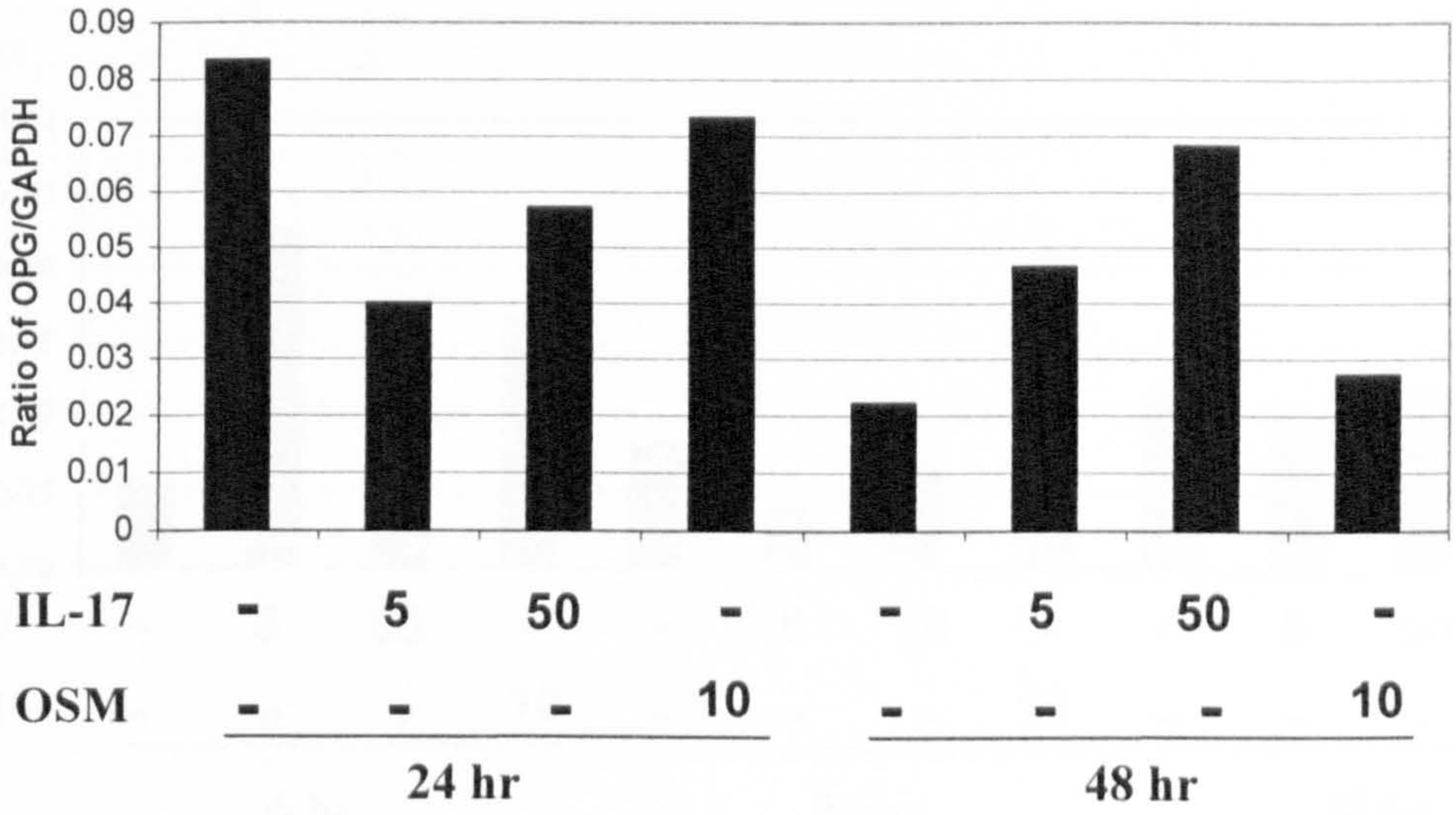


Fig 3.14 Ratio of OPG/GAPDH mRNA expression by SaOS-2 cells. SaOS2 cells were stimulated with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) for 24 hr and 48 hr. Total RNA was analyzed by Quantitative PCR analysis with OPG primers using the conditions described in Materials and Methods.

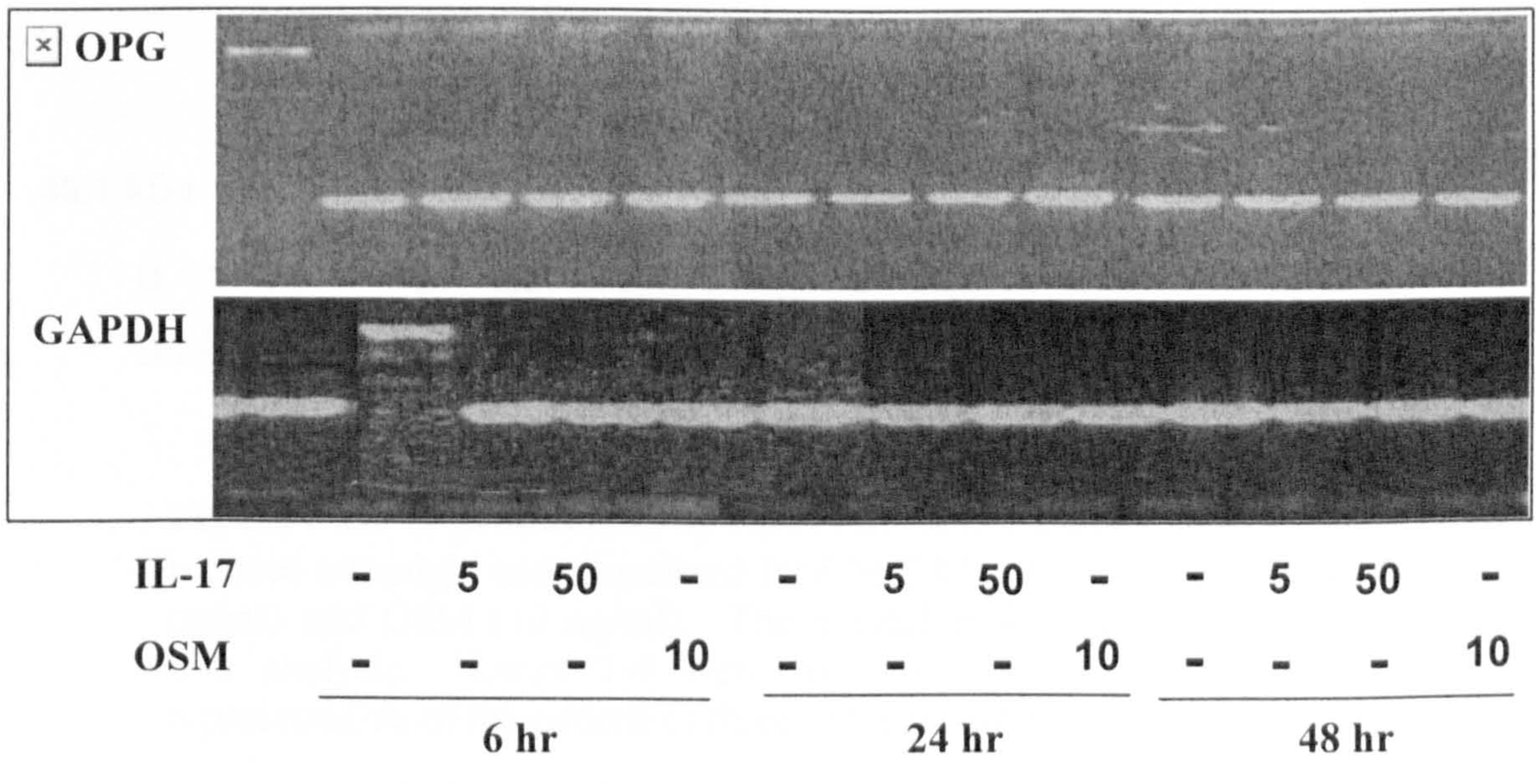


Fig 3.15 The expression of OPG mRNA by conventional PCR. SaOS-2 cells stimulated with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). The cells were cultured for the time points (6 hr, 24 hr and 48 hr). RNA was extracted by using trizol reagent. Total RNA was analyzed by RT-PCR with OPG primers using the conditions described in Materials and Methods. PCR was performed for 38 cycles. The PCR products were run on 1% agarose gel, stained with ethidium bromide and visualized under UV light.

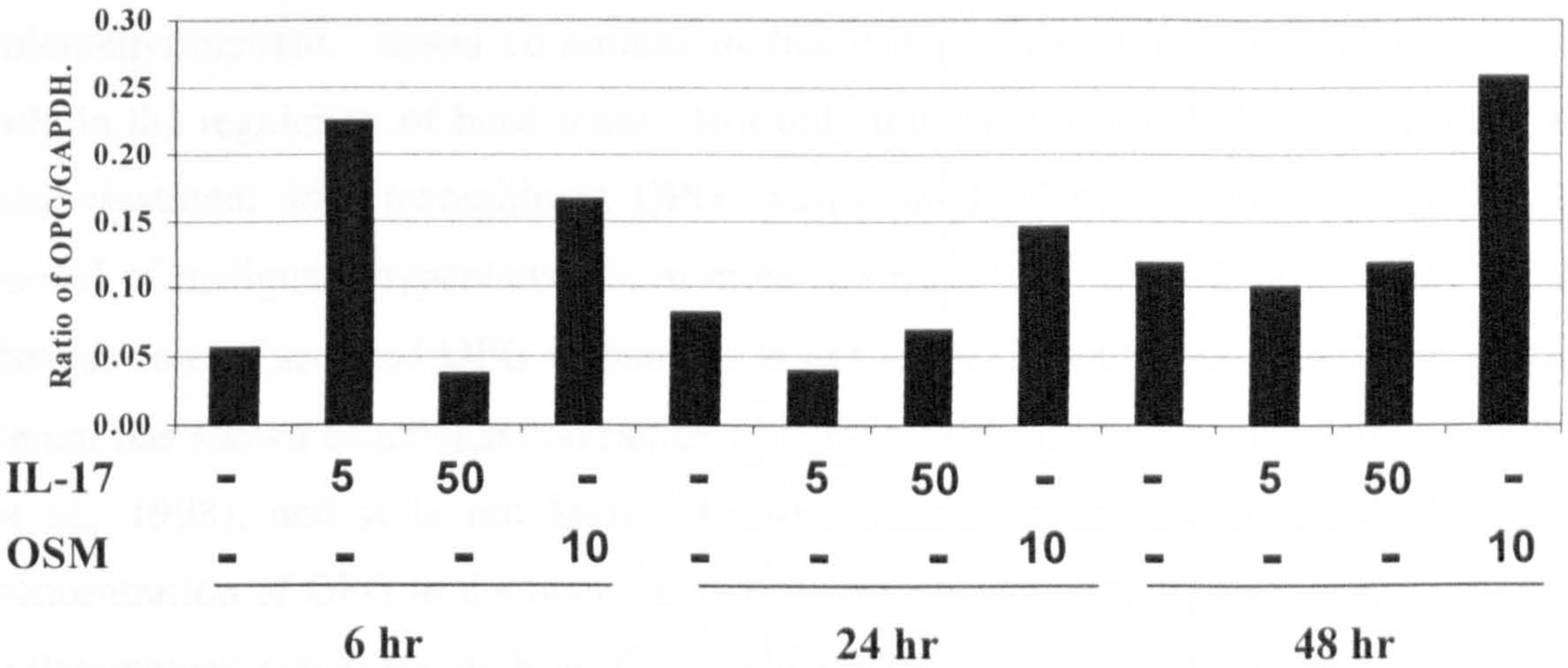


Fig 3.16 Ratio of OPG/GAPDH mRNA expression by SaOS-2 cells. SaOS-2 cells stimulated with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) for 6 hr, 24 hr and 48 hr. Total RNA was analyzed by Quantitative PCR analysis with OPG primers using the conditions described in Materials and Methods.

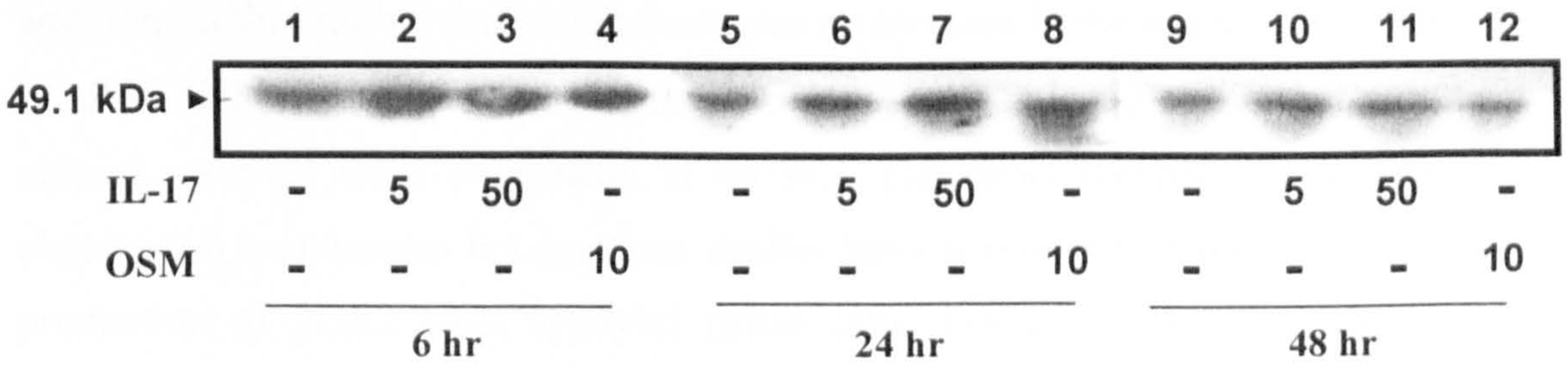


Fig 3.17 OPG production by SaOS-2. SaOS-2 cells were cultured, serum reduced overnight and stimulated for 6 hr, 24 hr and 48 hr with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). The conditioned media collected for Western blot analysis. Lanes 1-4 were run on a separate gel. This result is representative of the pattern of three independent experiments.

3.4 Discussion

OPG production is important for understanding the interaction between synovial fibroblasts, osteoblasts and osteoclasts and it is possible that a major step in the regulation of bone resorption lies in variations of OPG levels in the bone microenvironment. Based on animal studies it is postulated that OPG plays a crucial role in the regulation of bone mass. Not only transgenic animals have shown this but also treatment with recombinant OPG causes rapid effects on bone resorption in a model of malignant hypercalcemia in mice (Capparelli, C. et al. 2000). However, the precise role of secreted OPG in humans is not as well established. Measuring OPG in serum has shown somewhat contradictory data with regard to bone mass (Bornefalk, E et al., 1998), and it is not known to what extent serum levels reflect the actual concentration of OPG in the bone marrow microenvironment. Therefore, the effects of inflammatory cytokines such as IL-17 and OSM were assessed on the expression of OPG by synovial fibroblasts from RA and OA patients. These studies were contrasted with experiments on human osteosarcoma MG-63 and SaOS-2 cell lines.

For SFBs from patients with RA, OPG mRNA levels appear to increase in response to IL-17 in a biphasic way. The cells responded at 6 hr and 48 hr but did not respond at 24 hr while OPG mRNA was expressed in response to OSM at only 6 hr compared to the control. Furthermore, two samples from OA responded to IL-17 in the same way. In addition, in this study I found that there was no increase in the expression of OPG at 24 hr. The biphasic data suggest that the initial response to IL-17 at 6 hr is followed by a second wave of OPG expression at 48 hr. The exact mechanism underlying this observation is unknown but previous studies have demonstrated that IL-17 affects the production of PGE2 from synovial tissue cells. PGE2 increases the expression of RANKL whilst decreasing the expression of OPG. So, production of PGE2 may be responsible for the effects on OPG expression at 24 hr (Kotake S et al., 1999). Although another study reported that in primary cultures of human bone marrow stroma cells (hBMSC) PGE2 dose- and time-dependently down-regulated the mRNA levels of OPG after 4 hr of stimulation with PGE2 (1 mM), OPG mRNA levels were significantly decreased and the inhibitory effect was seen at and above 1 nM PGE2 (Brandstrom H. et al., 1998a). Furthermore the reduction in mRNA levels also results in a reduction of protein secretion. Brandstrom H et al., (2001) subjected hBMSC to stimulation with PGE2, and measured OPG in culture media. Inhibition of OPG secretion after treatment with PGE2 (1 μM) was detected after 16 hr of incubation and the inhibition was

sustained for 72 hr. In addition, expression of OPG in ST2 stromal cell line and human bone marrow stromal cells is downregulated by bone-resorbing factors such as PGE2 and glucocorticoids and upregulated by vit D3, Ca2 ions, or TGFβ (Hofbauer LC, 2000; Kong YY. and Penninger JM 2000; Brandstrom et al., 1998a,b; Huang L. at al., 2001). Alternatively, the expression of this gene in a biphasic way may be due to the secretion of insulin-like growth factor I (IGF-I) from the synovial tissue as a previous study reported that IGF-I has an inhibitory effect on OPG mRNA that is maximal by 24 hr. IGF-I in a dose- and time-dependent manner regulates OPG and RANKL in vitro and in vivo. These studies suggest that IGF-I may act as a coupling factor in bone remodelling by activating both bone formation and bone resorption; the latter effect appears to be mediated through the OPG/RANKL system in bone (Rubin J. 2002). However, in contrast to my findings Nagasawa T, et al., (2002) reported that the production of OPG was evident already after 6 hr and increased consistently to 24 hr while OPG decreased at 48 hr in human gingival fibroblasts in response to lipopolysaccharides (LPS).

In my study OPG mRNA expression was detectable in all samples from OA, but the general pattern of OPG expression in response to IL-17 between samples showed some variation. In some of them the expression occurred at 6 hr, 24 hr, and 48 hr, in others the expression was biphasic. While in a third group expression occurred at 24 hr. However, the expression of OPG from OA tissue was evident in response to IL-17 (50 ng/ml) from 4 samples at 6 hr out of 8 samples. Furthermore, the expression of the OPG mRNA level at 48 hr was in general slightly increased compared to the control.

The variation in response of synovial fibroblasts from OA patients as shown in table 3-1 suggests that there may well be considerable heterogeneity between patients, and that several different disease states were evident despite the general OA diagnosis. A previous study reported that OPG was highly expressed in RA synovial fibroblasts compared to OA and non-inflammatory synovial fibroblasts, and different levels of OPG expression were found among patients with RA (Kubota A. et al., 2004).

However, the variety in the expression of OPG in response to IL-17 and OSM may be due to the number and the affinity of the IL-17 receptor. The variations in some samples may be caused by differences in the number of IL-17R on the synovial fibroblasts from one patient to another. IL-17R is present on the surface of the cells and if IL-17R numbers decreased with increasing in time, the response to IL-17 by the cells may vary in different samples which may reflect the response of OPG expression to IL-

17. These different modes of regulation may reveal an unexpected complexity of the dynamics of IL-17R-mediated transcription.

In general, the production of OPG in response to IL-17 was decreased at 48 hr compared to the production of OPG at 24 hr. The decreases in OPG production if reproduced in vivo combined with RANKL levels that remained the same or where higher could lead to bone and cartilage degradation. However, inflammatory joint diseases such as RA tend to destroy joint cartilage and bone matrices. Since bone resorption is a common characteristic of osteoclasts, osteoclastogenic factors such as inflammatory cytokines and RANKL present within inflamed joints are thought to play a vital role in the destruction of these matrices (Kaplan C and Finnegan A. 2003). Furthermore, inflammation of knee joints in patients with RA was confirmed by significantly higher proinflammatory cytokine levels in the synovial fluid and the synovium than those seen in patients with OA (Sugiyama T.T. 2001).

Consistent with previous reports I have shown that both SaOS-2 and MG-63 cells express OPG (Kinpara K. et al., 2000; Dovio A et. al., 2003). Results from MG-63 and SaOS-2 suggested that there was some evidence of elevated OPG mRNA levels in response to IL-17 (50 ng/ml) and to OSM at 48 hr by MG-63 cells. There was enhancement in the level of OPG mRNA dose dependently in response to IL-17 at 48 hr by SaOS-2 cells while OPG mRNA was elevated in response to OSM at 6 hr by SaOS-2 cells. In contrast, Vidal ON et al., (1998a) reported that IL-1α induced OPG mRNA levels in MG-63 cells peaked at 4 hr and thereafter declined.

In addition, this study suggests that SaOS-2 cells after several passages responded differently to IL-17 and OSM treatments. This may be due to marked and consistent proliferation of the cells for several passages that leads to cellular stress and a lack of responsiveness to some cytokines and growth factors.

The production of OPG from MG-63 and SaOS-2 cells was detected in the conditioned media in response to IL-17 and OSM. Therefore enhancement of OPG at the mRNA and protein levels in MG-63 and SaOS-2 cells could be related to its stimulatory effects on osteoblastic differentiation. However, recent studies reported that OPG bound to TRAIL and inhibited TRAIL-induced apoptosis of Jurkat cells. They also showed that TRAIL blocked OPG in co-culture experiments. These results indicate a potential cross-regulatory mechanism involving OPG and TRAIL, which may also participate in the regulation of osteoclastic bone resorption (Emery JG. et al., 1998; Bucay N. et al., 1998).

In summary, this data indicate that IL-17 and OSM induces the secretion of OPG, a potent inhibitor of bone resorption. Thus, regulation of these cytokines in the bone microenvironment may be an important paracrine mechanism whereby OPG reduces bone resorption.

3.4.1 In this chapter I have demonstrated that:

- IL-17 regulates the expression of OPG mRNA in a biphasic manner in synovial cells isolated from RA.

- IL-17 regulates the expression of OPG mRNA in a variable manner in synovial cells isolated from OA patients.

- IL-17 affects the production of OPG in a dose-dependent manner in OA SFBs at 24 hr.

- IL-17 affects the expression of OPG mRNA in a time dependent manner in the human osteosarcoma cells SaOS-2 and MG-63.

- IL-17 and OSM affects on the production of OPG in the human osteosarcoma cells SaOS-2 at 24 hr

- IL-17 affects on the production of OPG in the human osteosarcoma cells MG-63. OPG production indicates in MG-63 cells in response to OSM.

CHAPTER-4

Results

4 The effect of IL-17 and OSM on the expression and production of RANKL

4.1 Introduction

Bone loss represents a major clinicaly unsolved problem in RA. The skeletal complications of RA consist of focal bone erosions and periarticular osteoporosis at sites of active inflammation, and generalized bone loss with reduced bone mass (Miossec P. 2001). Patients with RA frequently present not only with juxtaarticular osteopenia and bone erosions but also with generalized axial and appendicular osteoporosis at sites distant from inflamed joints. The pathogenesis of bone loss in RA is multifactorial; disease activity certainly is a major determinant of bone mass. Further pathogenic factors include effects of anti-inflammatory therapies (in particular glucocorticoids (Brosch S. et al., 2003). New evidence indicates that osteoclasts are key mediators of all forms of bone loss in RA. TNF-α is a pivotal in the pathogenesis of RA. In addition, to other proinflammatory cytokines in RA are largely produced by CD4 T-cells and include IFNγ, IL-17, RANKL, IL-1, IL-6, IL-11, PTHrP. Other cytokines produced by monocytes and macrophages such as IL-1, IL-12, IL-18 and OSM are also involved in RA (Funk JL et al., 1998; Horwood NJ. et al., 1998; Kotake S. et al., 1999; Quinn JM. et al., 2000; Ross FP. et al., 2000; Thomson BM. et al., 1987; Miossec P. 2001; Romas, E. 2002).

Osteoclast formation and function is regulated by cells of the osteoblastic linage. Recently, the molecular basis for the regulation was identified; osteoblastic cells induce osteoclastic differentiation and resorptive activity through expression of RANKL (Chamber TJ. 2000). Stromal cells supporting osteoclastogenesis are known to express RANKL (Yasuda H. et al., 1998). However, the stage at which stromal cell express RANKL can be regulated remains to be investigated. Therefore RANK, RANKL, and OPG have well-established regulatory effects on bone metabolism (Udagawa N. et al., 2002).

T cells infiltrating the synovium may play a pivotal role in the pathogenesis of rheumatoid synovium since both fibroblasts and activated T cells express RANKL and thereby promote osteoclast recruitment and activation. Thus, osteoprotegerin and RANKL appear to represent important molecular links between the immune system and bone metabolism in rheumatoid arthritis (Romas, E. 2002; Brosch S. et al., 2003).

A recent study in healthy human volunteers found that activated T cells expressing RANKL induced the formation of osteoclast-like cells from peripheral blood mononuclear cells (Kotake S, et al., 2001). Thus, the evidence indicates that T-cell activation in vivo results in bone loss via an effect that involves RANKL. This is consistent with a RANKL-mediated effect of T cells on bone resorption.

IL-17 is a T cell-derived proinflammatory cytokine almost certainly involved in physiological responses to infection, but also in immunopathology of autoimmune disorders. But their direct involvement in disease and joint destruction has been harder to demonstrate. Locally, they interact with other blood-derived and resident cells. IL-17 enhances the production of proinflammatory cytokines by monocytes and further increases their effects on matrix destruction (Miossec P. 2000). However, the increase of this cytokines in the synovial tissues of RA patients is suspected to be involved in the development of the disease. IL-17 increases RANKL expression by mouse stromal-osteoblasts (Kotake S. et al., 1999). The pathogenic roles of IL-17 in the development of RA, however, still remain to be elucidated. Previous studies have shown that, in vivo, IL-17 over-expression in the knee joints of type II collagen-immunized mice leads to bone erosion and synovial RANKL/RANK/OPG expression. Local IL-17 promoted osteoclastic bone destruction and also RANKL/RANK/OPG expression was found in the synovial infiltrate and at sites of focal bone erosion (Lubberts E. et al., 2003).

Previous studies, have hypothesized that, RANKL has a fundamental role in inducing osteoclastogenesis in RA, suggest the possibility that this (RANKL–OPG–RANK) system plays an important role in pathological bone destruction (Udagawa N. et al., 2002; Romas E. et al., 2002; Zheng MH. et al., 2001). In addition, abnormally high levels of RANKL are expressed in peri-implant tissues of patients with prosthetic loosening suggesting that RANKL may contribute significantly to aseptic implant loosening (Crotti TN. Et al., 2004).

In turn, RANKL has been shown to be expressed on T cells and fibroblast-like cells in synovium. This suggests that RANKL provides an important link between the action of hormones and physiological cytokines and bone resorption (Wong B. et al., 1997), where it induces dendritic cell survival, thereby enhancing T cell priming (Wong B. et al., 1997, Anderson, DM. et al., 1997). Therefore, RANKL may also regulate antigen presentation during an immune response.

Additional studies have confirmed that T lymphocytes produce cytokines other than RANKL, such as IL-17, GM-CSF and IFNγ, which have powerful regulatory effects on

osteoclastogenesis (Udagawa N. 2002). Experimental blockade of RANKL has been shown to be highly effective in preventing local bone erosion, but not synovial inflammation, in animal models of arthritis (Kong YY. et al., 1999; Pettit AR. et al., 2001). However, articular changes caused by chronic and excess of expression of TNFα are not totally blocked by monotherapies that target TNF, IL-1, or RANKL. Combined approaches, especially the combined blockade of TNF and IL-1 and, to a lesser extent, TNF and RANKL, lead to essentially a complete reduction of disease. Differences in abilities to block synovial inflammation, bone erosion, and cartilage destruction further support the rationale for using combined blockade of more than one proinflammatory pathway (Zwerina J. et al., 2004).

In summary, IL-17 acts on osteoblasts, resulting in PGE2 synthesis and RANKL expression, the latter directly inducing differentiation of osteoclast progenitors into mature osteoclasts by binding to RANKL receptors present on osteoclast progenitors (Kotake S. et al., 1999). However, IL-17 appears to be involved in not only immune responses but also osteoclastogenesis in RA patients. Excess production of IL-17 by memory T cells and excess expression and production of RANKL by synovial fibroblasts and osteoblasts may contribute to osteoclastic bone resorption in RA patients. The control of expression of IL-17, RANKL, or OPG in RA patients will provide direction to the development of new treatment strategies for bone destruction in RA patients.

4.1.1 The aims of this chapter are to:

- Determine the effects of IL-17 and OSM on the expression of RANKL by SFB derived from patients with RA.

- Establish the effects of IL-17 and OSM on the expression and production of RANKL by SFB derived from patients with OA.

- Establish the effects of IL-17 and OSM on the expression and the production of RANKL by osteoblast-like cells (human osteosarcoma) MG-63 cell line.

- Demonstrate the effects of IL-17 on the expression and the production of RANKL by osteoblast-like cells (human osteosarcoma) SaOS2 cell line.

4.2 Materials and Methods

Human synovial fibroblasts were derived from patientS with RA and OA. Human osteosarcoma cell line SaOS-2, was cultured in ∝MEM, and MG-63 cells were cultured in DMEM as mentioned in chapter 2. The cells were grown until they reached confluence at 37°C, 5% CO_2. Then the cells were serum-reduced in medium containing 0.5% (v/v) FCS overnight and stimulated as in chapter 2 (2.2.1).

After cytokine treatment, gene expression was assessed by analysis of mRNA levels as mentioned in chapter 2 section (2.3). RNA samples were treated with DNase I to remove genomic DNA contamination, see chapter-2 (2.4). RNA was reverse transcribed into complementary DNA (cDNA). Between 3 - 5 µg of the total RNA was used as template from each sample for cDNA synthesis in a final volume of 20 µl. The cDNA was diluted to 50 µl and stored at -20°C, in details are in section (2.4. 1 and 2. 4. 2). cDNA was amplified by PCR using primer pairs to generate products corresponding to specific gene products as listed in Table 2-1 section (2.4.3). The amplification of cDNA and a negative control reaction was run with each experiment and contained all the reagents except cDNA template. PCR was performed for 35 cycles for GAPDH, RANKL, and 38 cycles for IL-17R. Amplification products were identified by electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide and visualized under UV light

Agarose gel electrophoresis was used as outlined in chapter 2 section (2.6.1) for Northern blot analysis. Equal amounts of RNA (typically 15 - 20 µg for human osteosarcoma cells $SaOS_2$, MG-63 and human dermal fibroblast; 8 - 15 µg for human synovial fibroblast). The gel was run at 60 V for 1 – 2 hr in 1x running buffer, viewed under UV light to check the integrity of the RNA and photographed. RNA was transferred to Hybond-N with 200 ml 15x SSC overnight (section 2. 6. 2). The probe was typically labeled to $5 - 7 \times 10^6$ Bq/µg DNA. The blot was pre-soaked in 2x SSC and placed into a hybridization bottle. The blot was blotted semi-dry on filter paper, wrapped in cling film and exposed to a Storage phosphor screen (Molecular Dynamics,

Chesham, UK) overnight at room temperature. Radioactive bands were visualized by using a Phosphor Imager (Storm 860, Molecular Dynamics).

In addition, Real-Time PCR analysis was used in this chapter. Forward and reverse primers for the RANKL cDNA sequence were designed. The primers for RANKL had a calculated annealing temperature of 65°C and amplicon size of 116 bp for more details see section (2.8.2). Taqman GAPDH control reagents, primers and probe were used from R&D (part number 402869). The reverse transcription was performed in 20 µl reactions as outlined in section (2.8.5). The cDNA was then diluted 5 times in DEPC dH_2O for real-time PCR and 2 µl used as template for each reaction. PCR amplification was carried out in a 20 µl volume with 2 µl of the LightCycler DNA Mastermix as outlined in section (2.8.5).

In the relative standard curve method, standard curves with known amounts of total cDNA (relatively) are analysed for the gene of interest, RANKL. For each unknown experiment, the relative amount is calculated by division of the RANKL value with GAPDH value. Fold induction is calculated by dividing the treatment with the control.

4.3 Results

4.3.1 The effect of IL-17 and OSM on the expression of RANKL mRNA by SFB derived from patients with RA

The ability of synovial fibroblasts (SFB) from patients with RA to express RANKL mRNA was examined. In the first experiment, cells isolated from a RA sample were treated for 6 hr. In this experiment DNase treatment was not performed so the cDNA in some samples was contaminated with genomic DNA and the results from this experiment show that RANKL mRNA was expressed at 6 hr in response to IL-17 (5 ng/ml) but not in response to OSM (10 ng/ml) (Fig 4.1).

The results from a second RA SFB samples show that RANKL mRNA was expressed in a biphasic way; the expression of the gene was initially 2.8-fold over control at 6 hr in response to IL-17 (5 ng/ml). There was no expression of RANKL at 24 hr in the cells stimulated with IL-17 (5 and 50 ng/ml) compared to untreated cells. RANKL expression was increased at 48 hr when treated with IL-17 (50 ng/ml), about 10-fold compared to the expression of RANKL mRNA in the untreated cells. There was no

expression of RANKL mRNA in the cells stimulated with OSM (10 ng/ml) in this experiment (Fig 4.2).

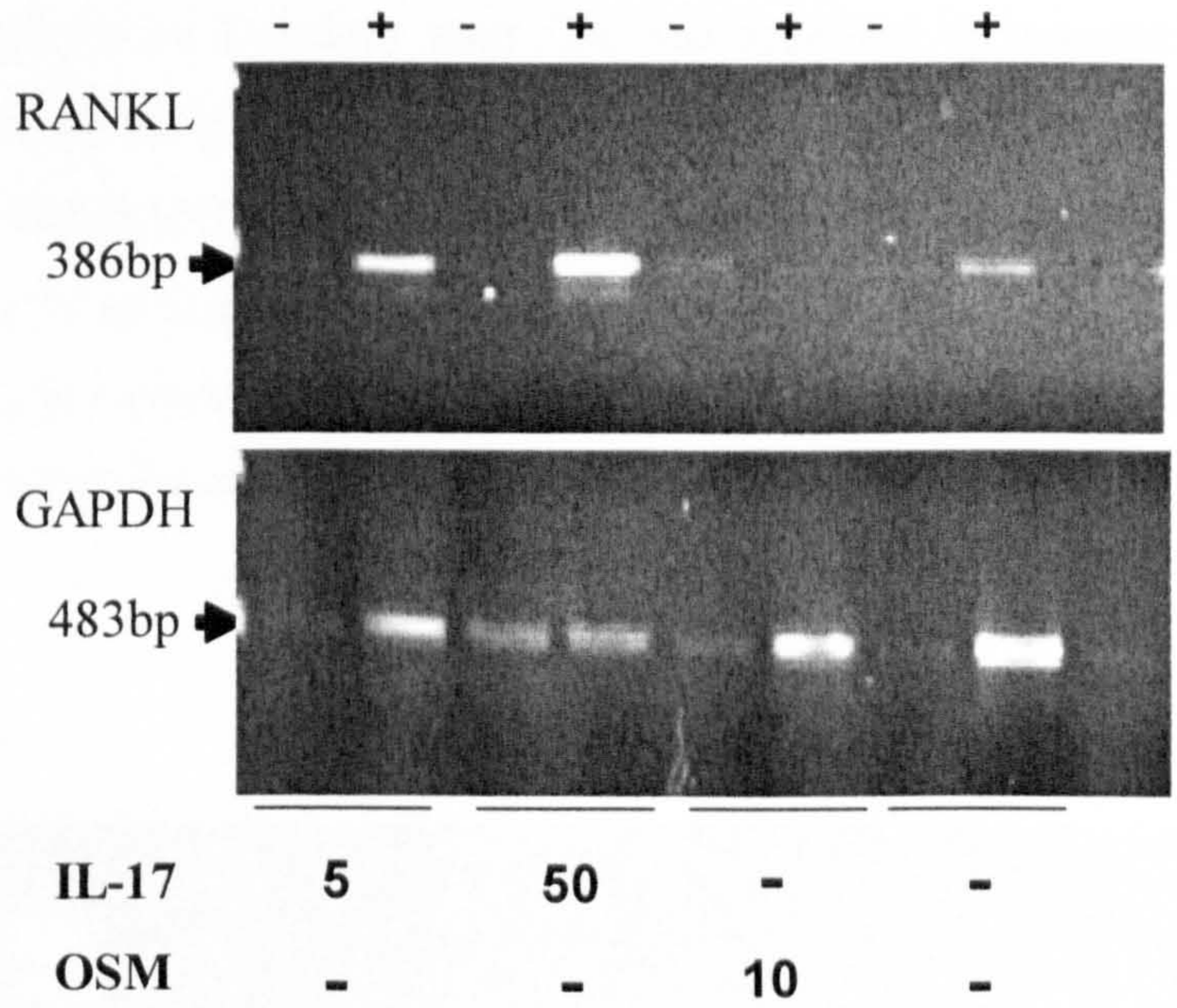


Fig 4.1 The effect of IL-17 on the expression of RANKL mRNA by conventional PCR in human SFB from rheumatoid arthritis. The cells was serum-reduced overnight and treated with IL-17 (5 and 50 ng/ml) or OSM (10 ng/ml) for 6 hr. Total RNA was analyzed by RT-PCR with specific primers using the conditions described in Materials and Methods. PCR was performed for 35 cycles. The PCR products were run on 1% agarose gel, stained with ethidium bromide and visualized under UV light. (+) is cDNA and (-) is negative control untranscribed total RNA.

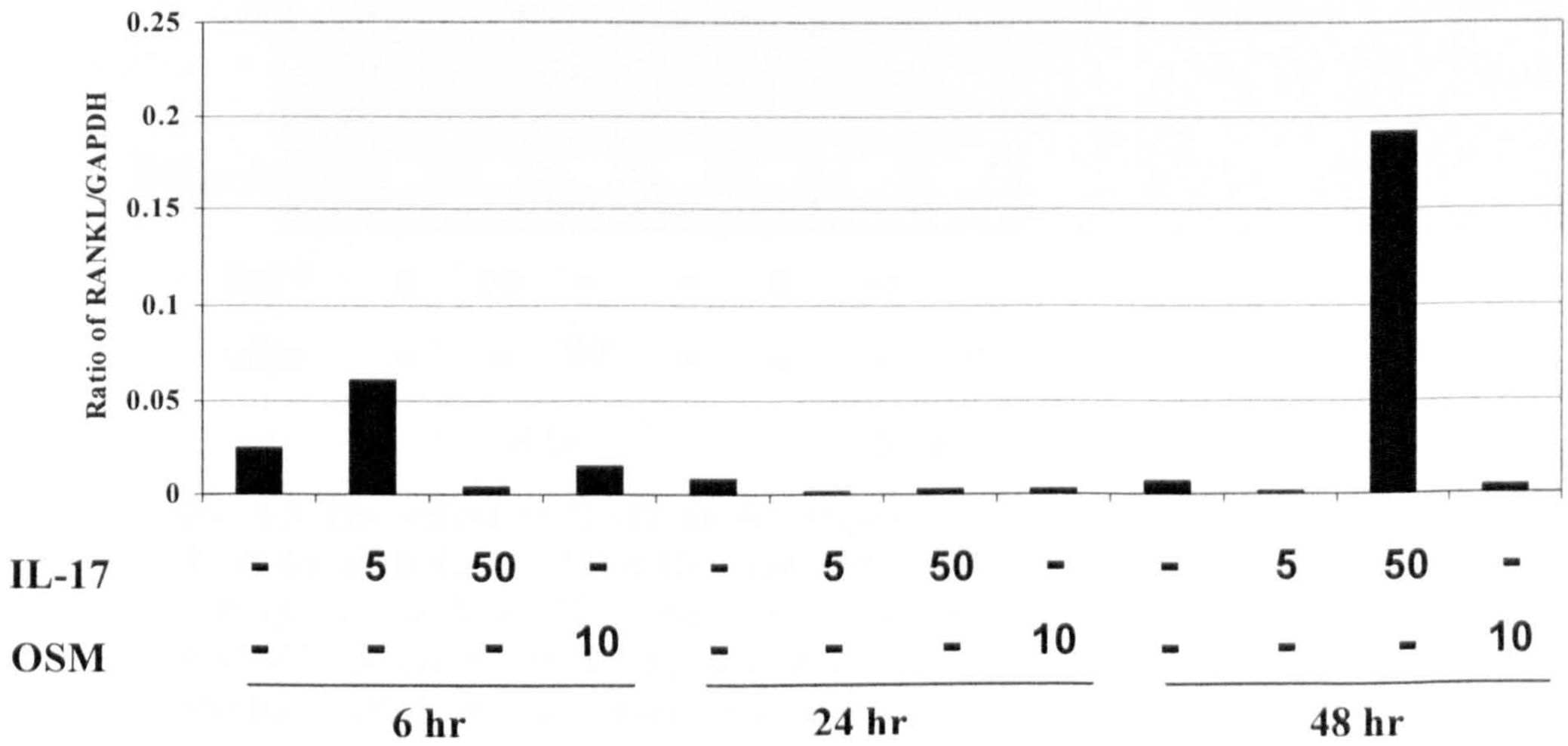


Fig 4.2 The effect of IL-17 on the expression of RANKL mRNA by SFB from RA. SFB cells were cultured, serum reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). Quantitative PCR analysis was as described in Materials and Methods section.

4.3.2 RANKL mRNA expression by SFB cultured from osteoarthritic tissue

The first sample from a patient with OA was assessed by conventional PCR. In this experiment, contamination with genomic DNA was avoided by DNase treatment. The results show that RANKL mRNA was not expressed by these cells when treated with IL-17 (5 and 50 ng/ml) at all time points. However, RANKL mRNA was expressed from the cells in response to OSM (10 ng/ml) at 6 hr and this increased at 24 hr but decreased at 48 hr (Fig 4.3).

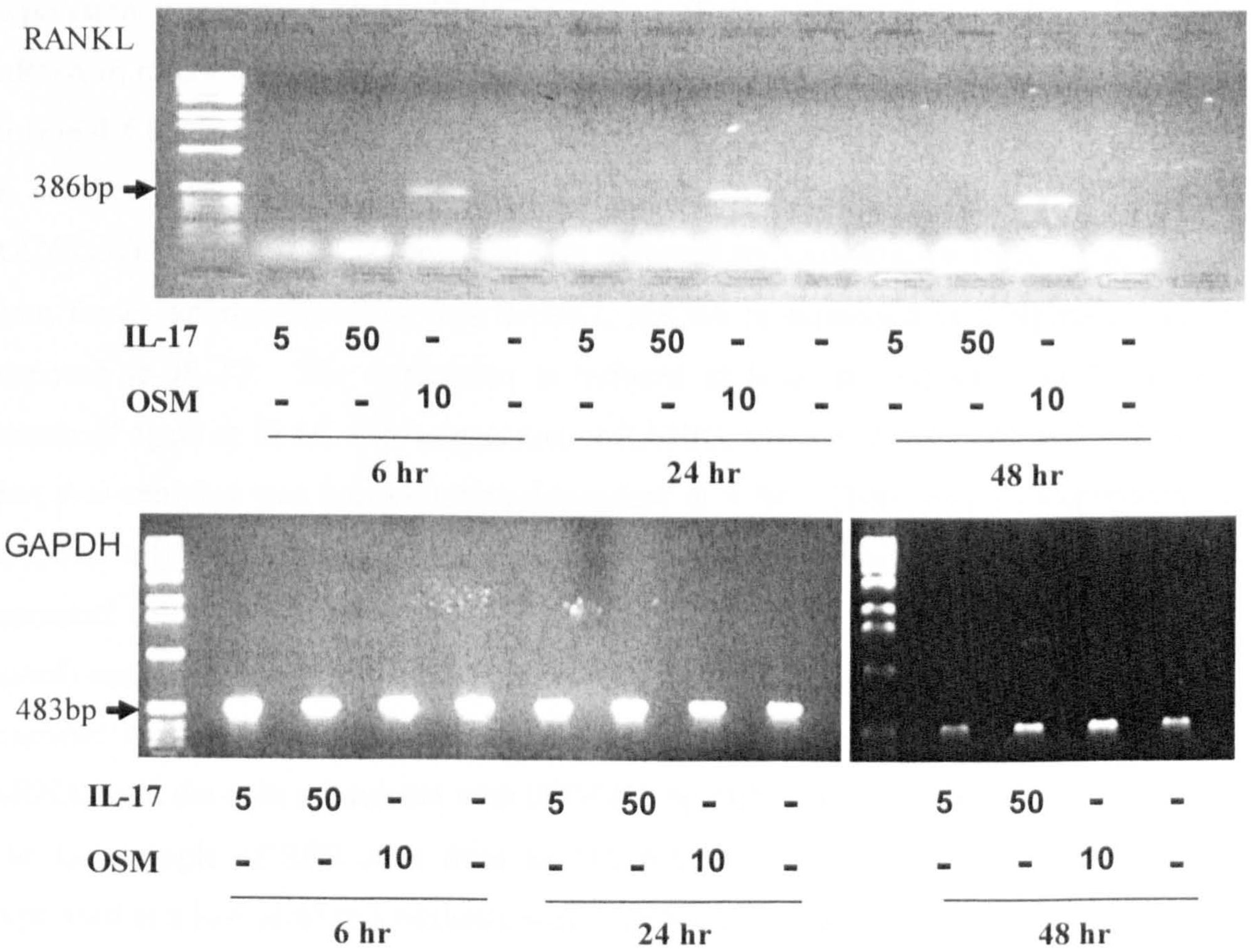


Fig 4.3 The effect of IL-17 on the expression of RANKL mRNA by conventional PCR by SFB OA. SFB cells were stimulated with IL-17 (5 and 50 ng/ml) or OSM (10 ng/ml) for 6 hr, 24 hr and 48 hr. Total RNA was analyszed by RT-PCR with RANKL primers for 35 cycles using the conditions described in Material and Methods. PCR products were run on 1% agarose gel, stained with ethidium bromide and visualized under UV light.

In total, five different patient samples were used for Quantitative PCR to detect RANKL mRNA expression in OA SFB. The results from these experiments show that RANKL mRNA expression was detectable in all five samples. In the first OA sample, quantitative PCR showed that RANKL mRNA was expressed in response to IL-17 (50 ng/ml) compared to control at 24 hr and this expression was increased about 30-fold after IL-17 (5 ng/ml) treatment compared to untreated cells at 48 hr. The expression of RANKL mRNA in response to OSM at 6 hr was a very low, and there was no expression of RANKL mRNA compared to control at 24 hr but the expression of RANKL mRNA was increased by 54-fold at 48 hr (Fig 4.4 A). In a second sample of OA SFB Quantitative PCR showed that there was a gradual increase of RANKL mRNA expression in response to IL-17 (5 ng/ml) at 48 hr compared to control. RANKL mRNA in respons to OSM in this experiment at 24 hr, but there was no expression at 48 hr (Fig 4.4 B).

In another three samples of synovial fibroblasts from patient with OA the ratio of RANKL/GAPDH mRNA expression was assessed by Quantitative PCR. The results from these experiments show that RANKL mRNA is expressed in a biphasic way in response to IL-17. The expression is induced at 6 hr, no expresion at 24 hr and enhanced again at 48 hr. The expression of RANKL mRNA in response to IL-17 in the first two samples was concentration-dependent at 6 hr. There was no expression of RANKL mRNA following IL-17 treatment (5 and 50 ng/ml) at 24 hr compared to untreated cells. While the expression of RANKL was increased at 48 hr by IL-17 (5 ng/ml) compared to control cells, there was no increase in the expression of RANKL in response to IL-17 (50 ng/ml) at 48 hr. Also, there was no expression of RANKL mRNA from the cells stimulated with OSM (10 ng/ml) as shown in (Fig 4.4 C and D)

The last sample of SFB cells from an OA patient shows that RANKL mRNA was expressed at a low level in a biphasic way. RANKL started to be expressed in response to IL-17 (5 ng/ml) at a higher level than IL-17 (50 ng/ml) treatment at 6 hr. There was no expression of RANKL mRNA in response to IL-17 at 24 hr compared to control. The expression of RANKL mRNA was increased again following IL-17 treatment in a dose dependent manner at 48 hr compared to the untreated cells. There was no expression of RANKL from the cells stimulated with OSM (10 ng/ml) at 6 hr and the expression was increased about 1.5 fold at 24 hr while no expression was observed at 48 hr (Fig 4.4 E).

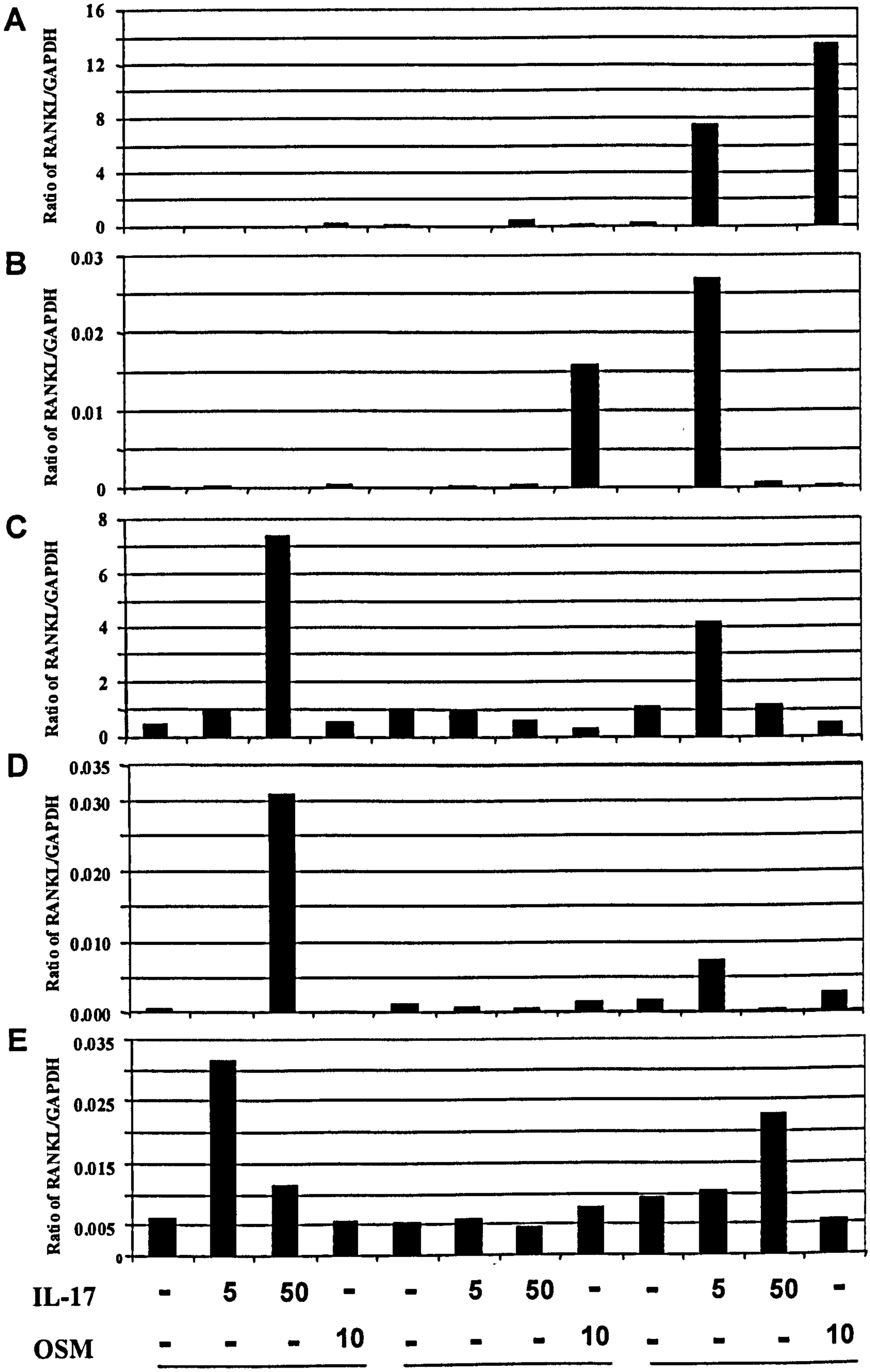


Fig 4.4 The effect of IL-17 and OSM on the expression of RANKL mRNA in OA assessed by Quantitative PCR. SFB cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). Total RNA was analyzed by Quantitative PCR analysis with RANKL primers using the conditions described in Materials and Methods. Five separate OA samples were analyzed (A-E).

4.3.3 The effects of IL-17 on the expression of RANKL mRNA by human osteosarcoma cell lines MG-63

The effect of IL-17 and OSM on the expression of RANKL mRNA in MG-63 human osteosarcoma cells was assessed by conventional and quantitative RT-PCR. The results (Fig 4.5) from these experiments show that RANKL mRNA is expressed at low levels in cells treated with IL-17 or OSM. The results shown are representative of three separate experiments. Northern blot experiments confirmed the low level of this transcript (data not shown).

To more accurately assess RANKL expression, quantitative PCR was performed. The ratio of RANKL/GAPDH mRNA expression was then plotted. The data show that RANKL mRNA is expressed in a biphasic way in response to IL-17 and OSM (Fig 4.6). RANKL mRNA was expressed in response to IL-17 (5 ng/ml) about 2. 5-fold at 6 hr but there was no expression of RANKL in response to IL-17 (50 ng/ml) at 6 hr compared to control. Expression of RANKL was increased about 1.5-fold by IL-17 (5 ng/ml) at 24 hr but there was no increased expression in response to IL-17 (50 ng/ml). At 48 hr the expression of RANKL mRNA increased by 1.5-fold in response to IL-17 (5 ng/ml) and 4.3-fold by IL-17 (50 ng/ml). The expression of RANKL mRNA was increased by 3.2-fold in response to OSM at 6 hr compared to untreated cells, decreased at 24 hr and increased by 3.2-fold at 48 hr.

4.3.4 The effects IL-17 on levels of RANKL mRNA in SaOS-2 cells.

The effects of IL-17 on the expression of RANKL mRNA by the SaOS-2 human osteosarcoma cell line were assessed by conventional and quantitative PCR. These experiments show that RANKL mRNA is detected by conventional PCR at low levels in SaOS-2 cells. RANKL is expressed but there were no differences between different treatments and time points (Fig 4.7). In addition, Northern blot analysis failed to detect RANKL expression (data not shown). Examination of IL-17 receptor expression showed that IL-17 R was expressed on SaOS-2 cells (Fig 4.8). Quantitative PCR results (Fig 4.9) show that the ratio of RANKL/GAPDH mRNA expression in SaOS-2 cells stimulated with IL-17 (50 ng/ml) is 11.5-fold higher than control at 24 hr but the level of expression was decreased at 48 hr. The expression of RANKL mRNA was increased by about 2-fold by IL-17 (50 ng/ml) compared to control at 48 hr. The expression level

of RANKL mRNA in response to OSM appears to be only enhanced at 48 hr (3-fold) compared to untreated cells. In addition, a subsequent experiment from these cells was performed after the cells had had several passages. The results show that there was no RANKL mRNA expressed by these cells compared to untreated cells. Also there was no expression for RANKL mRNA following OSM treatment compared to untreated cells (Fig 4.10). Both IL-17R and alkaline phosphatase mRNA levels were examined by conventional PCR for these cells; IL-17R and alkaline phosphatase mRNA were still expressed (Fig 4.11) and (Fig 4.12), respectively, suggesting that these cells have maintained their osteoblastic phenotype.

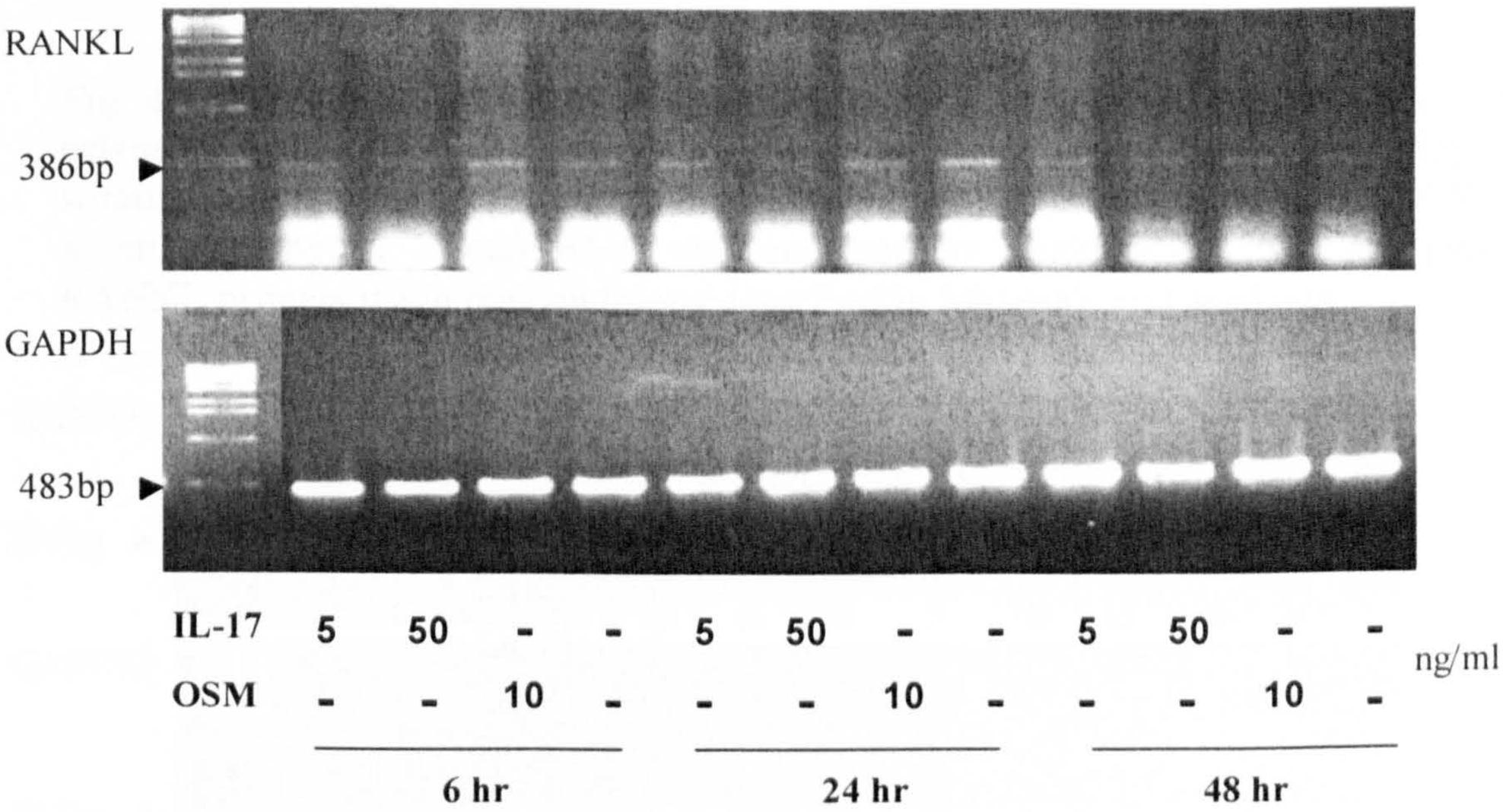


Fig 4.5 The effect of IL-17 on the expression of RANKL mRNA by human osteosarcoma cell line. MG-63 cells were cultured, serum reduced overnight and stimulated for 6hrs, 24hrs and 48hrs by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml). Total RNA was analyzed by RT-PCR with RANKL primers for 35 cycles and PCR products were run on 1% agarose gel, stained with ethidium bromide and visualized under UV light. These data are representative of three separate experiments.

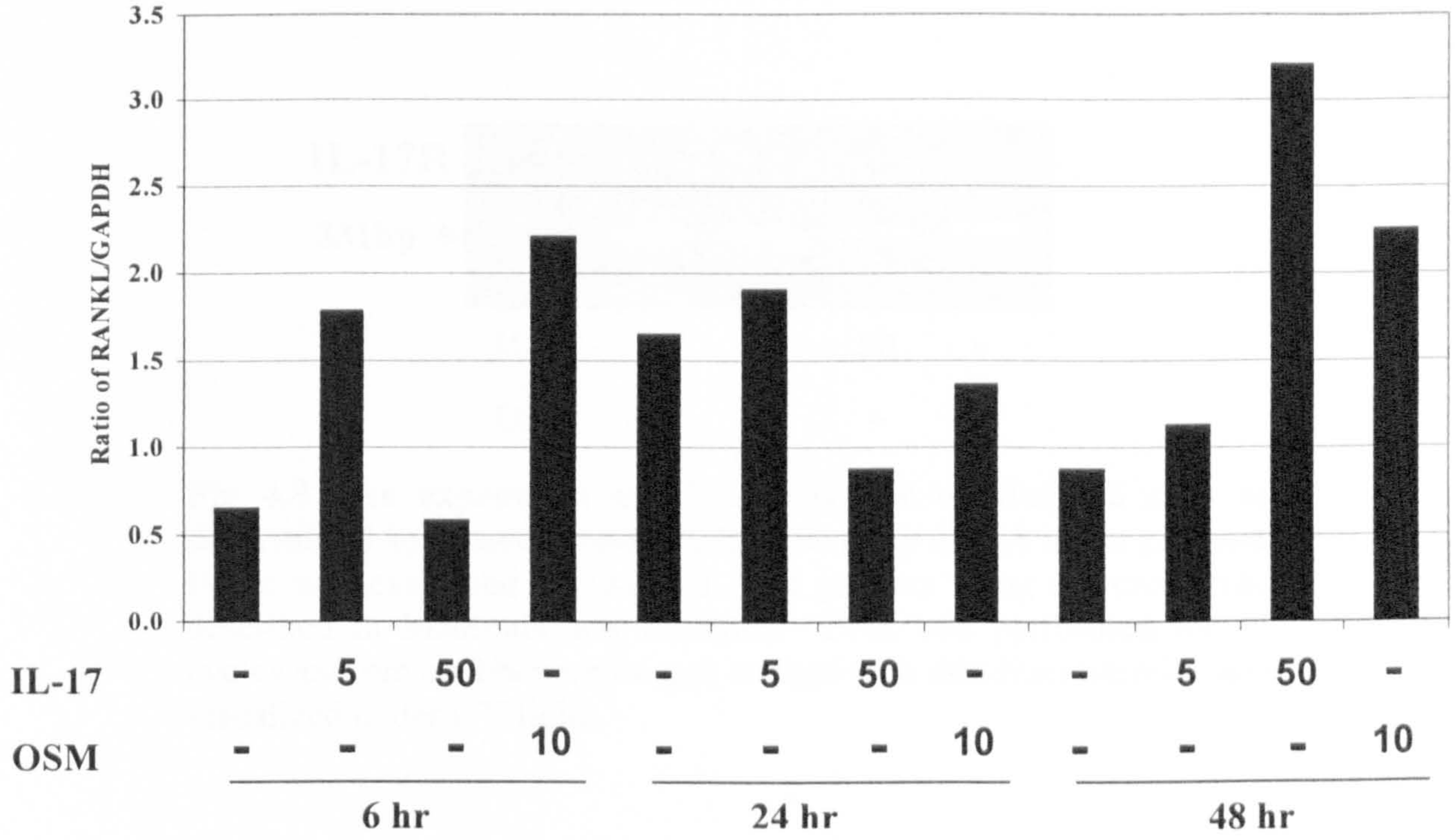


Fig 4.6 The effect of IL-17 on the expression of RANKL mRNA by human osteosarcoma cell line. MG-63 cells were cultured, serum reduced overnight and stimulated for 6 hr, 24 hr and 48 hr following IL-17 (5 and 50 ng/ml) or OSM (10 ng/ml) treatments. Total RNA was analyzed by Quantitative PCR analysis with RANKL primers using the conditions described in Materials and Methods.

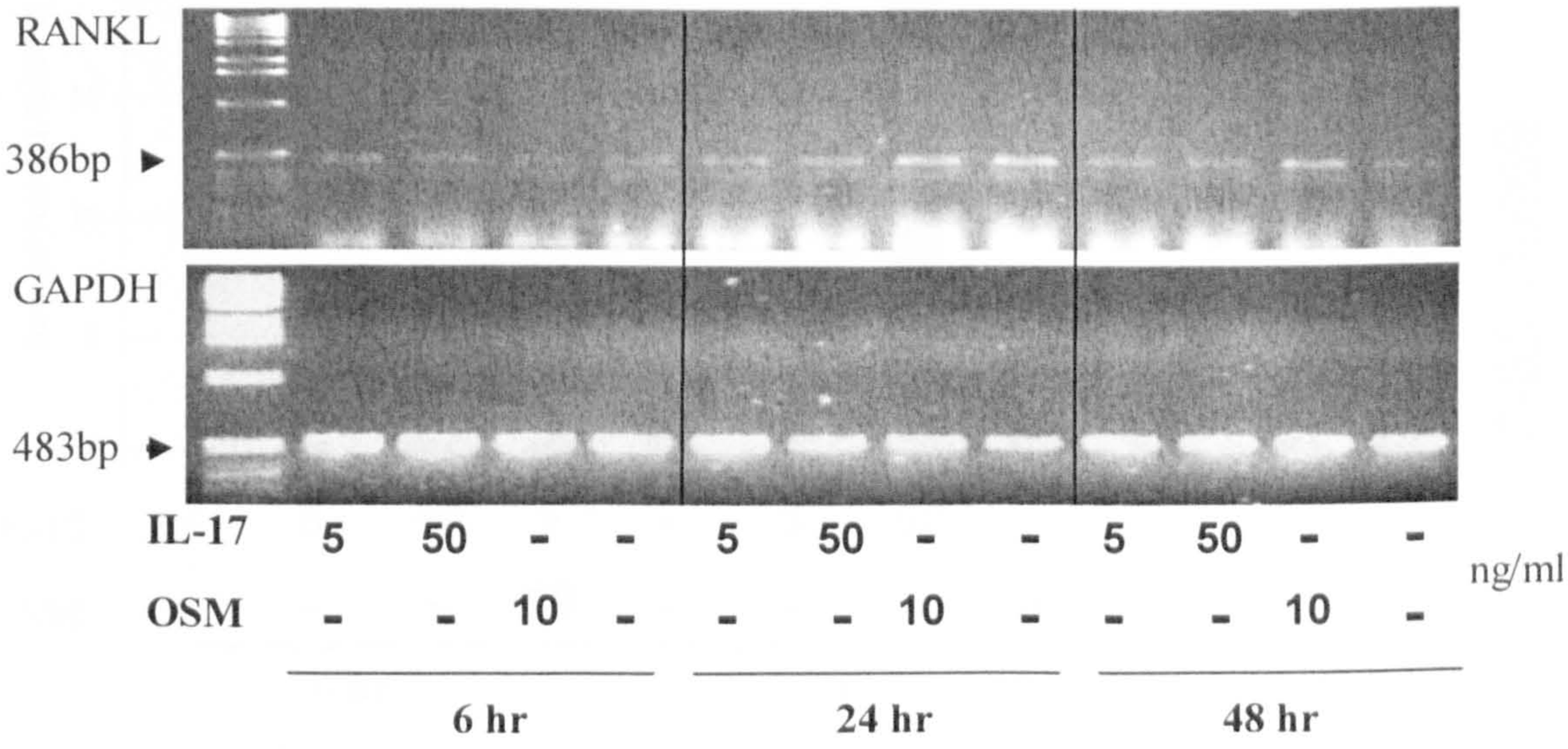


Fig 4.7 The effect of IL-17 on the expression of RANKL mRNA as determined by conventional PCR in SaOS-2. SaOS-2 cells were cultured, serum-reduced to 0.5% FCS overnight and treated with IL-17 (5 and 50 ng/ml) or OSM (10 ng/ml) and control for 6hrs, 24hrs and 48hrs. Total RNA was analyzed by RT-PCR with specific primers using the conditions described in Materials and Methods. PCR was performed for 35 cycles and run on 1% agarose gel, stained with ethidium bromide and visualized under UV light, stained with ethidium bromide and visualized under UV light. Data are representative of three independent experiments.

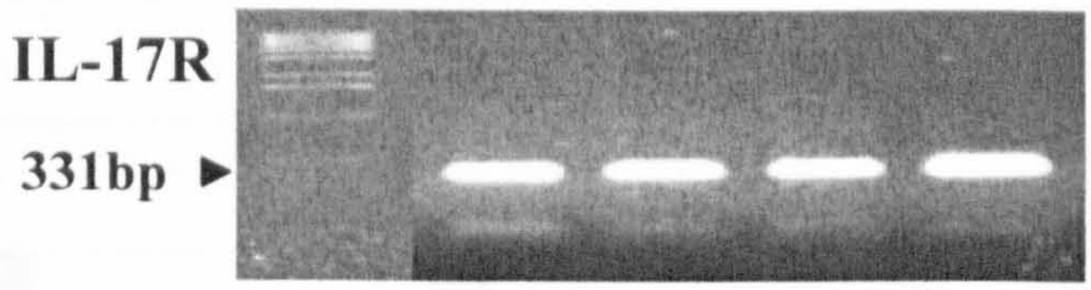


Fig 4.8 The expression of IL-17-receptor on SaOS-2 cells as determined by conventional PCR. The same cDNA in the previous figure was examined by using IL-17R primers using the conditions described in Materials and Methods. PCR was performed for 35 cycles and run on 1% agarose gel, stained with ethidium bromide and visualized under UV light.

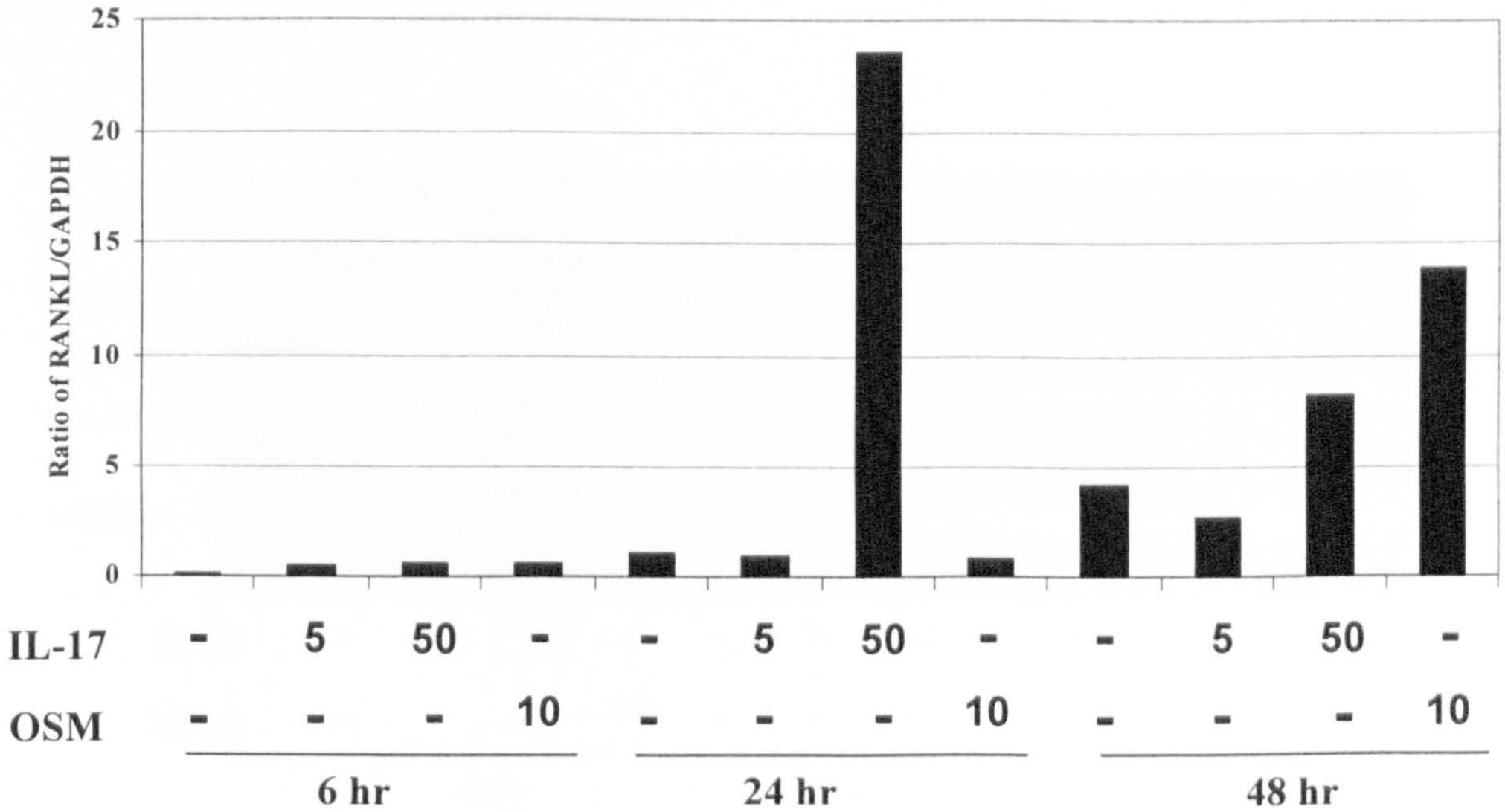


Fig 4.9 The effect of IL-17 on the expression of RANKL mRNA assessed by Quantitative PCR in SaOS-2. SaOS-2 cells were cultured, serum-reduced to 0.5% FCS overnight and treated with IL-17 (5 and 50 ng/ml) or OSM (10 ng/ml) and control for 6 hr, 24 hr and 48 hr. Total RNA was analyzed by RT-PCR with RANKL primers using the conditions described in Materials and Methods. This result is representative of the results from two experiments.

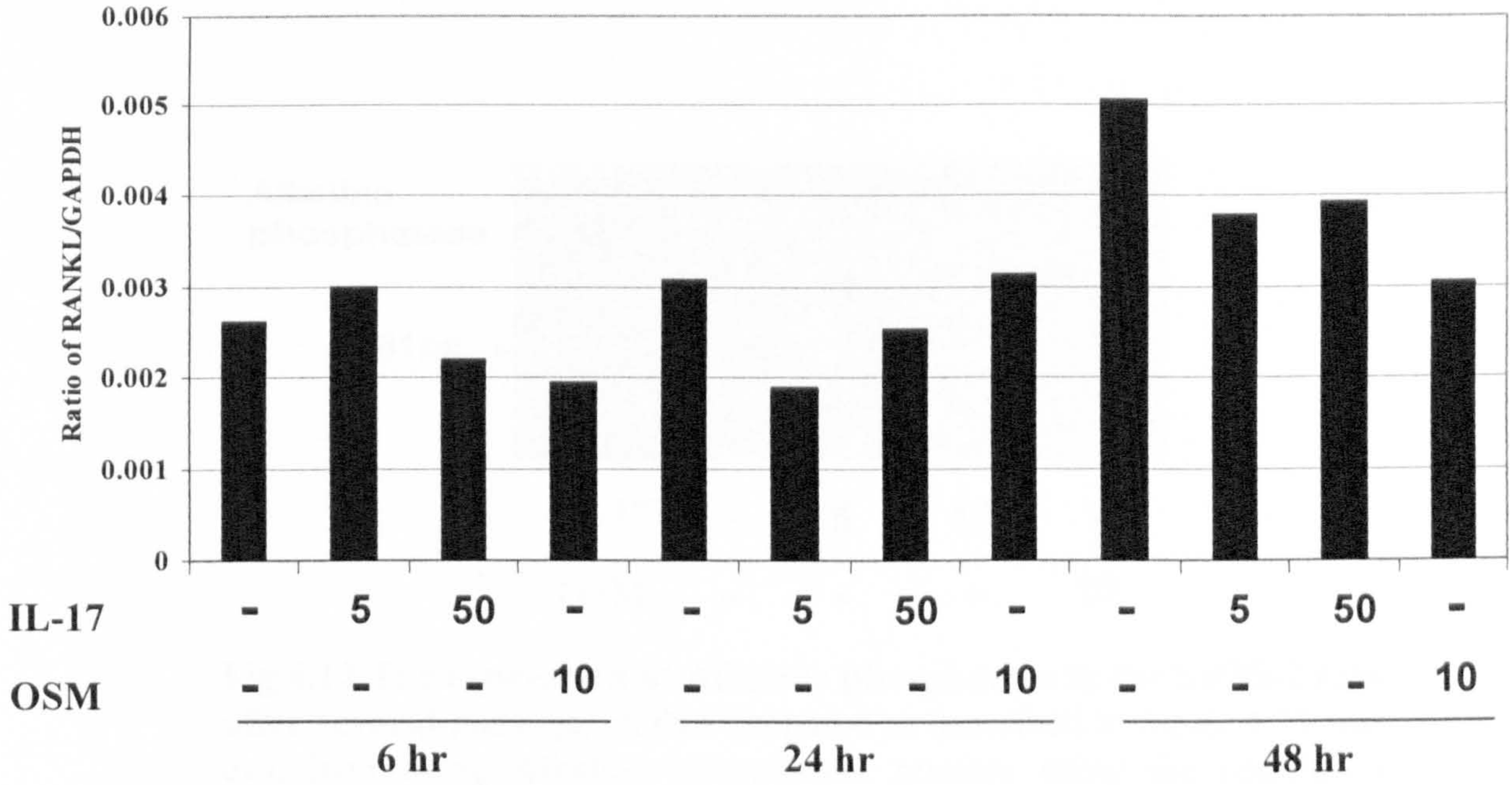


Fig 4.10 The effect of IL-17 on the expression of RANKL mRNA assessed by Quantitative PCR in human osteosarcoma cell line SaOS-2. Cells were cultured after several passage, serum reduced to 0.5% FCS over night and treated with IL-17 (5 and 50 ng/ml) or OSM (10 ng/ml) and control for 6 hr, 24 hr and 48 hr. Quantitative PCR analysis as described in Materials and Methods section.

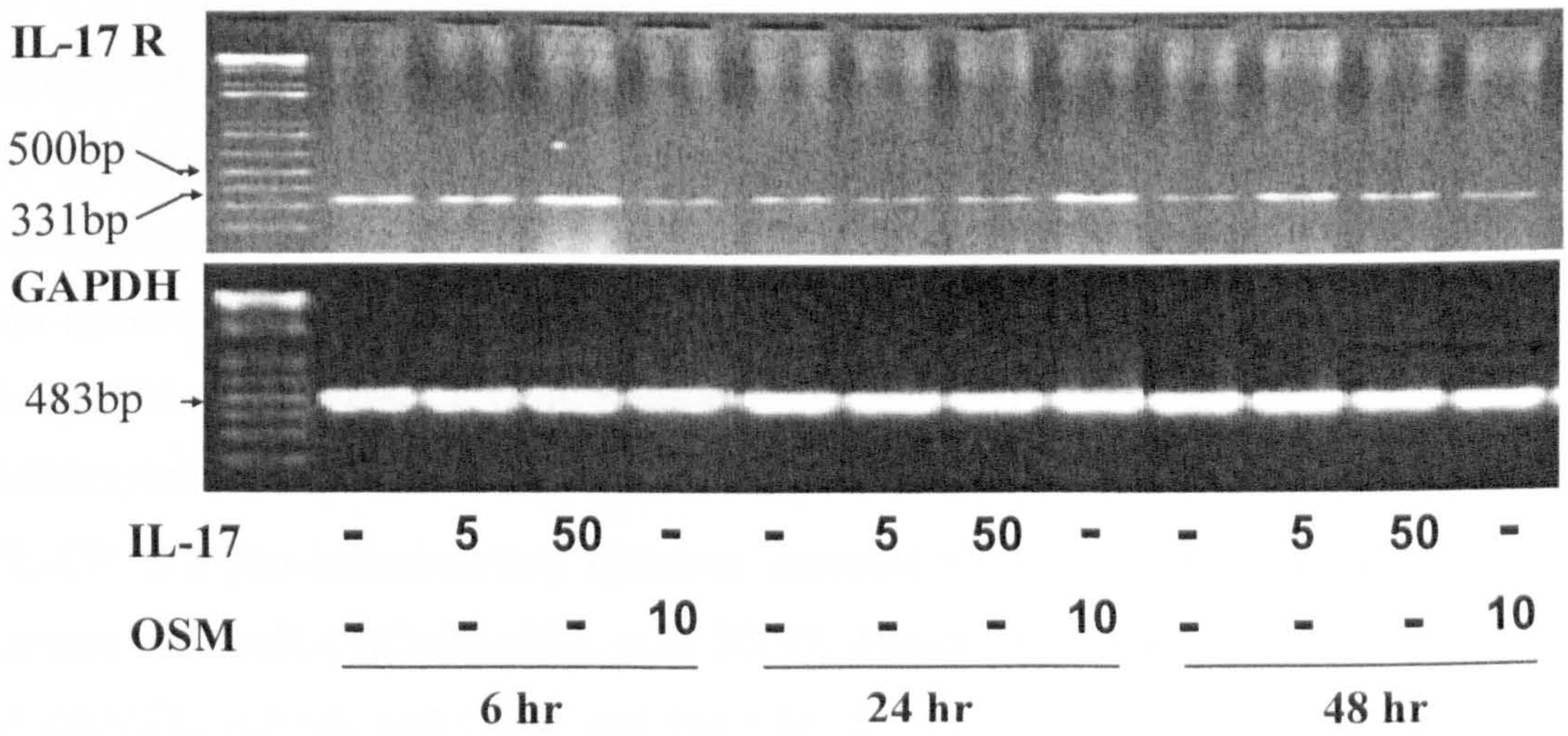
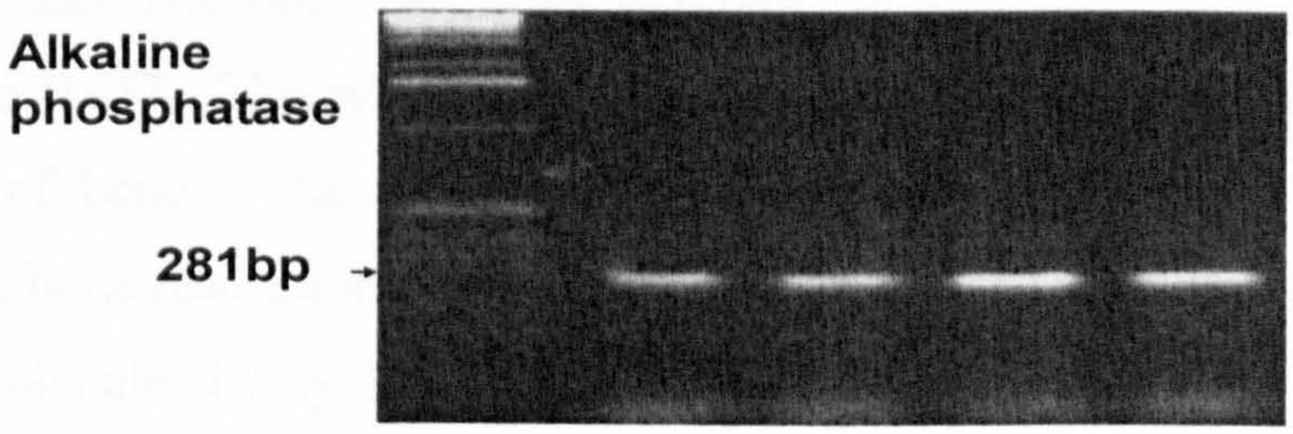


Fig 4.11 The expression of IL-17-receptor on SaOS-2 cells after several passages old. The expression of IL-17R as determined by conventional PCR SaOS-2 cells were cultured, serum-reduced to 0.5% FCS overnight and treated with IL-17 (5 and 50 ng/ml) or OSM (10 ng/ml) and control for 6 hr, 24 hr and 48 hr. Total RNA was analyzed by RT-PCR with IL-17R primers using the conditions described in Materials and Methods. PCR was performed for 35 cycles and run on 1% agarose gel, stained with ethidium bromide and visualized under UV light.



IL-17	-	5	50	-
OSM	-	-	-	10

Fig 4.12 The expression of Alkaline phosphatsae in the SaOS-2 cells after several passage. cDNA prepared as described in figure 4.11 was examined using Alkaline phosphatsae primers using the conditions described in Materials and Methods. PCR was performed for 35 cycles and run on 1% agarose gel, stained with ethidium bromide and visualized under UV light.

4.4 Discussion

Arthritis is a chronic inflammatory disease characterized by articular cartilage and bone destruction. The pathogenesis of inflammation and tissue destruction in RA results from complex cell–cell interactions between, among others, T lymphocytes, monocytes and synoviocytes. It was reported previously that numbers of activated T cells varied during RA. Activated T cells release a number of factors that may cause osteoclast differentiation, maturation and activation. Moreover, it was found that interleukin-17 (IL-17) is a pro-inflammatory cytokine secreted exclusively by activated T-cells and accumulates in RA (Chabaud M. et al 2001). However, recent studies and the discovery of RANKL, RANK and OPG, and the role of these molecules in promoting osteoclast differentiation suggest that these molecules may be the target of factors such as IL-17 and OSM. Furthermore, the molecular mechanisms that underlie this phenomenon have not been well understood.

A direct demonstration of the role of local RANKL and OPG in IL-17 action has been restricted to in vitro studies (Nakashima T et al., 2000 and Atkins GJ. et al., 2000). To confirm the role of these molecules and to characterize molecular mediators in the

physiology of abnormal bone resorption triggered by IL-17, studies that further examine the expression and regulation of these molecules are needed.

In the present chapter, I demonstrate that the expression of RANKL by synovial fibroblasts and osteoblast-like cells is rapidly and robustly enhanced within 48hrs in response to IL-17. This result indicates that IL-17 causes an increase in the pathogenic condition of bone. The changes in RANKL and OPG levels that may be lead to increase in bone resorption. These results provide further evidence that the disturbance of these molecules by cytokines such as IL-17 and OSM may interrupt the mechanism linking osteoblasts and osteoclasts. The quantitative PCR and conventional PCR studies presented here and elsewhere clearly establish that osteoblasts and synovial fibroblasts express RANKL and that it is regulated in vitro upon IL-17 and OSM treatment. The increase in RANKL confirmed that the balance of RANKL and OPG could be altered to favour bone resorption. This is consistent with an important regulatory role for these molecules in the initiation and maintenance of bone catabolism in normal physiology and of osteoclast differentiation. It has been demonstrated previously that an elevation of RANKL and a reduction of OPG occursadjacent to alveolar bone loss. This identifies the OPG/RANKL/RANK pathway of osteoclast differentiation as a possible target for regulating the bone destruction in patients with periodontitis (Crotti T et al., 2003). Also, it has been reported previously that RANKL mRNA is expressed in cells within RA tissues, and the ratio of RANKL: OPG mRNA expressed in RA tissues significantly correlates with the formation of functional osteoclasts (Haynes DR. et al., 2001).

However, its remains to be clarified whether RANKL is produced in joint cells, how its expression is regulated by systemic calciotropic hormones and local inflammatory mediators, and whether RANKL produced in the joint microenvironment plays any significant role in normal bone remodeling or pathological conditions such as osteoporosis. To address these issues, I examined expression of RANKL in SFB (RA and OA) and contrasted them with osteoblastic cells (human osteosarcoma cell lines MG-63 and SaOS-2). In the present study, I found that RANKL mRNA is expressed in these cells stimulated by IL-17 in vitro. These results indicate that IL-17 has effects in synovial fibroblasts and osteoblastic cells. In addition, the time course and dose response of RANKL mRNA expression in response to IL-17 show that IL-17 caused marked and transient induction of RANKL mRNA in SFB RA and OA cells and MG-63 and SaOS-2 cell lines. The increase in RANKL mRNA level was apparent in some

samples as early as 6 hr after the addition of IL-17, and RANKL mRNA level returned to baseline at 24 hr after addition of IL-17 while the expression was increased at 48 hr as shown in one sample from RA and in three samples from OA out samples. These results from RA and OA were similar to the results from MG-63. However, the earliest production of RANKL mRNA at 6 hr and decreased at 24 hr agreed with a previous study that demonstrated that the expression of RANKL mRNA in Porphyromonas gingivalis (P. gingivalis) infected mouse osteoblasts was enhanced at 6 hr after which mRNA levels decreased by 24 hr (Okahashi N et al., 2004). Also, increases in RANKL mRNA levels appeared as early as 6 hrs after the addition of IL-17. A similar finding has been reported for histamine in that it modulates RANKL expression and/or production of cytokines in stromal cells (Tasaka K. et al., 1993; Zheng T. et al., 1994). Histamine markedly increased the steady-state level of RANKL mRNA in a dose-dependent manner. The effect of histamine on the expression of RANKL mRNA by osteoblastic MC3T3-E1 cells was transient; with a peak at 6 hr while the increase in the production of RANKL protein by E1 cells was at 12 hr (Deyama Y. et al., 2002). In addition, another study showed that hPTH also induces RANKL mRNA expression from osteoblast cells in the early hours and hPTH cause a significant increase in bone resorption at 6 hr (Buxton EC et al., 2004).

In another two samples from OA the expression of RANKL mRNA was enhanced by IL-17 at only 48 hr. Furthermore the expression of RANKL mRNA by SaOS-2 was enhanced by IL-17 at 24 hr and at 48 hr. The expression of RANKL at 24hr and 48 hr in this study is agree with Frick KK and Bushinsky DA (2003) reported that cultured neonatal mouse calvariae in acidic and neutral medium for 24 hr and 48 hr. The results from this model of metabolic acidosis show significantly increased expression of RANKL mRNA at both 24 hr (2-fold) and 48 hr (5-fold) compared with the respective controls. Furthermore, the effect of IL-17 on RANKL mRNA was dose-dependent in MG-63 and SaOS-2 cell lines and in three samples (one RA and two OA) at 48hr. In general, RANKL mRNA was expressed by RA, OA, MG-63 and SaOS-2 cells treated with IL-17 at 48hr.

Overall, there was some variation between the results from different patients samples in the expression of RANKL mRNA by IL-17 and OSM. These variations may be due to the differences in the genetic and environmental conditions of the individual or the differences in the age, stage of disease or treatment regime. Therefore, mRNA levels may not mirror protein expression. More recently, it has been shown that OPG and

sRANKL levels did not show sex-related modifications but sRANKL/OPG plasma levels were age-dependent (Pulsatelli L et al., 2004). Furthermore, variations in the expression of RANKL mRNA in response to IL-17 may be due to difference in IL-17 receptor numbers.

However, as mentioned before very low concentrations of RANKL induced OCL formation in RA and OA as well as in Paget's disease, whereas much higher concentrations of RANKL mRNA were required for OCL formation in normal marrow cultures (Menaa C. et al., 2000).

In this study, IL-17 stimulated RANKL mRNA expression in both synovial fibroblast cells and osteoblasts. These results suggest that IL-17 may be directly involved in osteoclastogenesis through RANKL production by synovial fibroblast cells and osteoblasts. Moreover, IL-17R mRNA was expressed by theses cells so these finding suggests that the effect of IL-17 on RANKL mRNA expression in osteoblasts is mediated by stimulation of IL-17 receptors. However, this study provides further evidence that IL-17 may induce osteoclastogenesis by the stimulation of RANKL, which binds to RANK in osteoclast precursors. In conclusion, the results of the present study indicate that IL-17 induces expression of the RANKL gene in osteoblastic cells and synovial fibroblasts which leads to the production of sRANKL and non-soluble RANKL. These responses may influence osteoclastogenesis.

However, the cell-cell contacts in these phenomena are very important as shown in a recent study by real-time PCR in the breast cancer cell line, MDA-MB-231AL. Alone, these cells did not express RANKL while, co-culture of these cells with stromal or osteoblastic cells induced RANKL mRNA expression and decreased OPG mRNA expression (Park HR. et al., 2003). During bone resorption, T cells release IL-17, which induces production of RANKL by stromal cells, osteoblasts and SFB. Further studies are needed to elucidate the molecular mechanisms by which IL-17 regulates the expression of RANKL. In addition, study of the signal transduction pathways of IL-17, TNF, and RANKL and the cross-talk between them may enable us to limit their affects in disease.

4.4.1 In this chapter I have demonstrated that:

- IL-17 affects the expression of RANKL mRNA in a variable way in synovial cells from patients with RA and OA.

- There are no effects on the expression of RANKL mRNA by OSM in synovial cells from patients with RA.

- OSM affects the expression of RANKL mRNA in a variable way in synovial cells from patients with OA.

- IL-17 affects the expression of RANKL mRNA by increasing expression, time dependently in the human osteosarcoma cells SaOS-2 and in a biphasic way in MG-63 cells.

- OSM affects the expression of RANKL mRNA by increasing expression with time in the human osteosarcoma cells SaOS-2 and in a biphasic way in MG-63 cells.

Result

Chapter-5

5. The effect of IL-17 in combination with OSM on the expression and production of RANKL and OPG by synovial fibroblast and osteoblasts cells

5.1. Introduction

RA is a polyarticular disease characterised by chronic inflammation of the synovium with hyperplasia of synovial lining cells and pannus formation. The invasiveness and perpetuation of this pannus is maintained by new blood vessels that allow numerous immuno-modulatory cells such as T cells, B cells, monocytes and macrophages to infiltrate which may perpetuate elevated proliferation and activation of synovial fibroblasts (Chabaud. M., et al 2001; Firestein 1997). Cytokines are regulatory proteins secreted by white blood cells and a variety of other cells in the body. The pleiotropic action of cytokines includes numerous effects on cells of the immune system and modulation of inflammatory responses. Cytokine's are tools in modulating pathogenic responses; in RA inflammation the interaction between various cell types alters the joint cytokine population, leading to the initiation and propagation of an inflammatory response. Many pro-inflammatory cytokines are present in the joint, including TNF-α, IL-1, IL-17, IL-6 and LIF and these are thought to play a pivotal role in bone and cartilage degradation.

Other cytokines are secreted that can be considered anti-inflammatory; they promote matrix synthesis and decrease cartilage and bone degradation. These include IL-4, IL-10, IL-13, IL-1R antagonist, transforming growth factor TGF-β and insulin-like growth factor. In some cases, the cellular response is vastly altered after combinations of cytokines interact with the cells. This is regarded as synergism; meaning the response observed is greater than the sum of the cytokines individual effects. IL-17 has previously been shown to synergise with IL-1 in RA synoviocytes, enhancing the production of IL-6 (Chabaud., et al 1998). IL-17 mediated synergy with IL-1 and TNFα has also been observed in chondrocytes, osteoblasts and myoblasts (reviewed by Miossec, 2003).

Many pro-inflammatory cytokines have been show to be present in the synovial fluids of RA patients, including IL-1 and TNF-α, which represent key pro-inflammatory cytokines in

RA. Both cytokines elicit a broad spectrum of cellular responses including cell cell proliferation, migration, and apoptosis.

Studies from our laboratory have established that OSM acts synergistically with IL-1α to induce cartilage collagen breakdown and collagenase expression. IL-6 can mimic OSM in synergizing with IL-1α to induce chondrocyte-mediated cartilage collagen breakdown and collagenase production. Since OSM, IL-6, and sIL-6R levels have all been shown to be elevated in the rheumatoid joint, these studies suggest that these cytokines may be key mediators of cartilage collagen catabolism in inflammatory arthritides (Rowan AD., et al., 2001). In addition, another study demonstrated that IL-6/sIL-6R and OSM play an important role in the regulation of IL-6 expression within the central nervous system (CNS), particularly in conjunction with the proinflammatory cytokines TNF-α and IL-1β (Van Wagoner NJ., et al. 1999).

IL-17 induces the release of cytokines and prostaglandins from stromal cells and macrophages (Yao, ZS. et al., 1995; Jovanovic, DV. et al., 1998). Both TNF-α and IFN-γ have an additive effect on IL-17-induced secretion of IL-6, and the combination of IL-17 plus TNF-α was effective in promoting GM-CSF release from synovial fibroblasts (Fossiez, F. et al., 1996). Furthermore, IL-17 was shown to enhance cell surface expression of ICAM-1 on human fibroblasts (Yao, ZS. et al., 1995). Thus, a broad set of effects are induced by IL-17, and its action can be potentiated by other cytokines. Also IL-17 has been shown to induce NF-kB consensus sequence binding in mouse fibroblasts (Yao, Z., et al., 1995) and human macrophages (Jovanovic, DV., et al., 1998). Subsequently, the expression of several cytokines known to contain NF-kB recognition sites in their promoters were demonstrated to be regulated by IL-17. These cytokines include IL-8, IL-6, and GM-CSF (Starnes T. et al., 2002). Furthermore, TNF-α and IFN-γ have an additive effect on the stimulation of IL-6 expression by IL-17 (Fossiez, F. et al., 1996), and the combination of IL-17 and TNF-α can stimulate the expression of granulocyte-macrophage CSF by fibroblasts (Fossiez, F. et al., 1996). However, the IL-17-induced signal transduction events leading to the activation of NF-kB are not entirely known.

The understanding of different cytokines effects on cartilage and bone are confounded by their overlapping biological functions, resulting in redundancy, and moreover by their altered potency in the presence of other cytokines, which can be additive or synergistic

(Furst DE et al., 1999). In the present study, we investigated the combination of IL-17 and OSM on the expression and the production of RANKL and OPG, by synovial fibroblasts from patients with OA and RA and the human osteoblast-like cell lines MG-63 and SaOS2.

5.1.1 Therefore the aims of this chapter are to:

- Determine the effect of the combination of IL-17 and OSM on the expression of RANKL and OPG mRNA by synovial fibroblast cells from patients with RA.

- Investigate the effect of the combination of IL-17 and OSM on the expression of RANKL and OPG mRNA by synovial fibroblast cells from the patients with OA.

- Establish the effect of the combination of IL-17 and OSM on the expression of RANKL and OPG mRNA in human osteosarcoma, MG-63 and SaOS2 cell lines.

- Determine the effect of the combination of IL-17 and OSM on the production of OPG in synovial fibroblast cells from patients with OA and from the human osteosarcoma MG-63 and SaOS2 cell lines.

5.2 Materials and Methods

Human synovial fibroblasts were obtained from patients with RA and OA. The osteosarcoma cell lines used in this chapter were SaOS-2 and MG-63. Cells were cultured and serum-reduced as in section (2.2.1 and 2.2.2). After the old media was discarded then new media with the cytokines at different concentration IL-17 (5 or 50 ng/ml), OSM (10 ng/ml) and a combination of IL-17 (5 and 50 ng/ml with OSM 10 ng/ml respectively) were added for 6 hr, 24 hr and 48 hr. RNA was extracted after cytokine treatment; gene expression was assessed by Northern blot and quantitative PCR analysis as mentioned in section (2.7.2 and 2.8.5) respectively. Conditioned media was retained at -20°C for Western blot analysis as outlined chapter 2.

5.3 Results

5.3.1 Effect of IL-17 combined with OSM on the expression of RANKL and OPG by SFB cells from patients with RA.

The results from one patient only show that there was a down-regulation in the expression of RANKL and OPG in synovial fibroblasts at 6 hr and 48 hr respectively when OSM is combined with IL-17, in comparison to OSM alone or IL-17 alone (Fig 5.1).

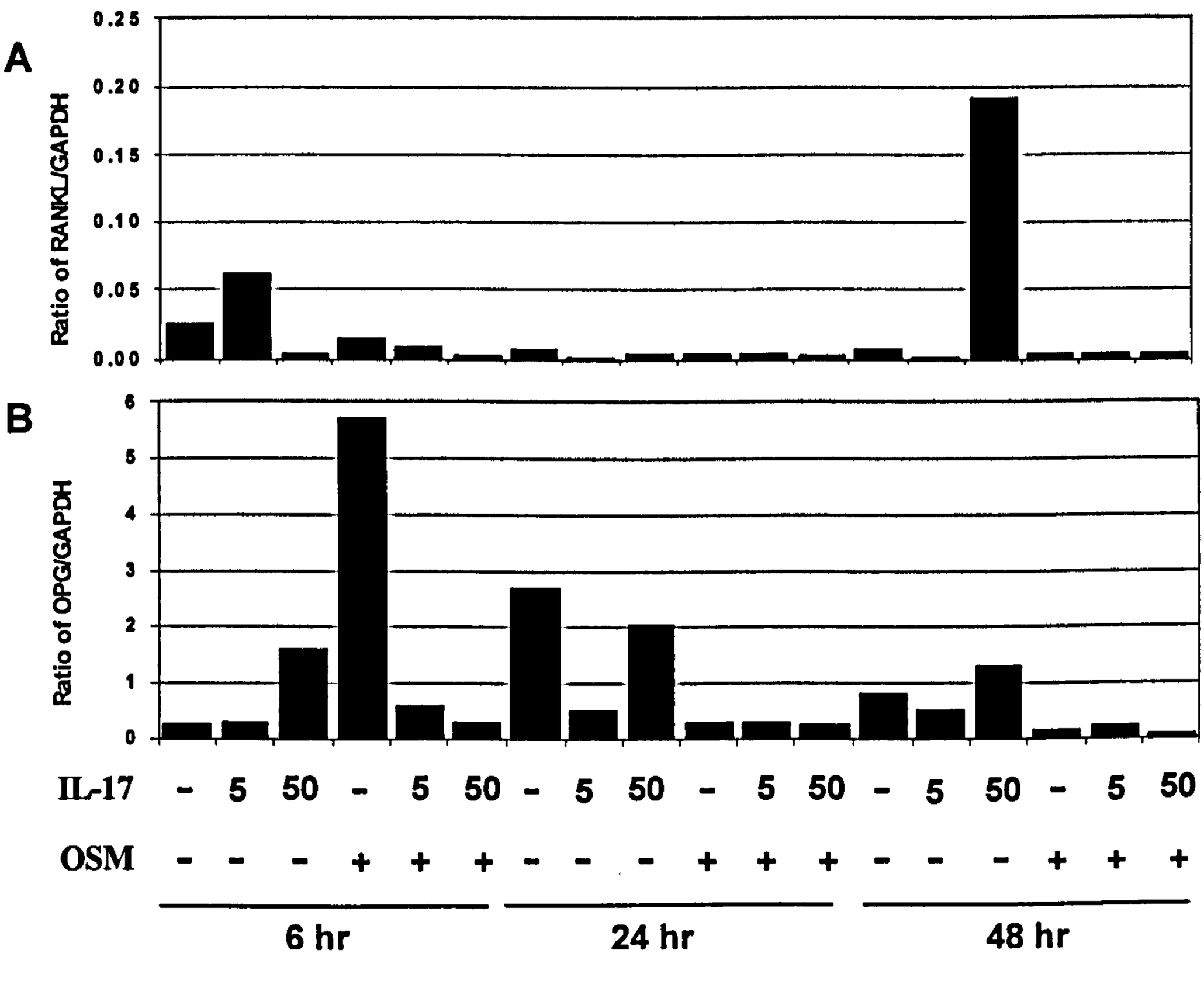


Fig 5.1 The effect of IL-17 combined with OSM on the expression of RANKL and OPG by RA SFB. SFB cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml) respectively. Total RNA was analyzed by Quantitative PCR analysis with (A) RANKL and (B) OPG primers using the conditions described in Materials and Methods. The data are presented relative to GAPDH.

5.3.2 Effects of IL-17 combined with OSM on the expression of RANKL and OPG mRNA and production of OPG by SFB cells from patients with OA

5.3.2.1 The effect of the combination of IL-17 with OSM on the expression RANKL in SFB (OA)

The effect of combining IL-17 with OSM on the expression of RANKL in synovial fibroblast cells cultured from four patients with OA was studied. The ratio of RANKL/GAPDH was quantified by quantitative PCR in first two samples the results show that RANKL mRNA expressed in response to IL-17 at 48 hr (Fig 5.2 A and B). The expression of RANKL in response to OSM in (Fig 5.2 A) was at 48 hr but in (Fig 5.2 B) at 24 hr. However, there was a down-regulation in RANKL transcription levels in response to the combination of both cytokines. In another two samples the results show that there was no induction of RANKL mRNA in cells treated with IL-17 combined with OSM (5 and 50 ng/ml IL-17 with OSM 10 ng/ml, respectively). While RANKL mRNA was expressed in response to IL-17 (50 ng/ml) at 6 hr and by IL-17 (5 ng/ml) at 48 hr (as previously described) but there was no RANKL mRNA induction in response to OSM alone or in combination with IL-17 (Fig 5.3 A). In one sample there was a very low level of RANKL mRNA expression in response to the combination of IL-17 with OSM at 24 hr (Fig 5.3 B). However the general pattern of the results shows that there is a down-regulation in the expression of RANKL mRNA by the combination of IL-17 and OSM.

5.3.2.2 Effect of the combination of IL-17 with OSM on the expression of OPG in SFB (OA)

The effects of IL-17 and OSM on the expression of OPG mRNA by synovial fibroblasts isolated from patients with OA were investigated by Northern blot and Quantitative-PCR analysis. The pattern of results shows that OPG mRNA is expressed in cells treated with IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) at 6 hr, but there was no increase in expression of OPG by the combination of IL-17 with OSM. The expression of OPG mRNA was increased dose dependently by IL-17 and OSM alone at 48 hr but there was no increase in the expression of OPG by the combination of IL-17 and OSM. In another sample OPG

mRNA was expressed in cells treated for 24 hr and there was down-regulation of OPG mRNA by the combination of IL-17 and OSM (Fig 5.4). Furthermore I have analysed by quantitative PCR another five samples from patients with OA. The cells were treated as previously and in general the results show that OPG mRNA is expressed in response to IL-17 alone but there was down-regulation when IL-17 and OSM are added together at 6 hr (Fig 5.5).

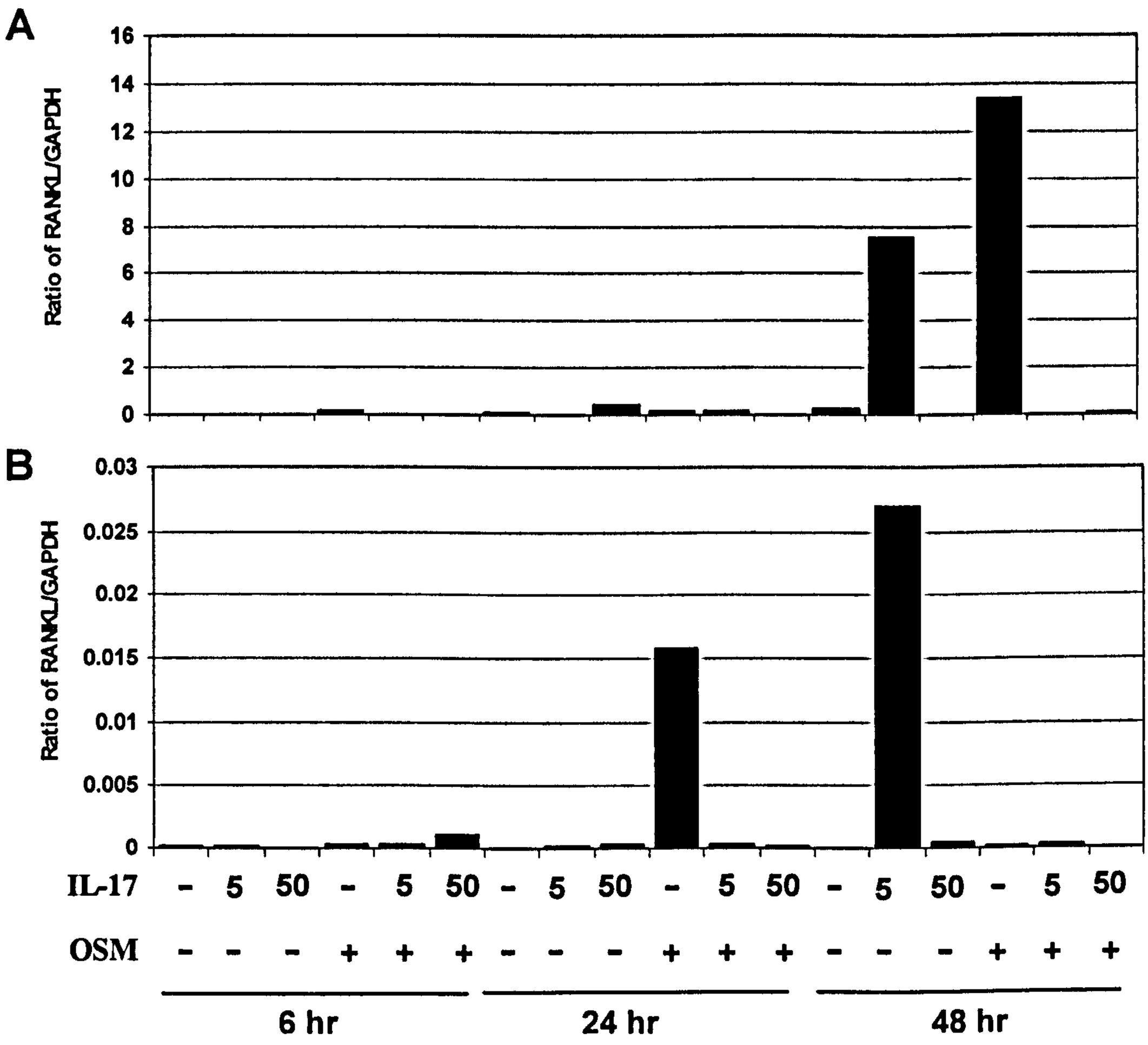


Fig 5.2 Effect of IL-17 combined with OSM on the expression of RANKL by OA SFB. SFB cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and IL-17 (5 or 50 ng/ml) combined with OSM (10 ng/ml). Total RNA was analyzed by Quantitative PCR analysis with RANKL primers using the conditions described in Materials and Methods. The data are presented relative to GAPDH. Two independent OA samples (A) and (B) were used.

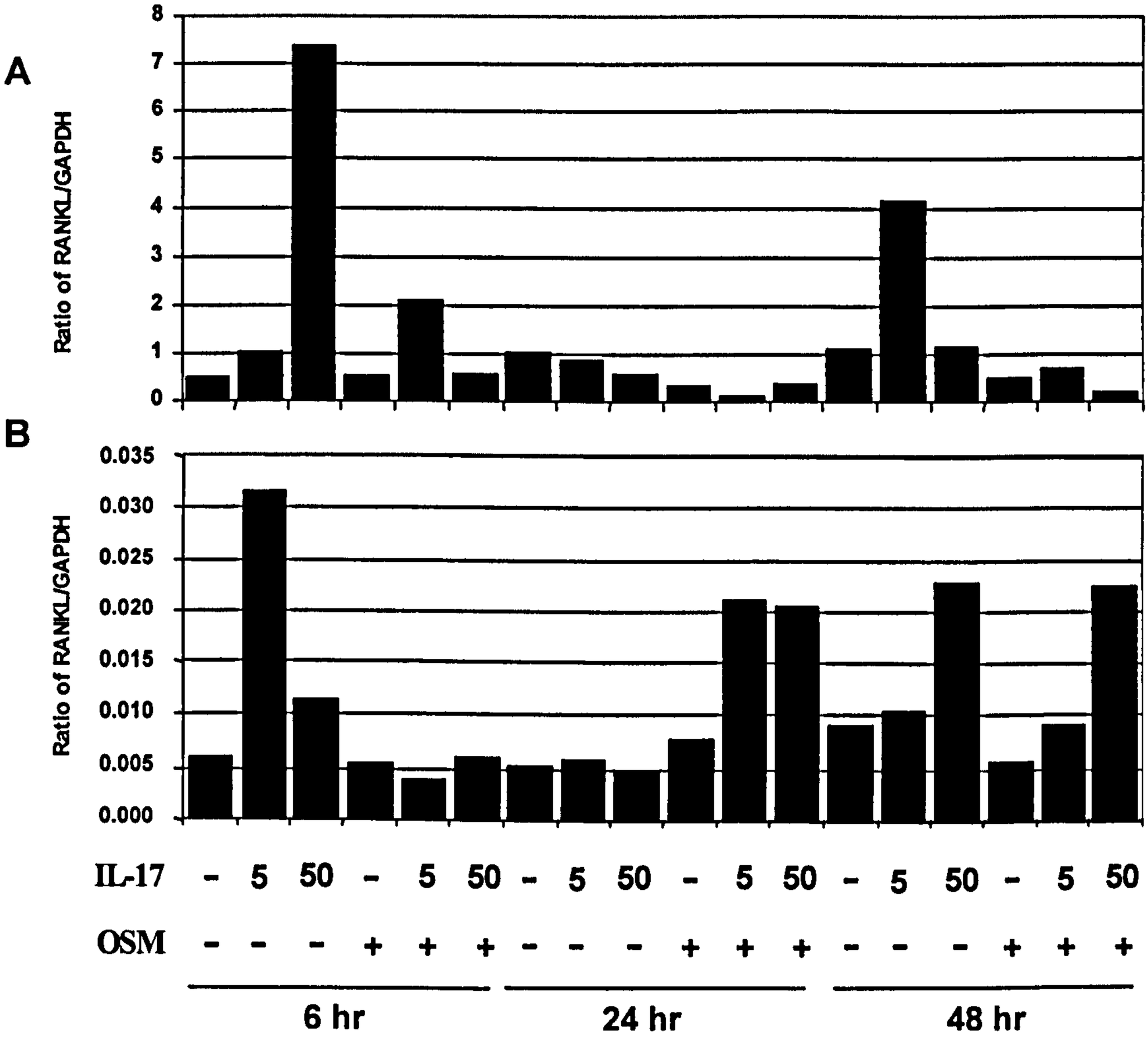


Fig 5.3 A and B Effect of IL-17 combined with OSM on the expression of RANKL by SFB cells from OA patient. SFB cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and by the combination of IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml). Total RNA was analyzed by Quantitative PCR analysis with RANKL primers using the conditions described in Materials and Methods. The data are presented relative to GAPDH.

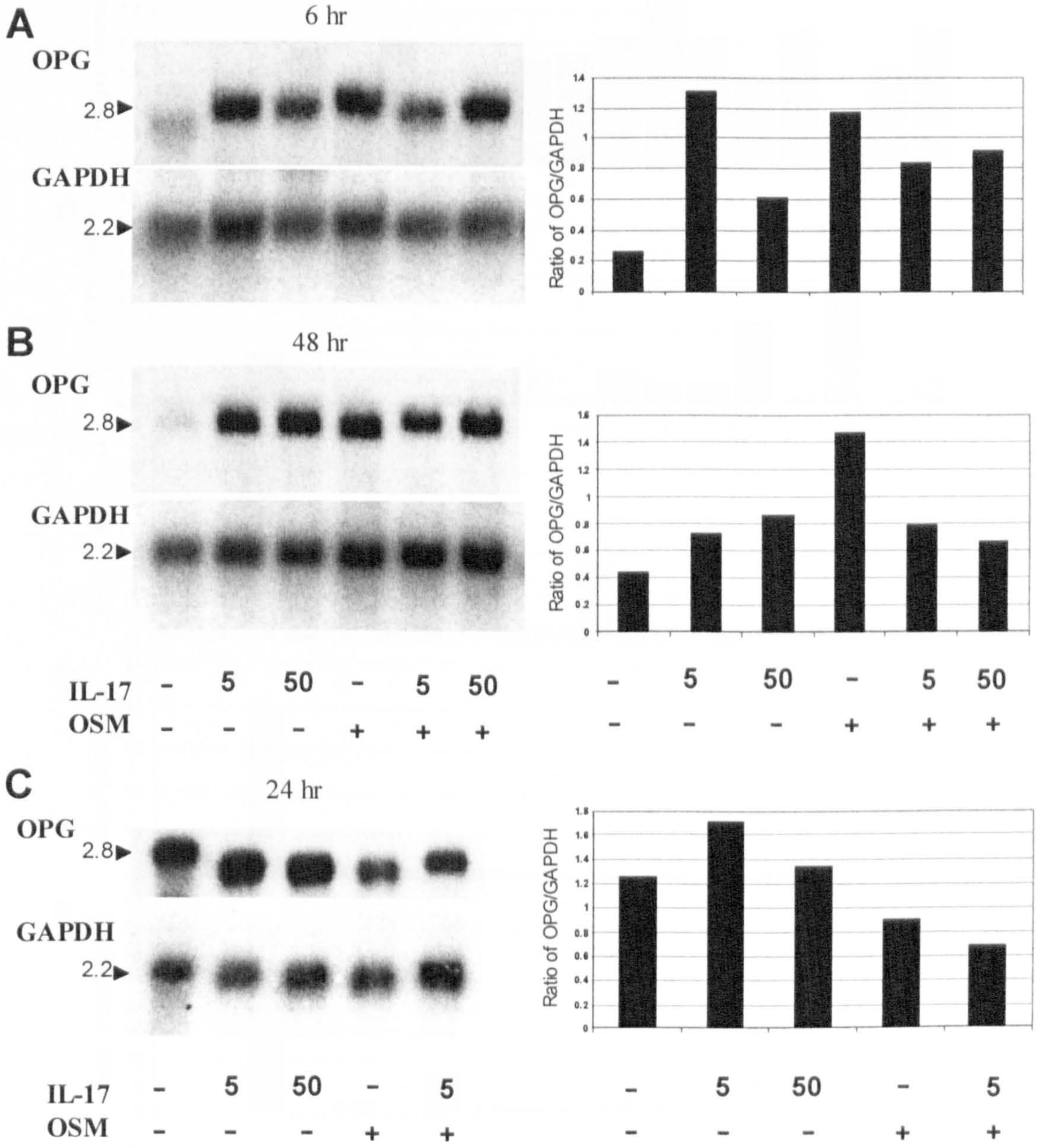


Fig 5.4 Effect of IL-17 and the combination of IL-17 and OSM on the expression of OPG by SFB from OA patients. SFB cells were cultured, serum reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and by the combination of IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml. RNA was extracted using trizol reagent. Equal amounts of RNA were electrophoresed using 1% agarose/0.4M Formalehyde and Northern analysis performed. GAPDH was used to normalise for RNA loading. **A** and **B** from the same patient, and **C** from a second patient.

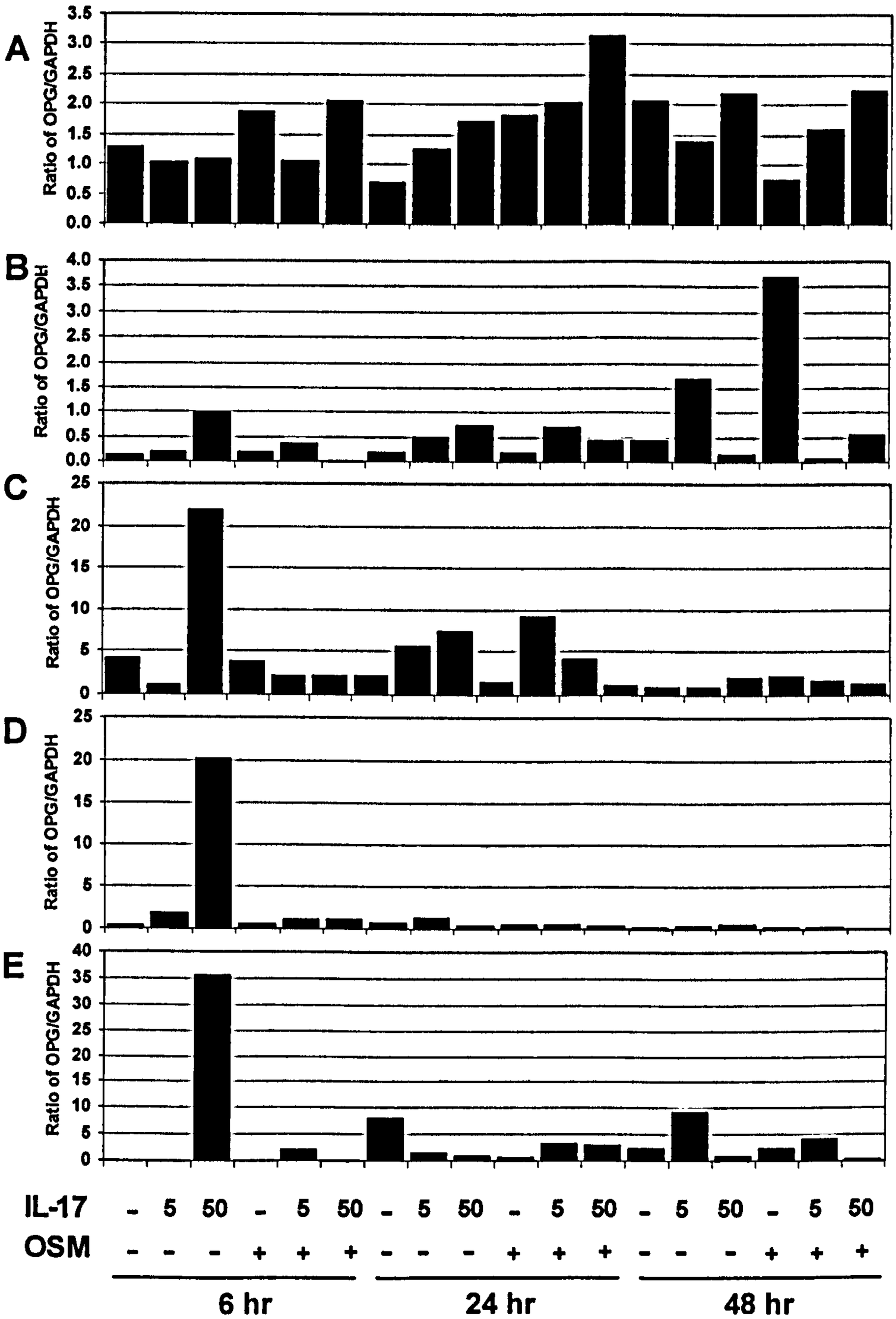


Fig 5.5 Effect of IL-17 and OSM on the expression of OPG by SFB from five different OA patients. SFB cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and by IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml). Total RNA was analyzed by Quantitative PCR analysis with OPG primers using the conditions described in Materials and Methods for five independent samples (A-E).

5.3.2.3 The effect of the combination of IL-17 and OSM on the production of OPG by SFB (OA)

The effects of IL-17 and OSM on the production of OPG by synovial fibroblasts (OA) were determined by Western analysis. The conditioned media from two independent experiments were compared. The pattern of results show that OPG production was increased in the conditioned media from cells treated with IL-17 (50 ng/ml) and OSM alone in both samples at 6 hr. OPG production was increased by IL-17 (5 ng/ml) but not by OSM in the first sample and by 17 (50 ng/ml) and OSM in the second sample at 24 hr. There was an increase in OPG production by IL-17 (5 and 50 ng/ml) by the first sample and by IL-17 (50 ng/ml) in the second sample but not by OSM at 48hr. However, there were no synergistic effects of IL-17 and OSM on the production of OPG by synovial fibroblast (Fig 5.6).

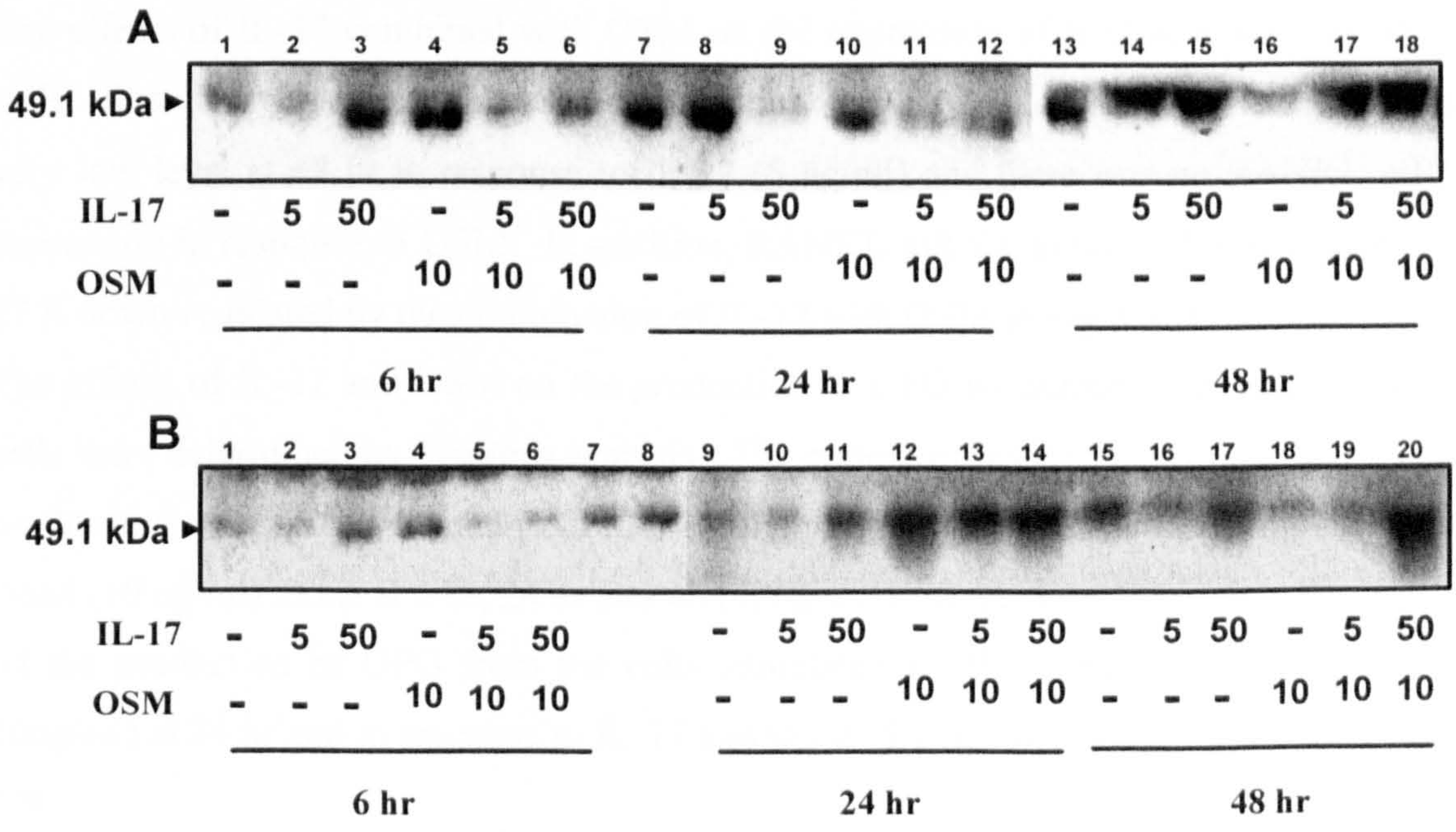


Fig 5.6 The effect of the combination of IL-17 and OSM on the production of OPG by SFB OA. SFBs were cultured, serum reduced overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and by the combination of IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml). The conditioned media collected for Western blot analysis. (**A**) First sample lanes 1-12 run on a separate gel. (**B**) Second sample lanes 1-8 were run on a separate gel and samples 7 and 9, and 8 and 10 are duplicate samples. These data were representative of the pattern of two independent experiments.

5.3.3 Effects of the combination of IL-17 with OSM on the expression of RANKL and OPG mRNA and production of OPG by MG-63

The effects of IL-17 combined with OSM on the expression of OPG mRNA by the human osteosarcoma cell line MG-63 was assessed by Northern blot analysis. The pattern of results show that OPG mRNA was expressed by the cells treated with IL-17 (50 ng/ml) and cells treated with OSM (10 ng/ml) at 48 hr but there was no increase in the expression by the combination of IL-17 with OSM (Fig 5.7). Furthermore I have analysed by quantitative PCR another experiment using MG-63 cells after several passages. The cells were treated as previously described and the patterns of results show that OPG mRNA is expressed in response to IL-17 alone at 48 hr but there were no synergistic effects by the combination of IL-17 with OSM (Fig 5.8 A).

The effects of IL-17 combined with OSM on the expression of RANK mRNA by MG-63 cells. Results by quantitative PCR analysis show that RANKL mRNA was expressed at a very low level at 48 hr in response to IL-17 (5 ng/ml) and there was no RANKL mRNA expression in response to OSM. In addition, RANKL mRNA expressed in response to IL-17 is down-regulated by the combination of IL-17 with OSM in Fig 5.8 B.

The effects of IL-17 and OSM on the production of OPG by human osteosarcoma MG-63 cells were determined by Western analysis. The pattern of results shows that the OPG was produced from the cells treated with IL-17 (50 ng/ml) alone at 6 hr, 24 hr and 48 hr and by OSM (10 ng/ml) alone at 6 hr, 24 hr and 48 hr. However there were little synergistic effects on the production of OPG from the cells stimulated by IL-17 and OSM (50ng/ml with 10ng/ml) at 24 hr and in response to IL-17 and OSM (5ng/ml with 10ng/ml) at 48 hr in (Fig 5.9).

5.3.4 Effects of the combination of IL-17 with OSM on the expression of RANKL and OPG mRNA and production of OPG by the human osteosarcoma cell line, SaOS-2

The effects of IL-17 and OSM on the expression of OPG mRNA by human osteosarcoma SaOS-2 cell line, was investigated by Northern blot analysis. The pattern of results show

that OPG mRNA was expressed from the cells treated with OSM (10 ng/ml) at 6 hr but there was no increased in the expression of OPG mRNA by the combination of IL-17 with OSM. The expression of OPG mRNA remains steady at 24 hr. OPG mRNA is expressed following treatment with IL-17 dose dependently and there was a little synergistic increase in its expression when both cytokines IL-17 (50 ng/ml) and OSM (10 ng/ml) are added at 48 hr (Fig 6.10). Further analysis was done by quantitative PCR, using SaOS-2 cells after several passages, treated as previously described. OPG mRNA is expressed following treatment with IL-17 (5 ng/ml) alone and in response to OSM alone at 6 hr while there was no increase in expression of OPG mRNA following treatment with IL-17 combined with OSM at 6 hr. OPG mRNA is expressed following treatment with OSM at 24 hr and there was a little synergistic effect in response to the combination of IL-17 (50 ng/ml) and OSM together at 24hrs. There was no increase in expression of OPG mRNA responses to IL-17 (5 and 50 ng/ml) but OPG mRNA is expressed following treatment with OSM at 48 hr and there was some synergism in response to the combination of IL-17 (5 ng/ml) and OSM at 48hr (Fig 6.11 A).

The effect of IL-17 and OSM on the expression of RANKL mRNA by human osteosarcoma SaOS-2 cell line was also studied. The results show that these cells did not respond to the treatment with changes in the abundance of RANKL mRNA (Fig 6.11 B).

The effects of IL-17 combined with OSM on the production of OPG by human osteosarcoma SaOS-2 cells were also studied by Western blot analysis. The pattern of results shows that OPG is produced following IL-17 (5 and 50 ng/ml) treatment and also in response to OSM (10 ng/ml) at 6 hr. The production of OPG was increased in response to IL-17 (5-50 ng/ml) at 24 hr and also at 48 hr. However, there were no synergistic effects of combining IL-17 and OSM on the production of OPG from these cells (Fig 5.12).

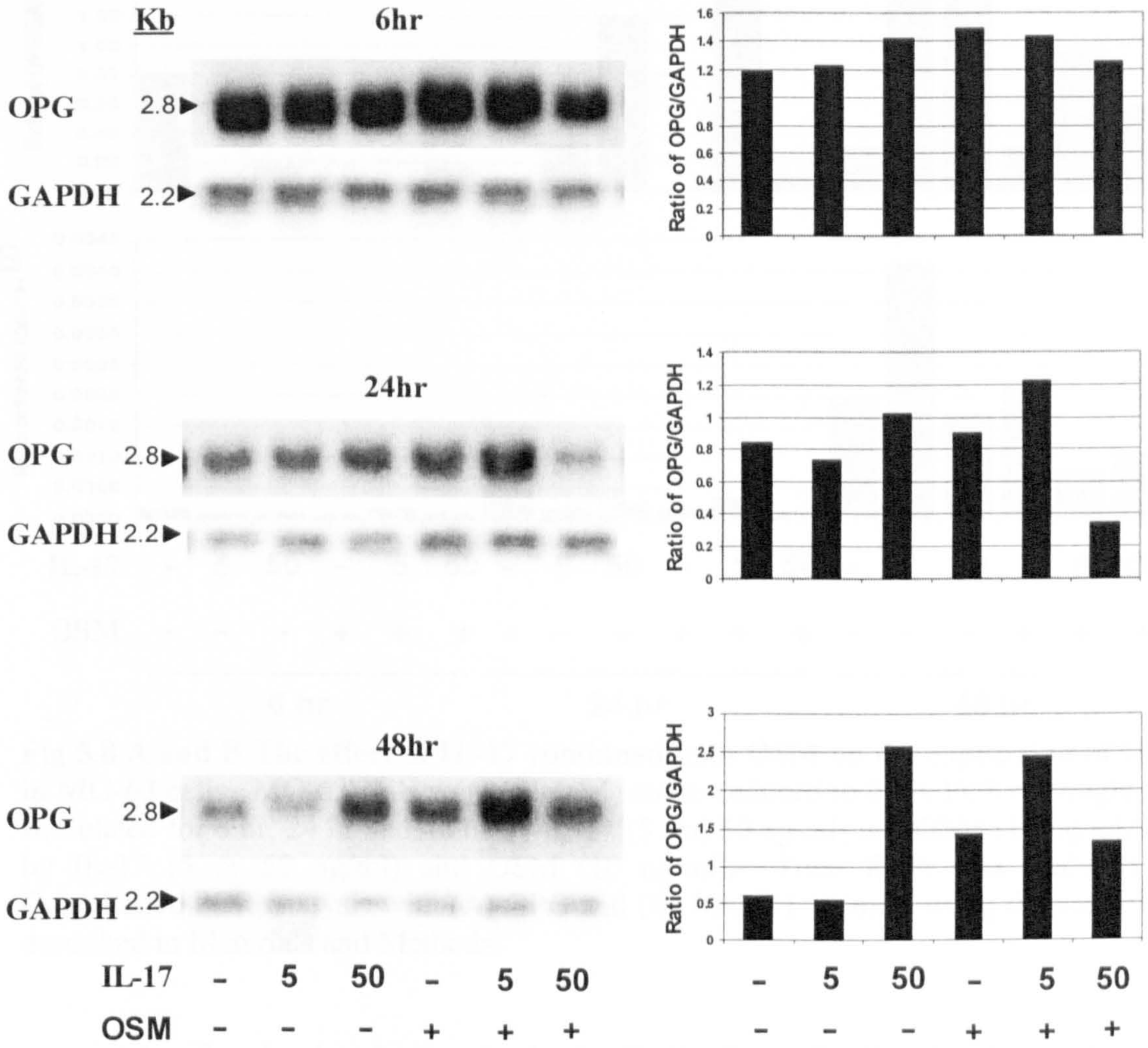


Fig 5.7 The effect of IL-17 and the combination of IL-17 with OSM on the expression of OPG by human osteosarcoma MG-63 cell line. MG-63 cells were cultured, serum reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and the combination of IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml. RNA was extracted using trizol reagent. Equal amounts of RNA were electrophoresed using 1% agarose/0.4M Formaldehyde and Northern analysis performed. GAPDH was used to normalise for RNA loading.

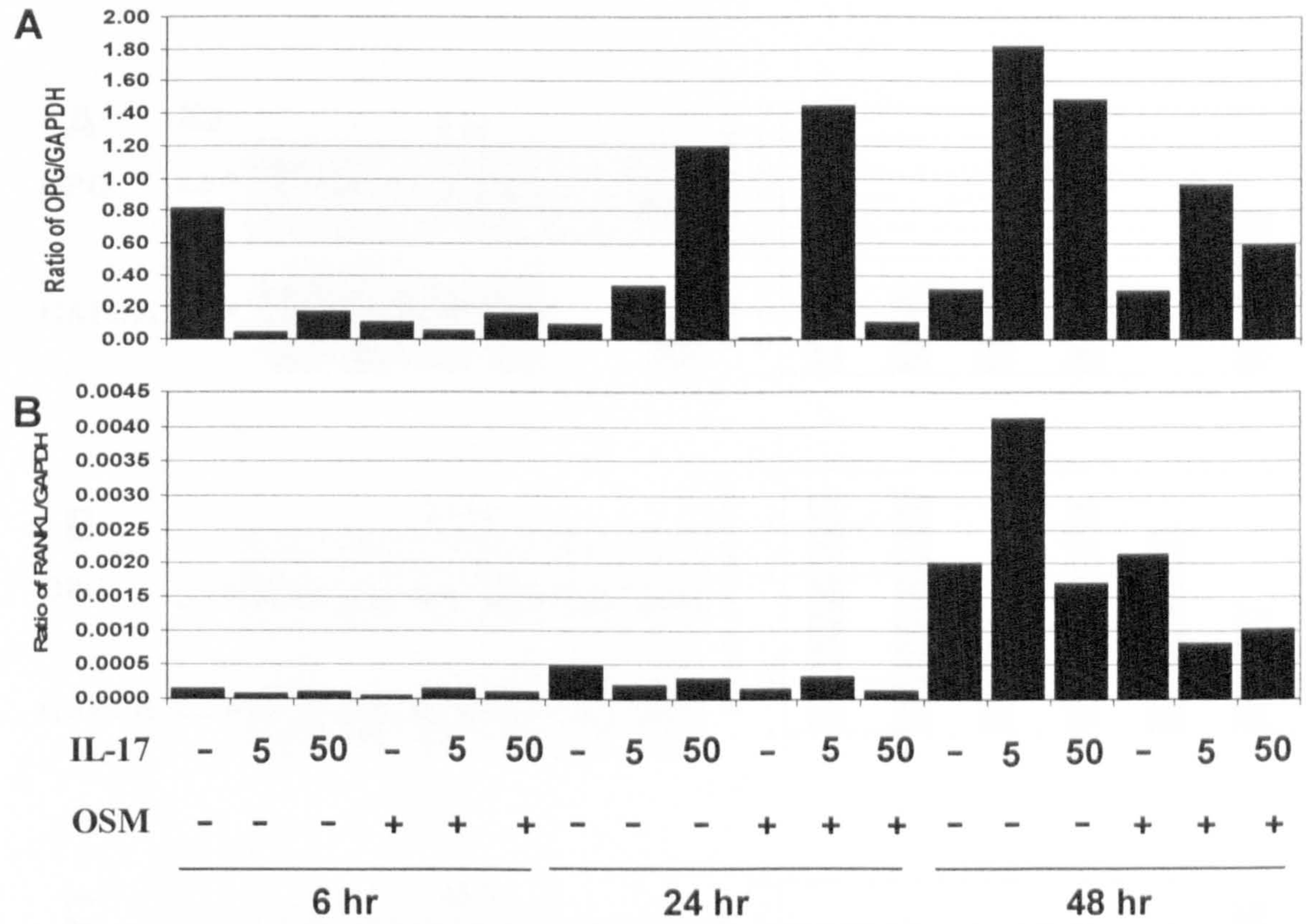


Fig 5.8 A and B The effect of IL-17 combined with OSM on the expression of OPG in MG-63 cells. MG-63 cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and by IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml). Total RNA was analyzed by Quantitative PCR analysis with (**A**) OPG and (**B**) RANKL primers using the conditions described in Materials and Methods.

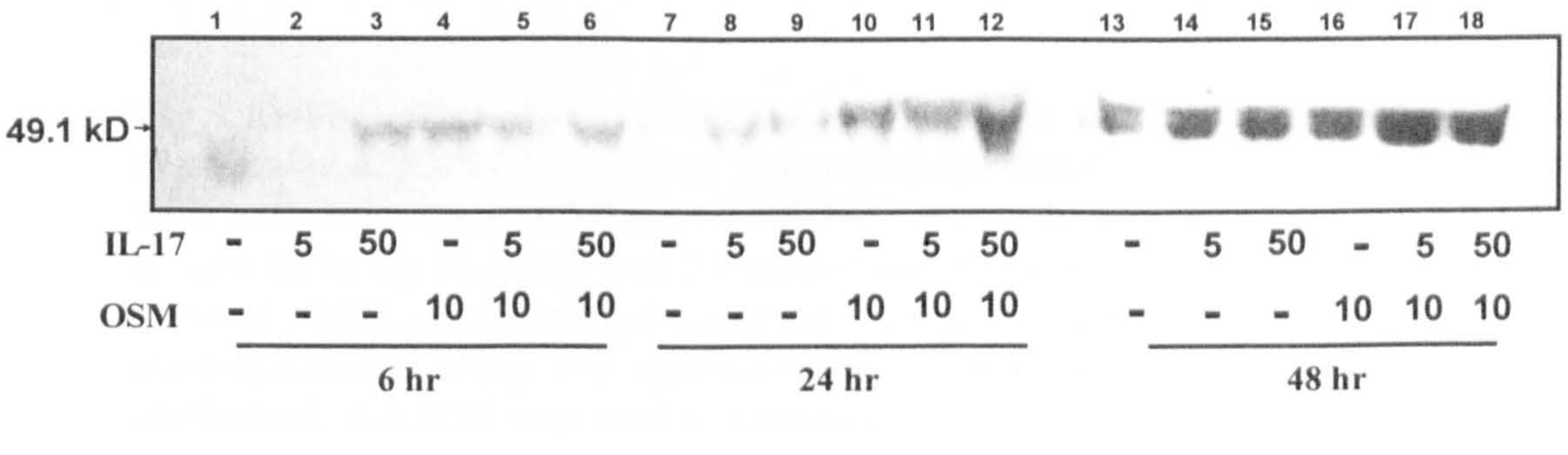


Fig 5.9 The effect of the combination of IL-17 combined with OSM on the production of OPG by MG-63. MG-63 cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and by the combination of IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml). The conditioned media were collected for Western blot analysis. Lanes 1-12 were run on a separate gel. These data were representative of the pattern of three independent experiments.

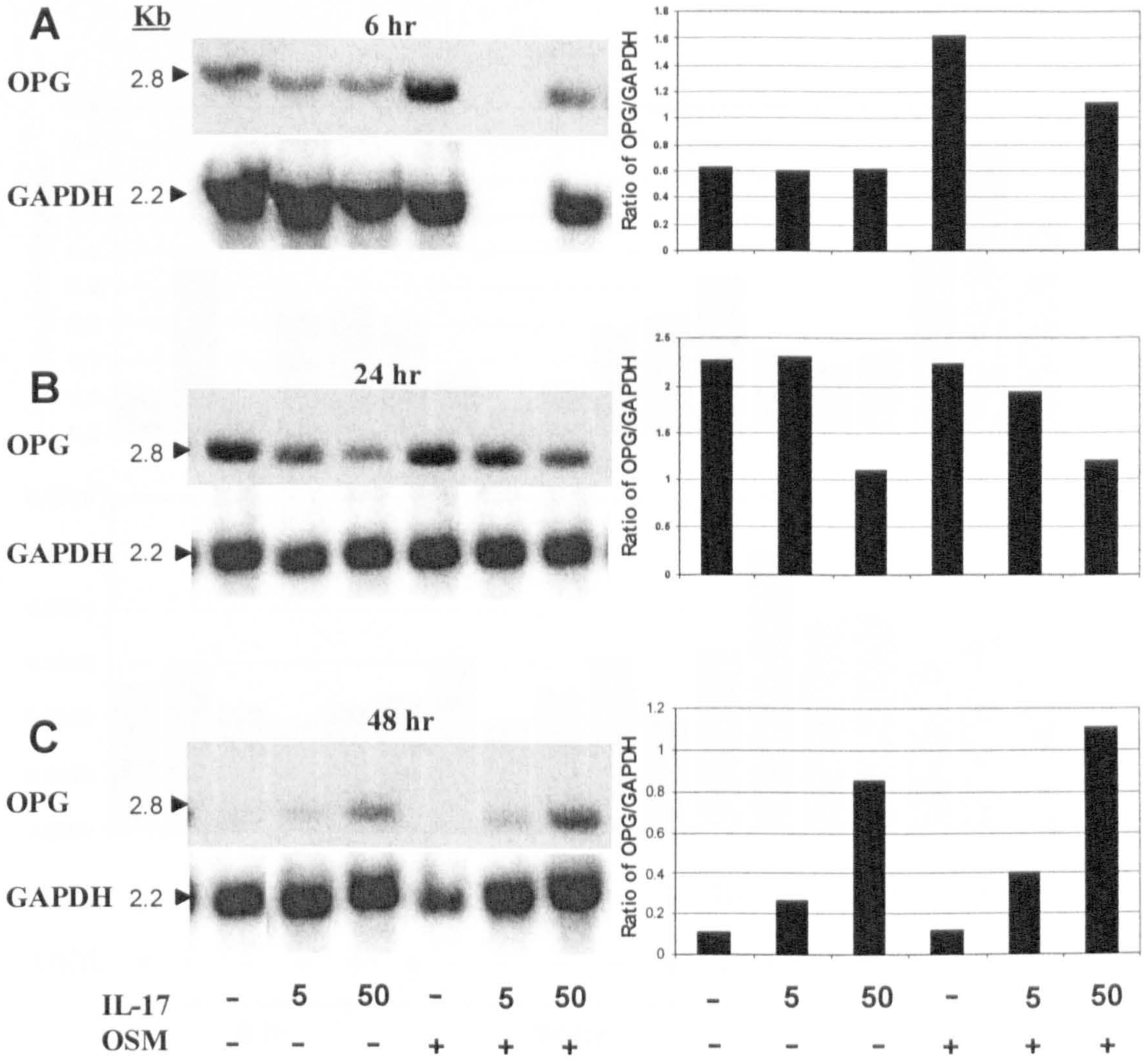


Fig 5.10 The effect of IL-17 and the combination of IL-17 with OSM on the expression of OPG by human osteosarcoma SaOS2 cell line. SaOS2cells were cultured, serum reduced to 0.5% FCS overnight and stimulated for 6.hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) or combinations these of. RNA was extracted using trizol reagent. Equal amounts of RNA were electrophoresed using 1% agarose/0.4M Formalehyde and Northern analysis performed. GAPDH was used to normalise for RNA loading.

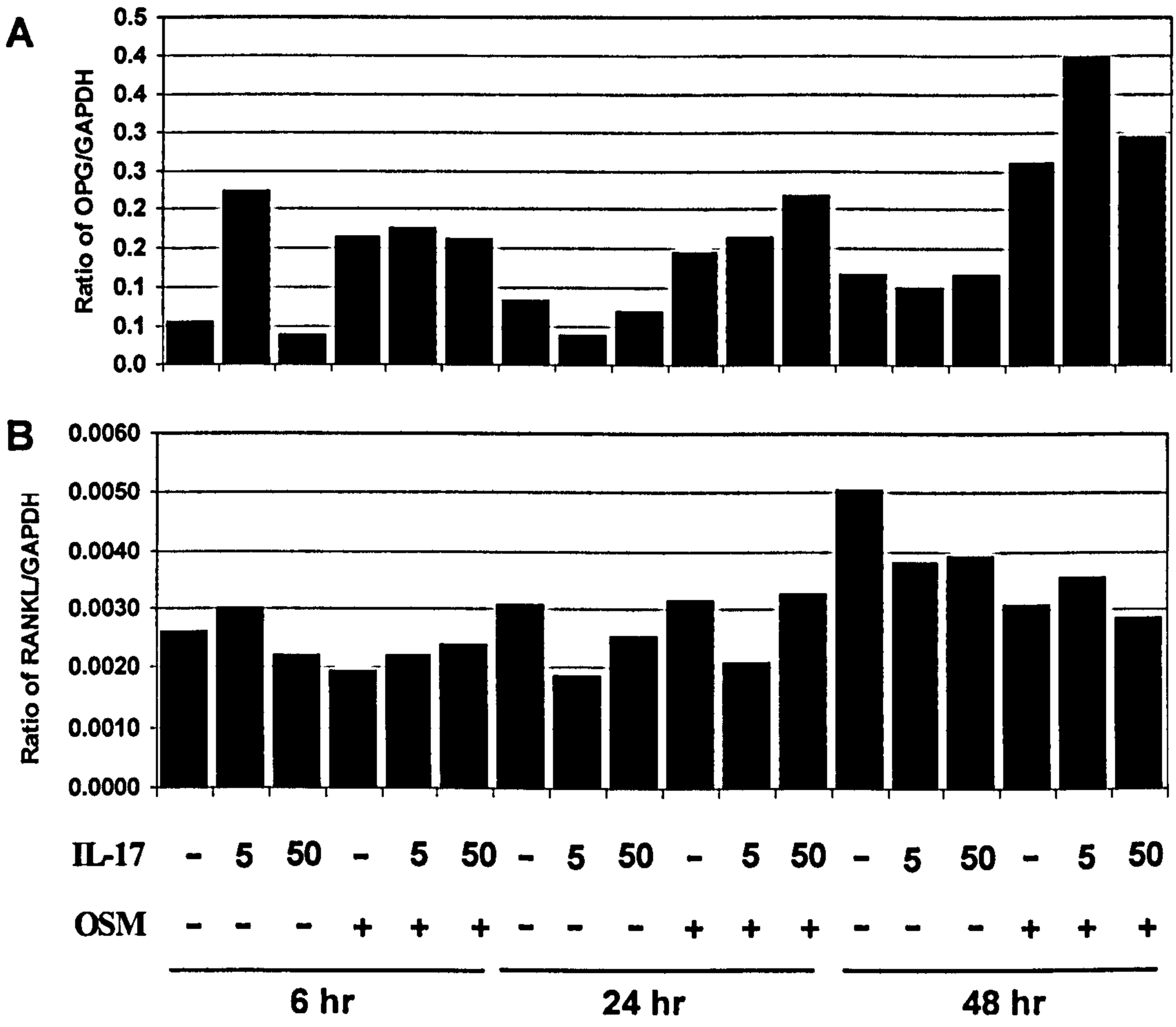


Fig 5.11 A and B The effect of IL-17 combined with OSM on the expression of RANKL in SaOS-2 cells. SaOS2 cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and by IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml). Total RNA was analyzed by Quantitative PCR analysis with (**A**) OPG and (**B**) RANKL primers using the conditions described in Materials and Methods. The results are expressed relative to GAPDH for imperative purposes.

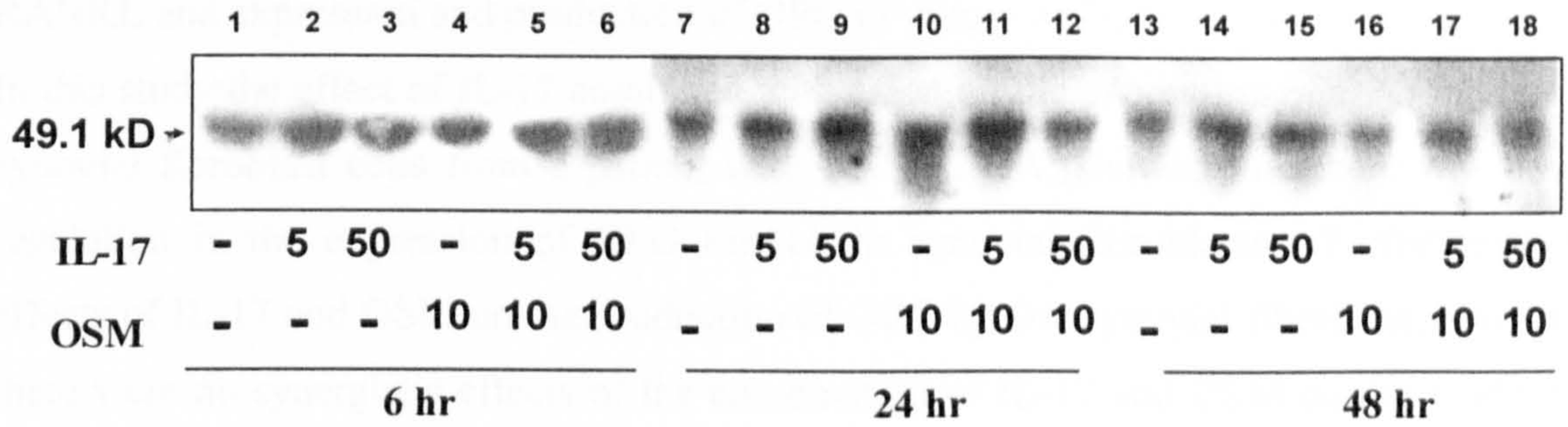


Fig 5.12 The effect of the combination of IL-17 combined with OSM on the production of OPG by SaOS-2 cells. SaOS2 cells were cultured, serum-reduced to 0.5% FCS overnight and stimulated for 6 hr, 24 hr and 48 hr by IL-17 (5 and 50 ng/ml) and OSM (10 ng/ml) and by the combination of IL-17 (5 or 50 ng/ml) and OSM (10 ng/ml). The conditioned media were collected for Western blot analysis. Lanes 1-6 were run on a separate gel.

5.4 Discussion

The activated T cell cytokine, IL-17 has been shown to promote bone and cartilage destruction, through PGE2, RANKL, IL-6, GM-CSF, IL-8 and M-CSF release in vivo and in vitro (Lubberts E et al., 2004; Yamamura Y et al., 2001; Chabaud M et al., 2001; Yao, Z. et al., 1995). However, IL-17 is not a solitary cytokine in the joints of arthritic patients, and a possible immuno-modulatory role has been suggested for IL-17 (Katz et al., 2001). Previous studies have shown that the destructive potential of IL-17 is possibly amplified in the presence of other pro-inflammatory cytokines, leading to a synergistic release of cartilage and bone degradation factors (Chabaud M et al., 2001). In addition, the response of chondrocytes is amplified after stimulation with two pro-inflammatory cytokines, including combinations of IL-1, OSM, IL-6/sIL-6R and TNFα (Cawston et al., 1995, 1998; Rowan et al., 2001; Hui et al., 2003).

As such, the present study has investigated the hypothesis; "IL-17 potentiates increased bone catabolism through interaction with other pro-inflammatory cytokines". The main

focus of this chapter is the effect of IL-17 combined with OSM on the expression of RANKL and expression and production of OPG in vitro.

In this study the effect of IL-17 combined with OSM on the expression of OPG mRNA by synovial fibroblast cells from a patient with RA and OA, showed that there was down-regulation in the expression of OPG mRNA in synovial fibroblasts. Furthermore the effects of IL-17 and OSM on the production of OPG by OA synovial fibroblasts show that there were no synergistic effects of the combination of IL-17 and OSM on the production of OPG by synovial fibroblast.

The effect of IL-17 combined with OSM on the expression of RANKL mRNA by RA and OA synovial fibroblast cells showed that there was down-regulation in the expression of RANKL mRNA.

From these results from RA and OA synovial fibroblasts, I suggest that the presence of both of these cytokines in the same area together may modulate effects of each other on the expression of RANKL and OPG and also on the production of OPG. The important finding in this study that the effect of the combination of IL-17 and OSM is that leads to the decreased expression of RANKL mRNA in arthritis (which may lead to an decrease in bone and cartilage degradation). In contrast, Koshy PJ. et al., (2002) reported that IL-17 can, alone and synergistically in combination with other proinflammatory cytokines such as TNF-alpha, IL-1, OSM and IL-6 promote chondrocyte mediated MMP dependent type II collagen release from cartilage and this effect is significantly reduced by IL-4, IL-13, TGF-beta1, and IGF-1. Koshy PJ. et al., (2002) suggested that IL-17 may act as a potent upstream mediator of cartilage collagen breakdown in inflammatory joint diseases. However, IL-17 may in combination with other proinflammatory cytokines have effects on cartilage and bone degradation through the release of factors that cause osteoclastogenesis (Kotake S. et al. 1999). In addition, Langdon C. et al., (1997) and Hui W et al., (1997) both demonstrated that OSM was detectable in variable levels in synovial fluids of RA patients but not in OA patients and Langdon C. et al., (1997) suggested that OSM has a potentially important function in the modulation of chemokine and MMPS production by synovial cells of the joint. In addition, adenoviral over expression of OSM induces increased inflammation and destruction in murine joints in vivo (Langdon et al., 2000; de Hooge AS et al., 2002). However, these observations suggest that OSM may have another role in

combination with IL-17 modulating the expression of RANKL and OPG. Because OSM favours bone apposition at periosteal sites instead of resorption in vivo. In spite of the expression of RANKL and its receptor RANK, in the periosteum, osteoclasts were not detected at sites of bone apposition. (de Hooge AS. et al., 2002)

In this study human osteoblast-like MG-63 and SaOS2 cell lines showed some synergestic effects on the expression of OPG mRNA in response to the combination of IL-17 and OSM. While the effect of the combination of IL-17 and OSM on the expression of RANKL mRNA by MG-63 and SaOS2 showed that RANKL mRNA is down-regulated. In addition, the effects of IL-17 and OSM on the production of OPG by MG-63 cells, showed a little synergistic effect but there were no synergistic effects of combining of IL-17 and OSM on the production of OPG by SaOS2 cells. From this study I concluded that synovial fibroblasts from patients with RA and OA and human osteosarcoma cell lines (MG-63 and SaOS2 cells) show that the combination of IL-17 and OSM down-regulated expression of RANKL mRNA and OPG mRNA in most of our samples from arthritis but there is some synergistic effect on the production of OPG by SFB (OA) at 48 hr and MG-63 at 24 hr and 48 hr.

However, previous studies demonstrated that IL-17 enhanced RANKL and OPG expression in mice fibroblasts and osteoblasts (Kotake S. et al. 1999). Also induction of IL-17 is considered as an early stage in the arthritis (Buch KA et al., 2001). Thus, a broad set of effects are induced by IL-17, and its action can be potentiated by other cytokines.

In this chapter, synergistic effects could be expected because both of the cytokines have different receptors belonging to different families. IL-17 activates NF-kB and/or AP1 while OSM transduces its signal via heterodimeric receptor complexes consisting of two subunits. The first is the glycoprotein gp130 which is universal for all of the IL-6 family members, and the second receptor complex subunit can be either the LIF receptor-β or OSM receptor-β (Mosley et al., 1996). In mice, OSM only utilizes the gp130/OSM receptor-β complex. Stimulation with OSM leads to activation of the mitogen activated protein kinase (MAPK) cascades and the Janus kinase signal transducer and activator of transcription (JAK/STAT) pathway, and modulation of AP-1 (Taga and Kishimoto 1997). In the cytoplasm, the receptor molecules transmit the signal to the ras–raf extracellular-response kinase 2 (ERK2) and MAPK pathway or Jak to STAT (Heinrich PC. et al., 2003;

Lin SK. et al., 2004; Palmqvist N. et al., 2002). After receptor activation and oligomerization, signal transduction pathways become activated. Subsequently, STAT factors translocate to the nucleus and initiate transcription of acute phase response genes (Mori S. et al., 1999). In my studies there was no synergism but there was decreased expression of RANKL and a little or no increased expression and production of OPG in MG-63 and SaOS-2 respectively. These finding could be due to the production of some other regulatory cytokines such as IL-4, IL-13 or IL-10 from these cells. Alternatively, the downstream signaling pathway of IL-17 cross through ras as reported in IEC-6 cells that IL-17-mediated activation of extracellular signal regulated kinase mitogen-activated protein kinases was mediated through ras, c-Jun N-terminal kinase activation was dependent on functional TRAF6. These data suggest that NF-kappa B-inducing kinase serves as the common mediator in the NF-kappa B signaling cascades triggered by IL-17, TNF-alpha, and IL-1 beta in intestinal epithelial cells (Awane M, et al., 1999). Furthermore, previous studies have highlighted the activation of MAPK cascades, STAT protein, NF-kB and AP-1 binding complexes in IL-17 signaling, although their exact roles can differ between cell types and gene production of interest (Reviewed by Miossec P. et al., 2003; Shalom-Barak T et al., 1998)

In addition, an initial study reported that OSM regulates the expression of Tissue Factor (TF) by vascular smooth muscle cells (SMCs) in a biphasic manner leading to sustained pattern of TF mRNA. The effect of OSM on TF mRNA expression is regulated at the transcriptional level. These authors suggest that the induced expression of TF by OSM is primarily through the activation of NF-kappaB and that activation of NF-kappaB is regulated in part by the MEK/Erk-1/2 signal transduction pathway (Nishibe T. et al., 2001). In addition OSM induces chemokine (C-C motif) ligand 2 (CCL-2) expression in osteoblasts. Activation of the MEK/ERK and STAT pathways, which leads to c-Fos expression and AP-1-DNA binding, is involved in this process (Lin SK et al., 2004).

Both of cytokines, used in this study, signal through the same pathway which may lead to hyper-phosphorylation of the same signal pathway proteins leading to down-regulation of other downstream signal pathways (Fig 5.13). The exact proportion and function of IL-17 and OSM remain to be determined. Conversely, control of such an effect may lead to an anti-inflammatory therapeutic.

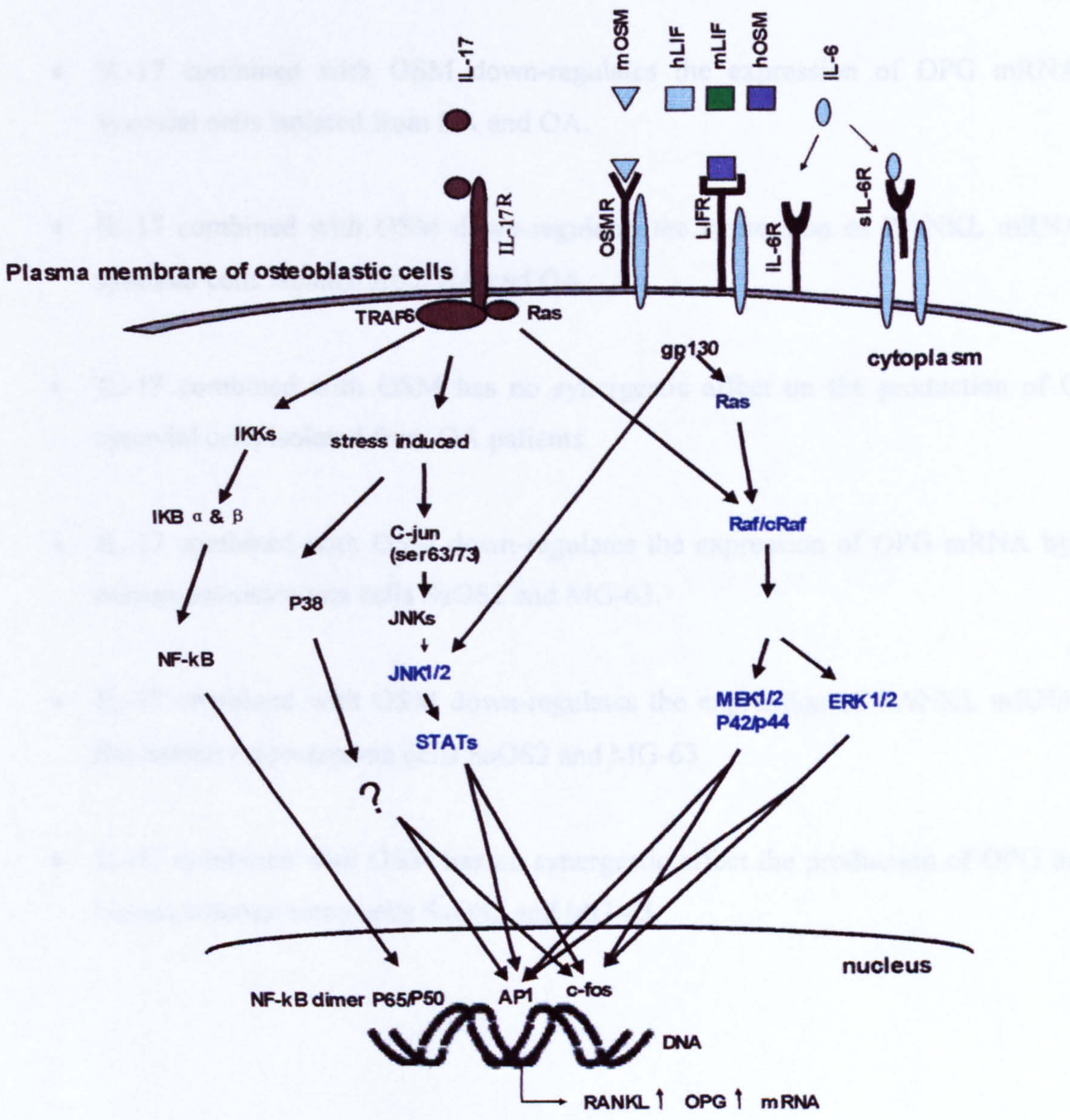


Fig 5.13 Possible mechanisms of signal transduction of IL-17 and OSM. IL-17 and OSM have been shown to activate TRAF6, NF-kB and MAPKs cascade pathway. These factors may influences RANKL and OPG gene expression (Schwandner R. et al., 2000; Shalom-Barak et al., 1998; Martel-Pelletier et al., 1999; Van Bezooijen RL. et al., 2001; Aggarwal S. and Gurney AL. 2002; Palmqvist N. et al., 2002).

5.4.1 In this chapter I have demonstrated that:

- IL-17 combined with OSM down-regulates the expression of OPG mRNA in synovial cells isolated from RA and OA.

- IL-17 combined with OSM down-regulates the expression of RANKL mRNA in synovial cells isolated from RA and OA.

- IL-17 combined with OSM has no synergestic affect on the production of OPG synovial cells isolated from OA patients

- IL-17 combined with OSM down-regulates the expression of OPG mRNA by the human osteosarcoma cells SaOS2 and MG-63.

- IL-17 combined with OSM down-regulates the expression of RANKL mRNA by the human osteosarcoma cells SaOS2 and MG-63

- IL-17 combined with OSM has no synergestic affect the production of OPG in the human osteosarcoma cells SaOS2 and MG-63.

CHAPTER-6

Osteoclastogenesis

6 Osteoclastogenesis

6.1 Introduction

Bone loss is a major unsolved problem in RA, a common autoimmune disease characterized by chronic synovial inflammation and hyperplasia culminating in joint destruction (Feldmann, M., 1996; Mulherin, D., 1996). The skeletal complications of RA consist mainly of erosions of marginal and subchondral bone, juxtaarticular osteoporosis, and universal bone loss with reduced bone mass. The consequences of this profound bone loss are pain, joint deformity, progressive functional disability, an increased risk of bone fractures, and increased mortality rates. The skeleton is constantly remodelled and regenerated by a dynamic process of resorption and replacement. Osteoclastic activation is the initial step in the remodelling sequence. Osteoclasts are large, multinucleated cells that arise by fusion of committed precursors that originate from haematopoietic progenitors of the monocyte-macrophage lineage. Osteoclasts form a ruffled membrane rim against the bone surface and secrete a multiplicity of specialized factors, including proteases, collagenases, phosphatases, and protons that break down the bone matrix, releasing mineral ions into the extracellular fluid (Suda, T. et al. 1992, Roodman, G 1996, Itonaga, I. et al. 2000; Weilbaecher 2000).

Numerous other adult skeletal diseases are due to inappropriate osteoclastic activity, leading to an imbalance of bone remodelling, which favours resorption. Such diseases include osteoporosis, periodontal disease, multiple myeloma and metastatic cancers. For individuals with osteoporosis, bone fractures represent life-threatening events, and today there are large numbers of people worldwide at danger. Recently, our understanding of osteoclast differentiation and activation has advanced rapidly with the discovery of a family of biologically related TNFR/TNF-like proteins: OPG, RANK and RANKL, which together profoundly control osteoclast function (Matsuzaki K. et al. 1998; Itonaga, I. et al. 2000; Wong BR. et al., 1997; Anderson DM. et al., 1997). The study of this pathway is providing a deeper understanding of how diverse physiological and pathophysiological signals exert their effects and induce osteoclastogenesis, bone resorption and skeletal remodeling, and so manage bone mass. RANKL can also stimulate mature osteoclasts to resorb bone and increase the fusion of mononuclear precursor osteoclasts and the survival of multinucleated osteoclasts (Burgess, TL. et al., 1999; Jimi, E. et al., 1999).

Much of our understanding of osteoclastogenesis has come from in vitro studies in which cells are grown in the presence of osteoblasts, stroma, growth factors, and/or hormones. These studies demonstrated that close contact between stromal and bone marrow cell types was essential for osteoclastogenesis, and suggested that stromal-derived factors stimulate this process. It is now known that this organization allows production of two haematopoietic factors that are both required and sufficient for osteoclastogenesis, the TNF-related cytokine, RANKL and the polypeptide growth factor, M-CSF. Together, M-CSF and RANKL are necessary to stimulate expression of genes that characterize the osteoclast lineage, including those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK) and the calcitonin receptor leading to the development of mature osteoclasts (Matsuzaki K. et al. 1998). RA is characterized as both an autoimmune reaction initiated by lymphocytes and proliferation of inflamed synovial membrane accompanied by inflammatory cell infiltration and bone damage. Bone erosion at the site of RA lesions is caused by osteoclasts, and this site is the so-called bare area (Goldring SR., 2002; Bromley M.; Wooley DE. 1984).

TNF-α is a pro-inflammatory cytokine believed to play a key role in the inflammatory response in RA. Sabokbar A. et al. (2003); Kudo O. et al., (2002) established that TNF- α in the presence of M-CSF is sufficient to induce human osteoclast differentiation from arthroplasty cells derived from tissue isolated at joint replacement surgery and that TNF-α acts synergistically with IL-1-α to stimulate lacunar resorption in mouse cultures. This process is distinct from the RANK/RANKL signaling pathway and is likely to operate in periprosthetic tissues when there is heavy wear particle deposition and cytokine production. Interleukin-17 is a recently identified proinflammatory cytokine that displays a number of pleiotropic features. IL-17 is produced by activated human memory T lymphocytes ($CD45^{+}RO^{+}$), as well as by $\alpha\beta TCR^{+}$; $CD4^{-}/CD8^{-}$ (double negative) murine thymocytes activated with CD3mAb, and IL-17 is found excessively in the pannus of inflamed synovium of rheumatoid suffers (Fossies et al., 1996; Shin et al., 1999; Kennedy et al., 1996 and Chabaud et al., 1998). A number of cytokines and inflammatory mediators present in the rheumatoid joint could be induced by IL-17. These include the expression of IL-1β, IL-6, G-CSF, PGE_2, TNF-α, LIF and RANKL in fibroblast, osteoblasts, endothelial and epithelial cells (Chabaud et al., 1998; Kotake et al., 1999; Kotake et al., 2001). In

addition, IL-17 in combination with TNF-α, but not IL-1β, augments osteoclastic formation in vitro (Van Bezooijen RL et al., 1999). Therefore, IL-17 may play a role in regulating bone degradation characterized by the existence of activated T cells and TNF-α production in pathologies such as RA and loosening of bone implants (Van Bezooijen RL et al., 1999).

6.1.1 The aims of this chapter were:

- To establish the effects of IL-17 on the formation of TRAP positive cells in cultures of mononuclear cells (PBMCs).

- To establish the effects of IL-17 on the formation of TRAP positive cells in cultures of mononuclear cells (PBMCs) on extracellular matrix.

- To establish the effects of IL-17 on the resorption of mineralized matrix by long term cultures of mononuclear cells (PBMCs).

- To establish the role of RANKL in IL-17-induced osteoclastogenesis.

- To study the role of IL-17 in the regulation of the interaction between synovial cells (both RA and OA) and PBMCs leading to osteoclastogenesis in vitro.

6.2 Materials and Methods

6.2.1 Multinucleated cell formation

PBMCs were isolated and cultured in eight-well multi-chambered slides. Cells were seeded at (5 x 10^5) cells/well for 24 hr in RPMI 1640 (chapter 2, sections 2.8.9 and 2.8.9.1). Two sets of experiments were used, one with ECM (sections 2.8.8.1 and 2.9.1) and the other without ECM. Then fresh untreated medium was added as –ve control, and to the treated wells; medium/FCS with different growth factor combinations, IL-17 (5 and 50 ng/ml) with or without M-CSF (25 ng/ml), RANKL and M-CSF (30 + 25 ng/ml, respectively), were added to duplicate wells. The cultures were maintained for 21 days;

culture medium was replenished every three days with fresh medium supplemented with the agents described above. The entire experiment was repeated three times.

6.2.2 Cytochemical and Functional Assessment of osteoclast Formation

Cells were fixed in 4% paraformaldehyde solution for 10 min then washed twice in DBPS. After staining for TRAP activity, using a commercial kit (Sigma) according to the manufacturer's instructions, TRAP-positive cells containing more than three nuclei were counted as multinucleated cells by light microscopy. DAPI stain was also used to further confirm the presence of multinucleated cells as described in the materials and methods section (2.9.3). An example for TRAP activity and DAPI stain is shown in Fig 6.1.

Functional assessment of osteoclast formation was determined at the end of the co-culture period using a resorption assay. Cells were stained for TRAP activity to identify osteoclast-like cells, grown on the ivory wafers. Then the cells were removed from the ivory slices by immersing them in sodium hypochlorite as described in materials and methods (2.9.3). Then the ivory slice and/or commercial dentine slice surfaces were examined for evidence of lacunae resorption. The slices were then stained with or without 0.5% (w/v) toluidine blue for 3 min, air dried, and examined by light microscopy. The extent of resorption was determined by measuring the percentage of the eroded area on each slice by image analysis.

6.2.3 Mineralised matrix resorption assay.

The ivory slices were placed in (96 or 48) well tissue-culture plates. 5×10^4 or 1×10^6 PBMCs were seeded in each well and allowed to settle on the ivory slices for 2 – 4 hr. Nonadherent cells and debris were removed by pipetting after 2 hr; RPMI 1640 medium/FCS was used to wash debris, or the ivory slice was moved to a new tissue culture plate with a fresh medium. Then culture medium/FCS containing OPG (100 ng/ml) were used as a negative control, medium/FCS with RANKL + M-CSF (100 + 30 ng/ml, respectively) as a positive control and treatments with IL-17 (5 and 50 ng/ml) with or

without (30 ng/ml) of M-CSF were added. Each experimental condition was repeated in five replicates.

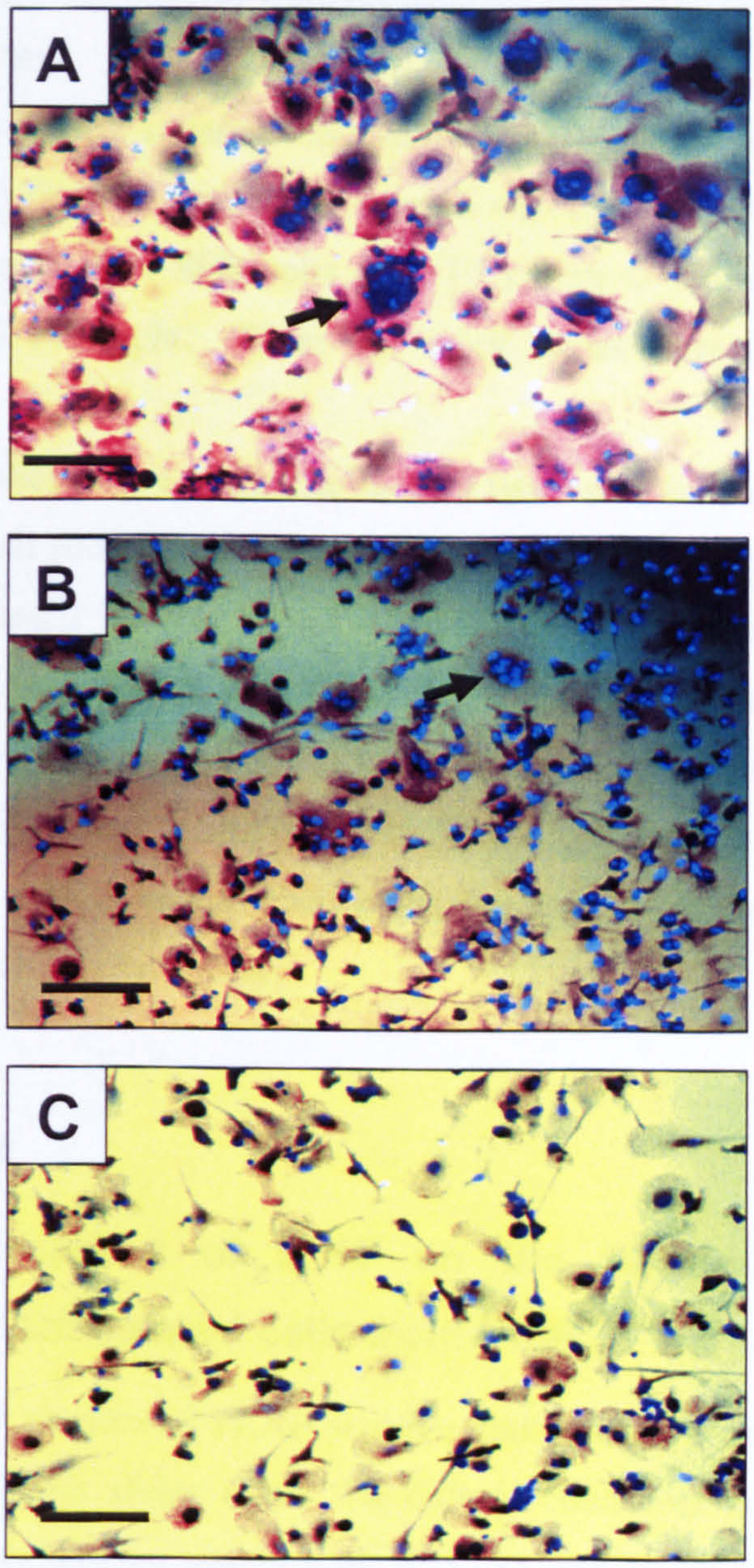


Fig 6.1 TRAP-postive and DAPI staining identification of multinucleated cells (M) in the PBMC culture. (**A**) +ve-control PBMC cells were treated with RANKL and M-CSF (30 + 25 ng/ml, respectively). (**B**) IL-17 (5 ng/ml) with M-CSF (25 ng/ml). (**C**) – ve control cells were cultured in RPMI-1640 with 10%FCS. Cells were cultured for 21 days before fixing with 4% Paraformaldehyde and stained for TRAP-positive and DAPI stain as described in Materials and Methods. The nuclei of multinuclear cells appeared clear with the DAPI stain to count more than three nuclei in TRAP-positive cells . Magnification bar is 100μm for all image. The intense blue staining clearly identifies multinucleated cells (marked by the arrows).

The cultures were maintained for 21 days, during which time the medium and added factors (including cytokines/growth factors) were replenished every 3 days. The whole experiment was repeated three times.

Ivory slices (0.4 cm x 0.4 cm squares, 0.6 mm thick) were placed in 48 wells tissue-culture plates. Then (1×10^6) PBMCs were added to each well and cultured on slices for (2 – 4 hr) as previously mentioned, then SFB cells (2×10^4) from OA or from RA patients were added and incubated overnight. Then the ivory slices were washed and treated as mentioned before.

Commercial dentine slices were placed in 96-well tissue-culture plates. PBMCs (5×10^5) were added to each well and cultured on slices for 2 hr. Then SFB cells (1×10^4) from RA were added and incubated for overnight. Then the ivory slices were washed with fresh medium and treated medium/FCS with OPG (100 ng/ml) was added as negative control, medium/FCS with RANKL + M-CSF (100 + 30 ng/ml, respectively) was added as a positive control, IL-17 (5 and 50 ng/ml) with M-CSF (30 ng/ml) and IL-17 (50 ng/ml) without M-CSF were added in duplicate wells for each treatment. The cultures were maintained for 21 days as stated before.

6.3 Results

6.3.1 The effect of IL-17 on the formation of TRAP+ve multinucleated cells from PBMC

The results show that a number of TRAP-positive multinucleated cells were formed in the eight-well multi-chambered slides under the culture conditions used. The cultures of PBMCs from healthy volunteers treated with IL-17 (5 and 50 ng/ml) with or without M-CSF (25 ng/ml) were compared to negative control (untreated cells) and also compared to the positive control wells which were treated with RANKL and M-CSF (30 and 25 ng/ml, respectively). The number of TRAP-positive multinucleated cells were increased in the chambers treated with IL-17, with and without M-CSF, and in the positive control RANKL with M-CSF compared to negative control (compare Fig 6.2 C-F with A).

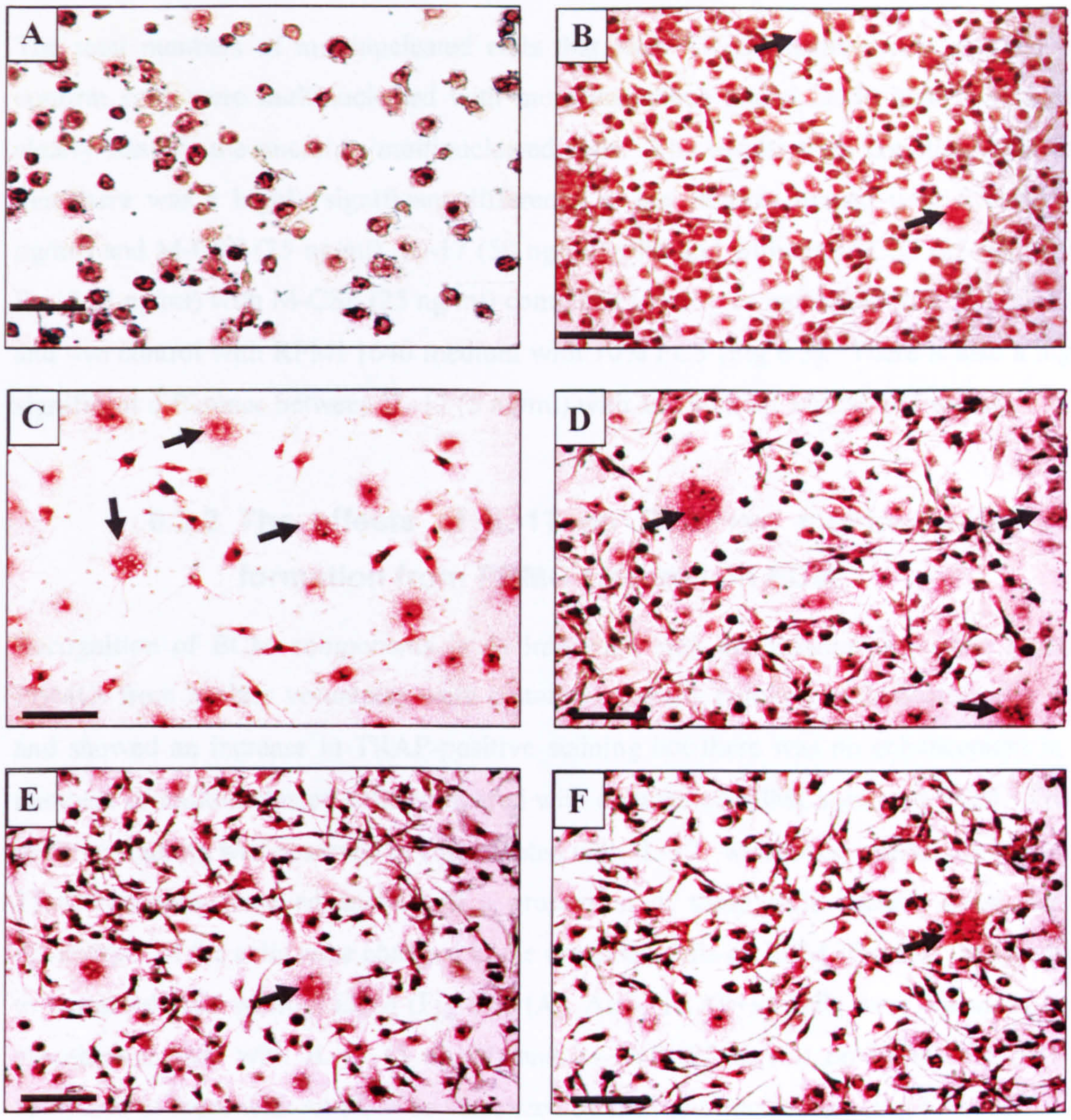


Fig 6.2 Formation of TRAP-postive multinucleated cells in PBMC culture. Cells were cultured in 8 well multi-chambered slides. (**A**) –ve control which is treated with RPMI-1640 with 10% FCS. (**B**) +ve-control that is RANKL and M-CSF (30 + 25 ng/ml, respectively). (**C**) IL-17 (5 ng/ml). (**D**) IL-17 (50 ng/ml). (**E**) IL-17 and M-CSF (5 + 25 ng/ml, respectively). (**F**) IL-17 and M-CSF (50 + 25 ng/ml, respectively). Cells were cultured for 21 days before fixing with 4% Paraformaldehyde and stained for TRAP-activity as described in Materials and Methods. Magnification bar is 100μm for all images. Multinucleated cells are marked by the arrows. Each image is equivalent to 1 field.

The total numbers of multinucleated cells that were TRAP-positive were counted. To confirm cells were multinucleated with more than three nuclei DAPI stain was used to clearly identify the nuclei in multinucleated cells. The results from counting cells show that there was a highly significant difference in the cultures treated with RANKL (30 ng/ml) and M-CSF (25 ng/ml), IL-17 (50 ng/ml) with and without M-CSF (25 ng/ml) and IL-17 (5 ng/ml) with M-CSF (25 ng/ml) compared to cultures incubated with M-CSF alone and –ve control with RPMI 1640 medium with 10% FCS (Fig 6.3). There is also a highly significant difference between IL-17 (5 ng/ml) with and without M-CSF (25 ng/ml).

6.3.2 The effects of IL-17 on TRAP+ve multinucleated cell formation from PBMC cultured on ECM.

Recognition of ECM components is an important step in initiating osteoclast function. PBMCs from healthy volunteers were cultured on tissue culture plastic coated with ECM and showed an increase in TRAP-positive staining but there was no enhancement in the numbers of multinucleated cells compared with cultures of PBMCs without ECM. TRAP-positive staining was observed in cells treated with IL-17. Moreover, overall the visualized TRAP+ve cells were enhanced and a proportion of these cells became large and the phenotypes of the cells were changed in the untreated cells on ECM (Fig 6.4) (B) compared to untreated cells without ECM (Fig 6.4) (A). Also, TRAP+ve cells were enhanced in the chambers treated with IL-17 (5 ng/ml) and M-CSF (25 ng/ml) on ECM (Fig 6.4) (D) compared to those without ECM as visualized by the light microscopy (Fig 6.4) (C).

6.3.3 The effects of IL-17 on bone resorption activity of PBMC cultured on ivory.

Bone resorption by PBMCs cultured with IL-17 was also examined. The percentage-eroded area on the surface of ivory slices was determined by reflected light microscopy and image analysis. The results from these experiments show a highly significant increase in the percentage of resorption areas in cultures treated with IL-17 (5 and 50 ng/ml) with M-CSF and in the +ve control (RANKL with M-CSF) compared to OPG –ve control. Also, there is a significant increase in bone resorption in cultures treated with IL-17 (50 ng/ml)

with M-CSF compared with IL-17 (50 ng/ml) alone. Furthermore, the resorption observed with IL-17 (50 ng/ml) alone is significantly increased in comparison to OPG alone and IL-17 with OPG (Fig 6.5).

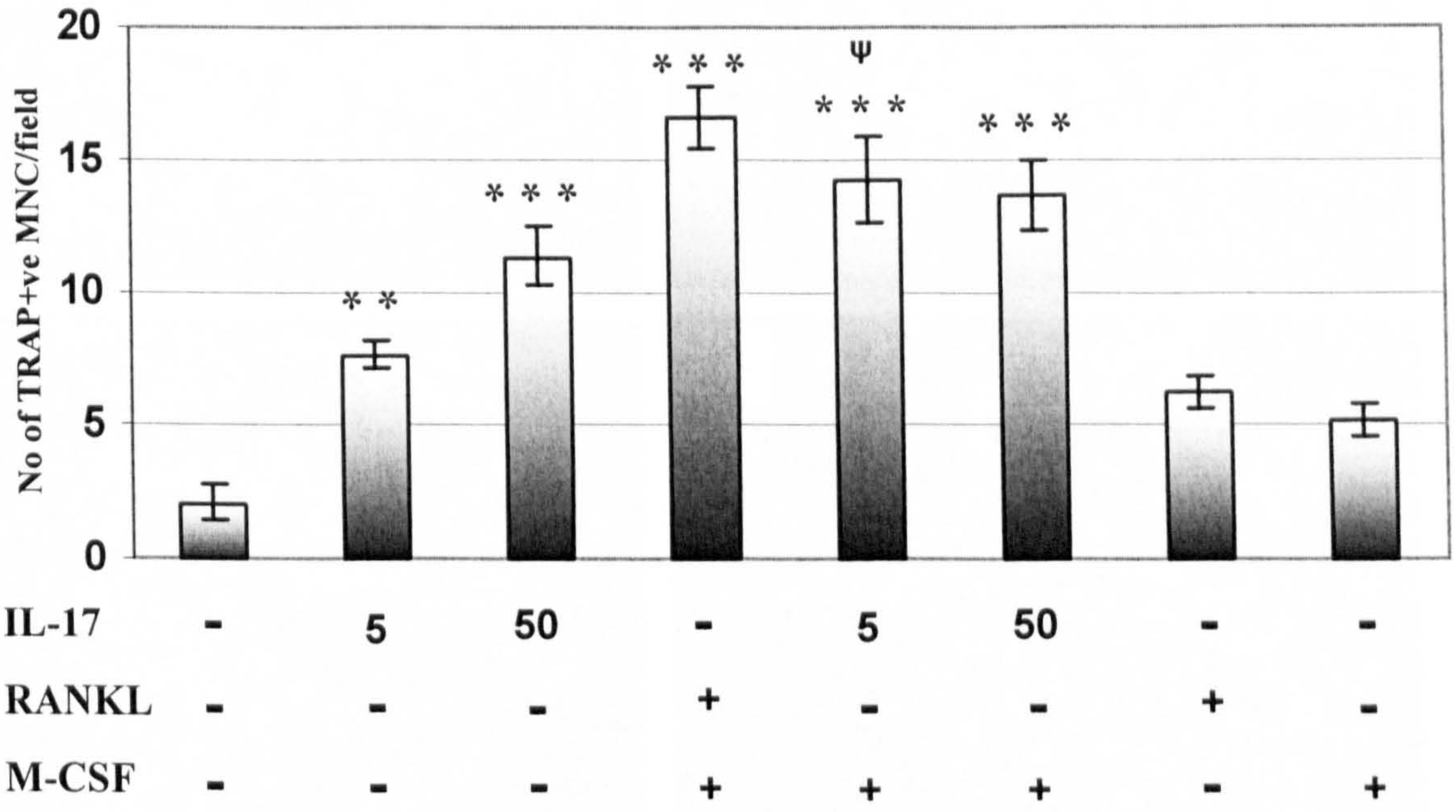


Fig 6.3 TRAP+ve multinucleated cells (MNCs) formed in vitro from PBMC. PBMC cells were cultures in the presence of IL-17 (5 or 50 ng/ml) with or without M-CSF (25 ng/ml). The positive control was RANKL and M-CSF (30 and 25 ng/ml respectively) and negative control was fresh RPMI with 10% FCS. RANKL and M-CSF alone were also assessed. TRAP-positive MNCs containing more than three nuclei were counted, ten fields for each treatment per chamber slides. The results shown are the means of 10 fields from each duplicate cultures of PBMC. The difference between mean was assessed using one way analysis of variance (ANOVA), Tukey and Duncan post test. Where * indicates comparisons with –ve control and ψ with IL-17 (5 ng/ml). * ($P \leq 0.05$), *** ($P \leq 0.001$).

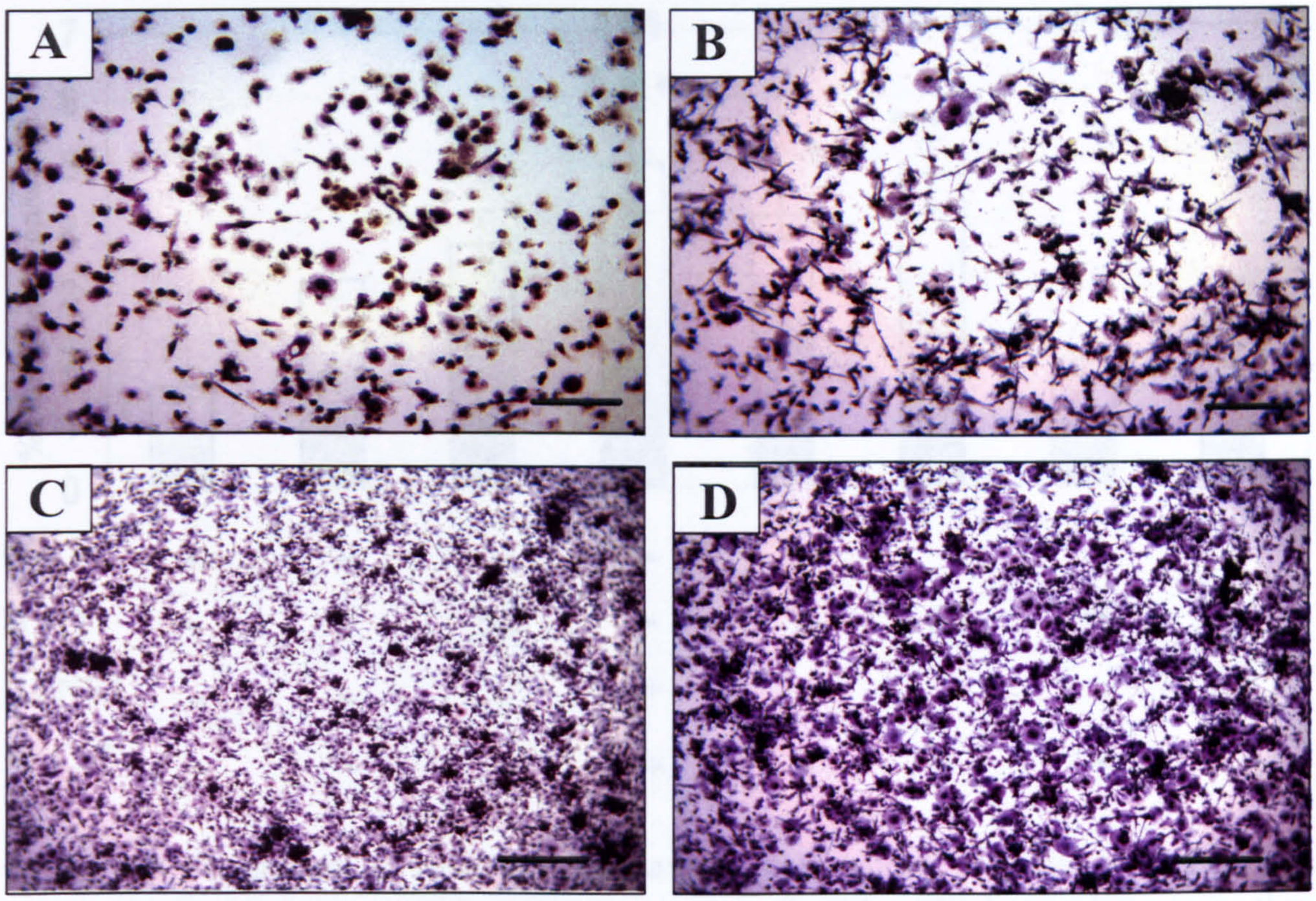


Fig 6.4 Formation of TRAP-postive multinucleated cells in the PBMC culture on ECM. PBMC cells were cultured in (**A**) –ve control which is treated with RPMI-1640 with 10%FCS. (**B**) –ve control which is treated with RPMI-1640 with 10%FCS and the cells were cultured on the ECM from MG-63 as described in materials and methods. (**C**) PBMCs were treated with IL-17 with M-CSF (5 + 25 ng/ml, respectively) and cultured without ECM. (**D**) Cells were treated with IL-17 and M-CSF (5 + 25 ng/ml, respectively) and cultured on ECM. Cells were cultured for 21 days before fixing with 4% Paraformaldehyde and stained for TRAP activity as described in Materials and Methods. Magnification bar is 50μm.

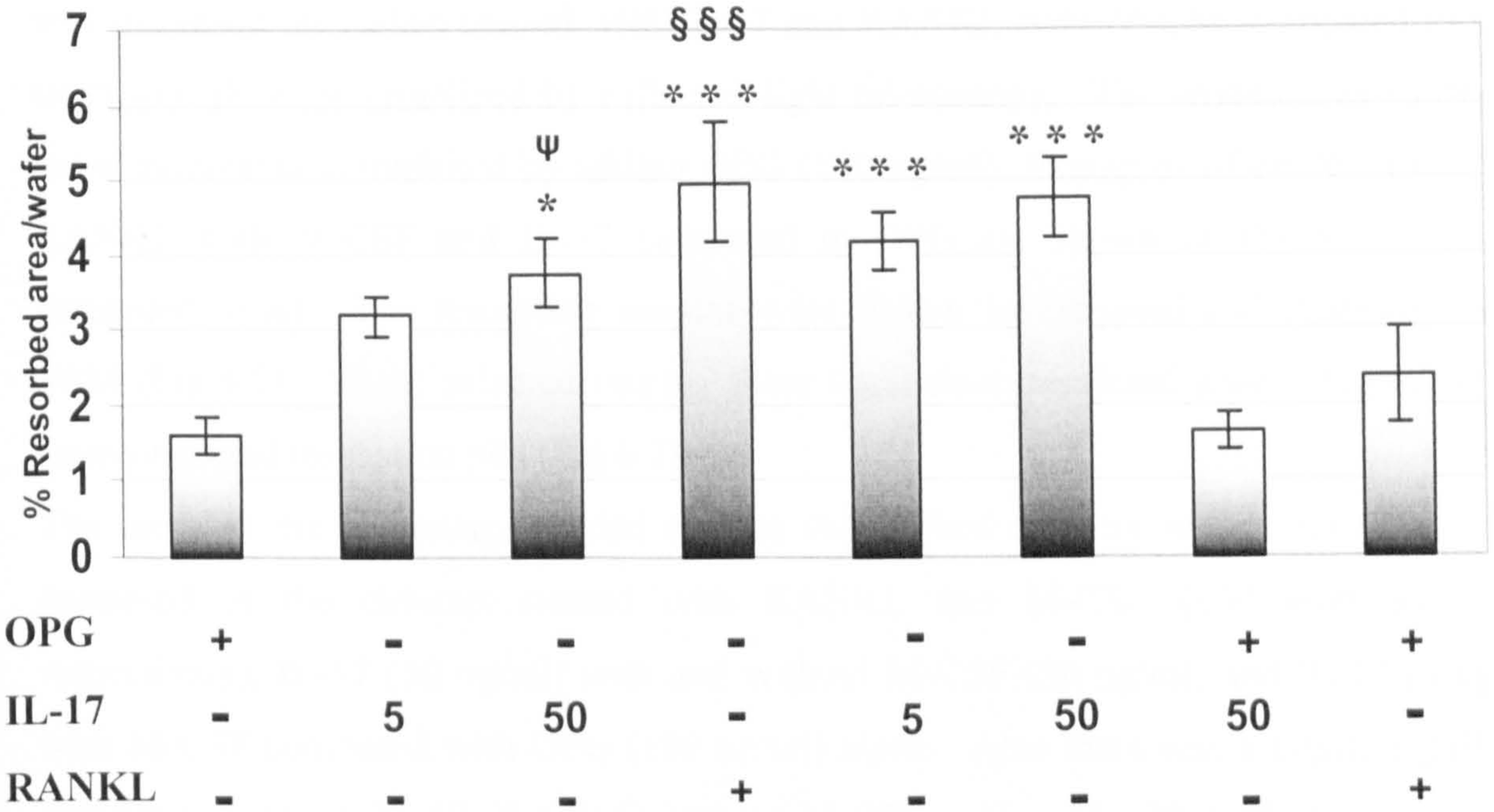


Fig 6.5 Formation of resorption areas on ivory slices cultured with PBMCs. PBMCs (1.5×10^6) were plated on ivory slices for 2 hr. The ivory slices were then transferred to 48-well plates and incubated for 24 hr in fresh medium with M-CSF then washed and the cultures were treated with IL-17, RANKL (100 ng/ml), M-CSF (30 ng/ml) and OPG (100 ng/ml). The cultures were grown for 21 days and the media replenished every 3 days. Resorption pits formed on ivory slices were visualised under reflected light microscopy. The percentage of resorbed area was determined per wafer. Each bar represents the mean (n=5) ±SE. Statistical significant was determined by one way analysis of variance, Tukey and Duncan post test.

Where * indicates comparisons with the OPG -ve control, **ψ** with IL-17 (50 ng/ml)+ M-CSF, **§ § §** with RANKL + M-CSF + OPG.

* ($P \leq 0.05$), *** ($P \leq 0.001$).

6.3.4 The effects of IL-17 on bone resorption by PBMC co-cultured with SFB (RA) on ivory.

Unstimulated PBMCs (1.5 x 10^6) derived from a volunteer and SFB from a RA patient (1 x 10^4) were co-cultured for 21 days on ivory slices. The results show that the eroded area was increased on wafers treated with IL-17 and RANKL with M-CSF compared to OPG treatment alone as visualized by reflected light microscopy. The eroded area caused by these treatments is inhibited by adding OPG (100 ng/ml). Examples of eroded areas from RANKL with M-CSF and IL-17 compared to OPG are shown in Fig 6.6 (B and C compared to A). The resorption lacunae were further investigated and characterized by SEM (Fig 6.7). These selected images show focal de-mineralized zones (Fig 6.7A) and interconnected resorption pits (Fig 6.7B).

The mean of the percentage-eroded area on the surface of ivory wafer was significantly increased in the cultures treated with RANKL and M-CSF (100 with 30 ng/ml, respectively), IL-17 (50 ng/ml) with and without M-CSF (30 ng/ml) and IL-17 (5 ng/ml) with M-CSF compared with OPG (100 ng/ml) alone. Also there was a highly significant difference between IL-17 (5 ng/ml) without M-CSF compared with OPG treatment. The eroded area on ivory slices treated with RANKL, M-CSF and OPG is highly significantly inhibited compared to RANKL and M-CSF. In addition, the eroded area is highly significantly inhibited in response to OPG and IL-17 (50 ng/ml) compared to IL-17 alone (Fig 6.8).

Additionally, to contrast between ivory and the commercial dentine slices, PBMCs were co-cultured with RA synovial fibroblasts on dentine slices. Examples of resorption areas visualized by reflected light microscope are shown in Fig 6.9. The results from this experiment show that the percentage-eroded area on the surface of dentine slices was highly significantly increased in the cultures treated with RANKL and M-CSF (100 with 30 ng/ml), IL-17 (5 or 50 ng/ml) with M-CSF (30 ng/ml), and IL-17 (50 ng/ml) compared to the cultures treated with OPG (100 ng/ml) alone. Also, there was a significant difference between IL-17 (50 ng/ml) without M-CSF and IL-17 (50 ng/ml) with M-CSF as shown in (Fig 6-10). The experiment was in duplicate and only one experiment was performed.

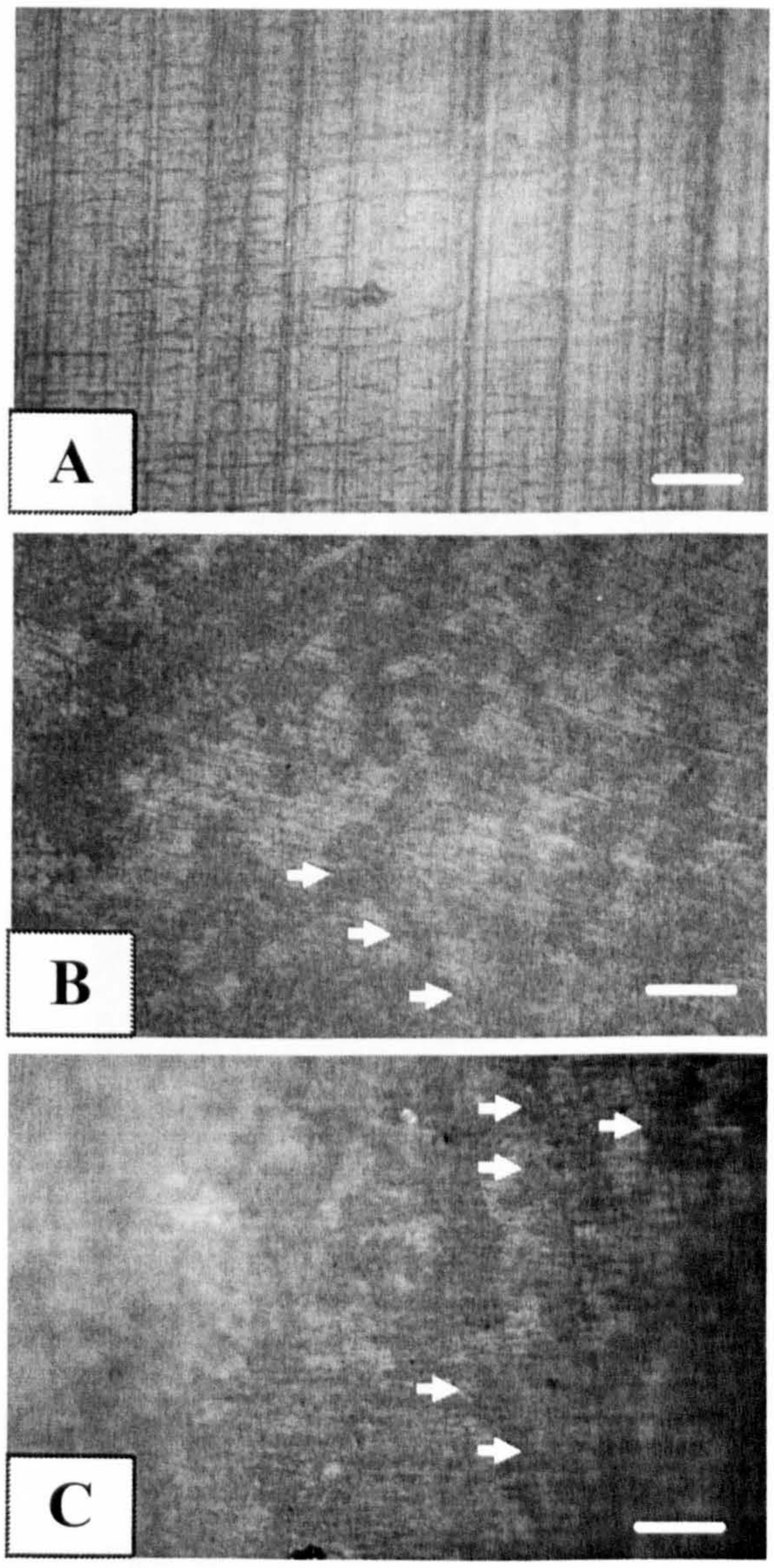


Fig 6.6 Formation of resorption area on ivory slices in the cultures of PBMC with synovial fibroblasts from a rheumatoid arthritis patient. **A** Cells were treated with OPG (100 ng/ml). **B** Cells were treated by RANKL and M-CSF (30 + 25 ng/ml, respectively) as +ve-control. **C** Cells were treated with IL-17 with M-CSF (5 + 25 ng/ml, respectively). Cells were cultured for 21 days. before fixing with 4% Paraformaldehyde and staining for TRAP activity as described in Materials and Methods. After the cells were removed, images were taken by reflected light microscopy. Magnification bar is 50 μm for all images. Resorption pits are marked by the arrows.

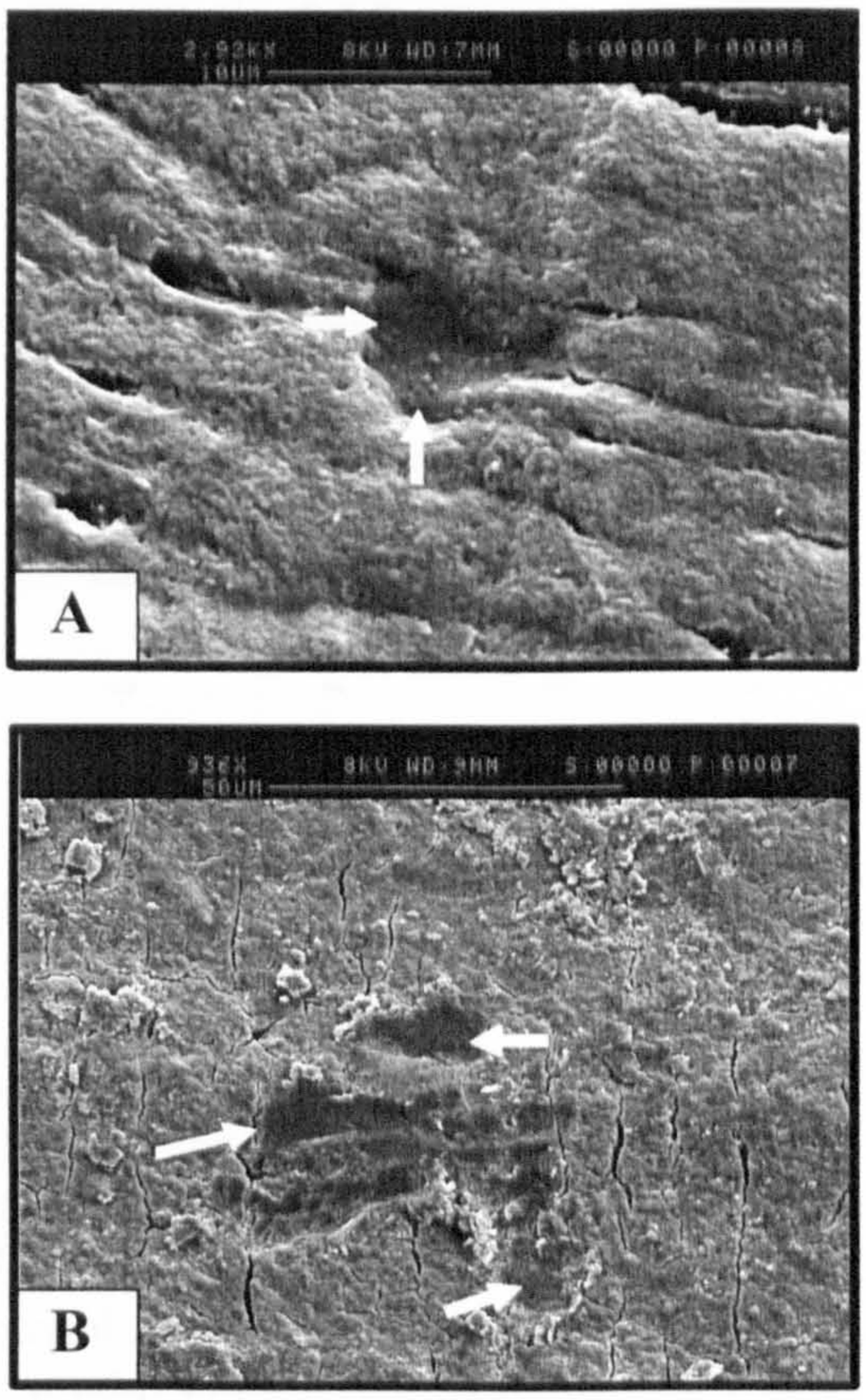


Figure 6.7 Formation of resorption pits on ivory slices by SEM. PBMC co-cultured with RA synovial fibroblasts. **A** Cells were treated with RANKL and M-CSF (100 + 25 ng/ml, respectively). **B** cells were treated with IL-17 and M-CSF (50 + 25 ng/ml, respectively). Cells were cultured for 21 days and medium and and added cytokines were replenished every three before fixing with 4% Paraformaldehyde and staining for TRAP activity as described in Materials and Methods. After the cells were removed, the slices were visualized by SEMR pits cells are marked by the arrows

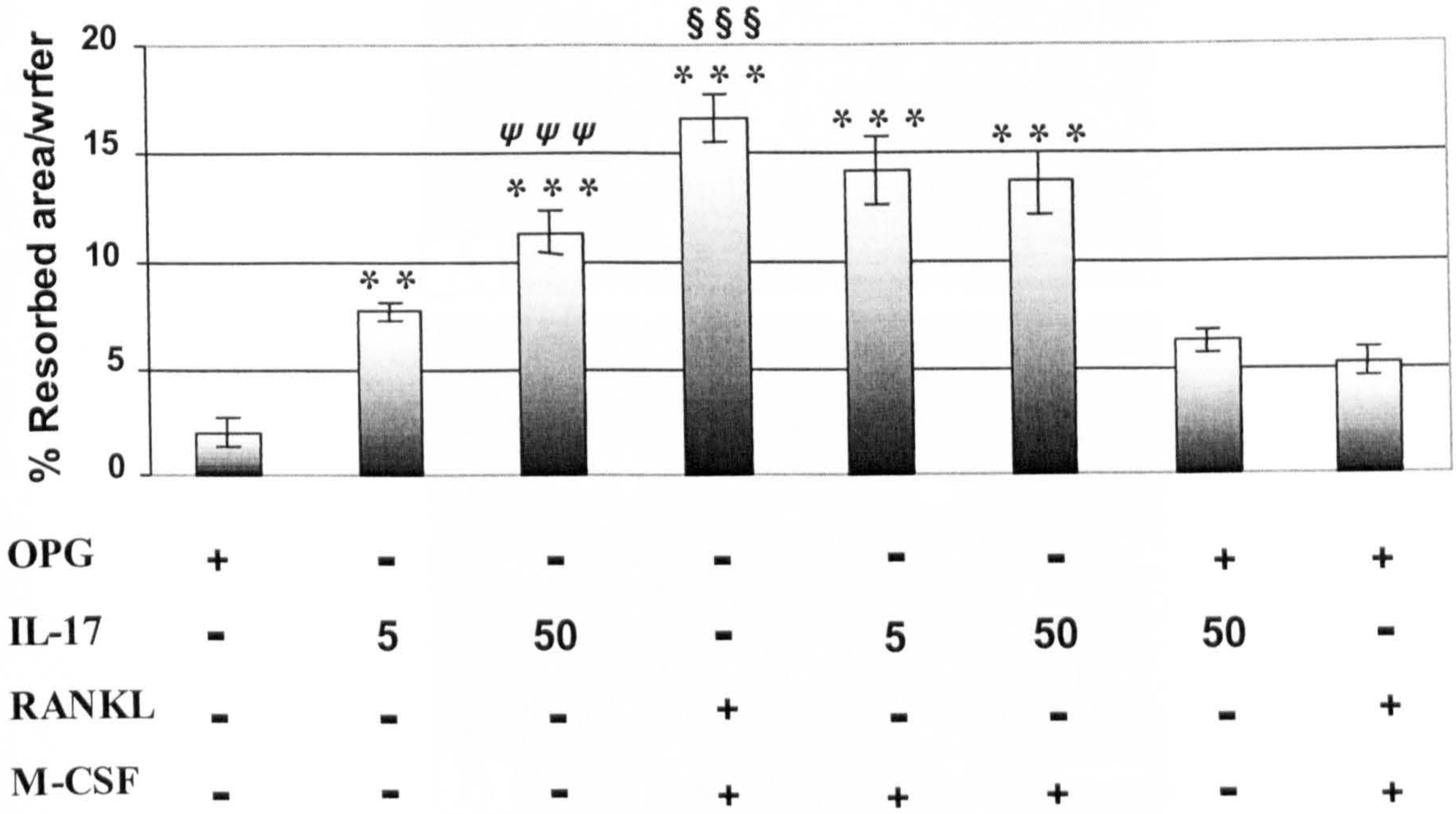


Fig 6.8 Formation of resorption area on ivory slices in the co-cultures of PBMC and Synovial fibroblasts from RA. PBMCs (1.5 x 10^6) were plated on ivory slices for 2 hr and then transferred to 48-well plates and incubated for 24 hr in in fresh medium with M-CSF as indicated in materials and methods. After one day RA synovial fibroblasts (2 x 10^4) cells were co-cultured with the PBMCs in the presence of IL-17 (5 and 50 ng/ml), RANKL (100 ng/ml), M-CSF (30 ng/ml) and OPG (100 ng/ml). Then the cultures were further incubated for 21 days. Resorption pits formed on ivory slices were visualised under reflected light microscope, The percentage of resorbed area was determined. Each bar present the mean (n=5) of independent ivory slices.
Statistical significance was determined by one way analysis of variance and Tukey and Duncan post test . Where ** indicates comparisons with OPG -ve control, **§ § §** with RANKL + M-CSF + OPG. ***ψ ψ ψ*** with IL-17 + OPG.
** ($P \leq 0.01$), *** ($P \leq 0.001$).

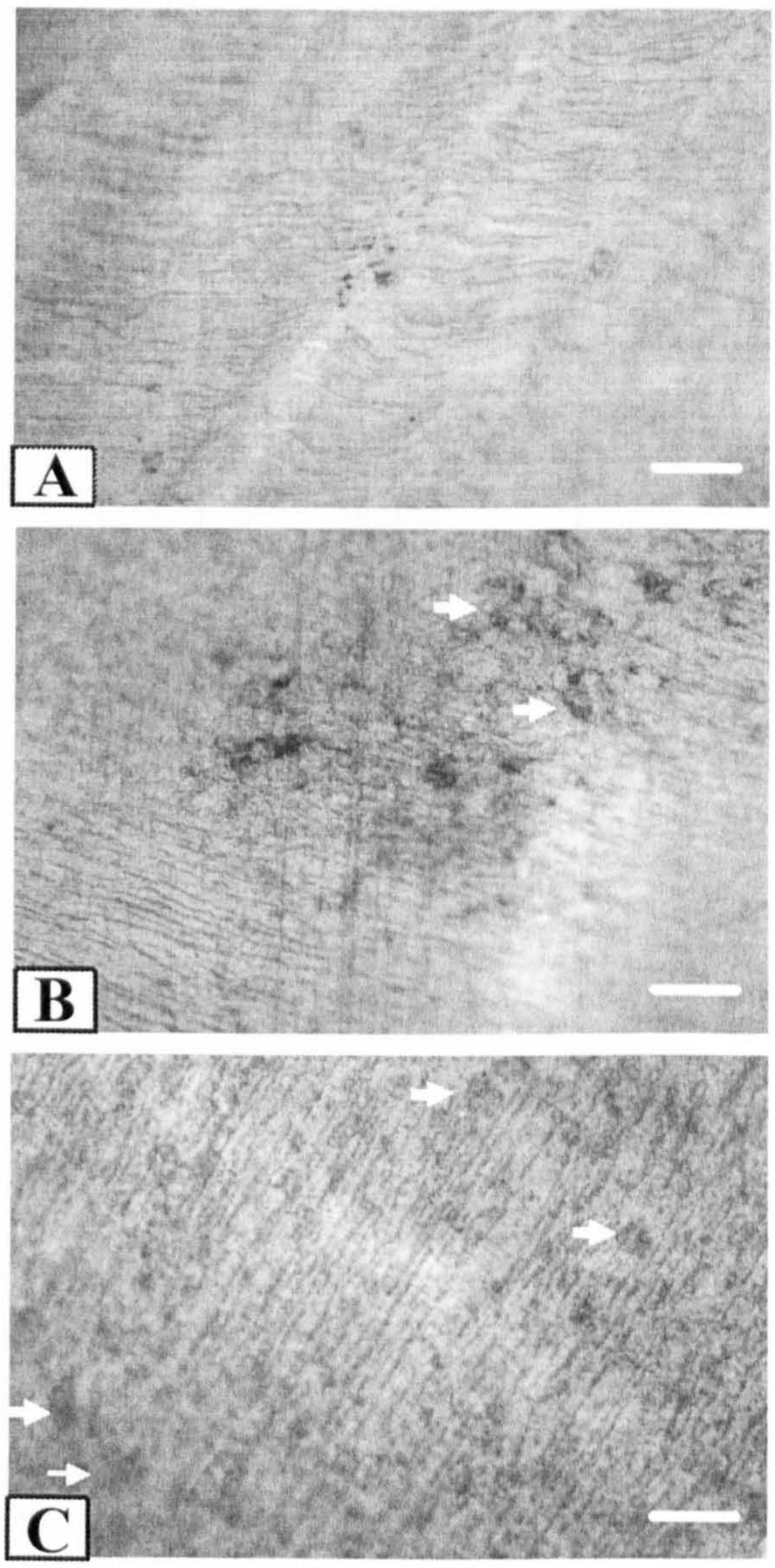


Fig 6.9 Formation of resorption pits on commercial dentine slices in the cultures of PBMCs co-cultured with synovial fibroblasts from RA patient by reflected light microscope. **A** OPG (100 ng/ml). **B** IL-17 (50 ng/ml). **C** IL-17 (50 ng/ml) with M-CSF (30 ng/ml). Cells were further cultured for 21 days. before fixing with 4% Paraformaldehyde and staining for TRAP activity as described in Materials and Methods. After the cells removed as described in material and methods and the pictures was taken by reflected light microscope. Magnification bar is 50μm for all images. Resorption pits cells are marked by the arrows

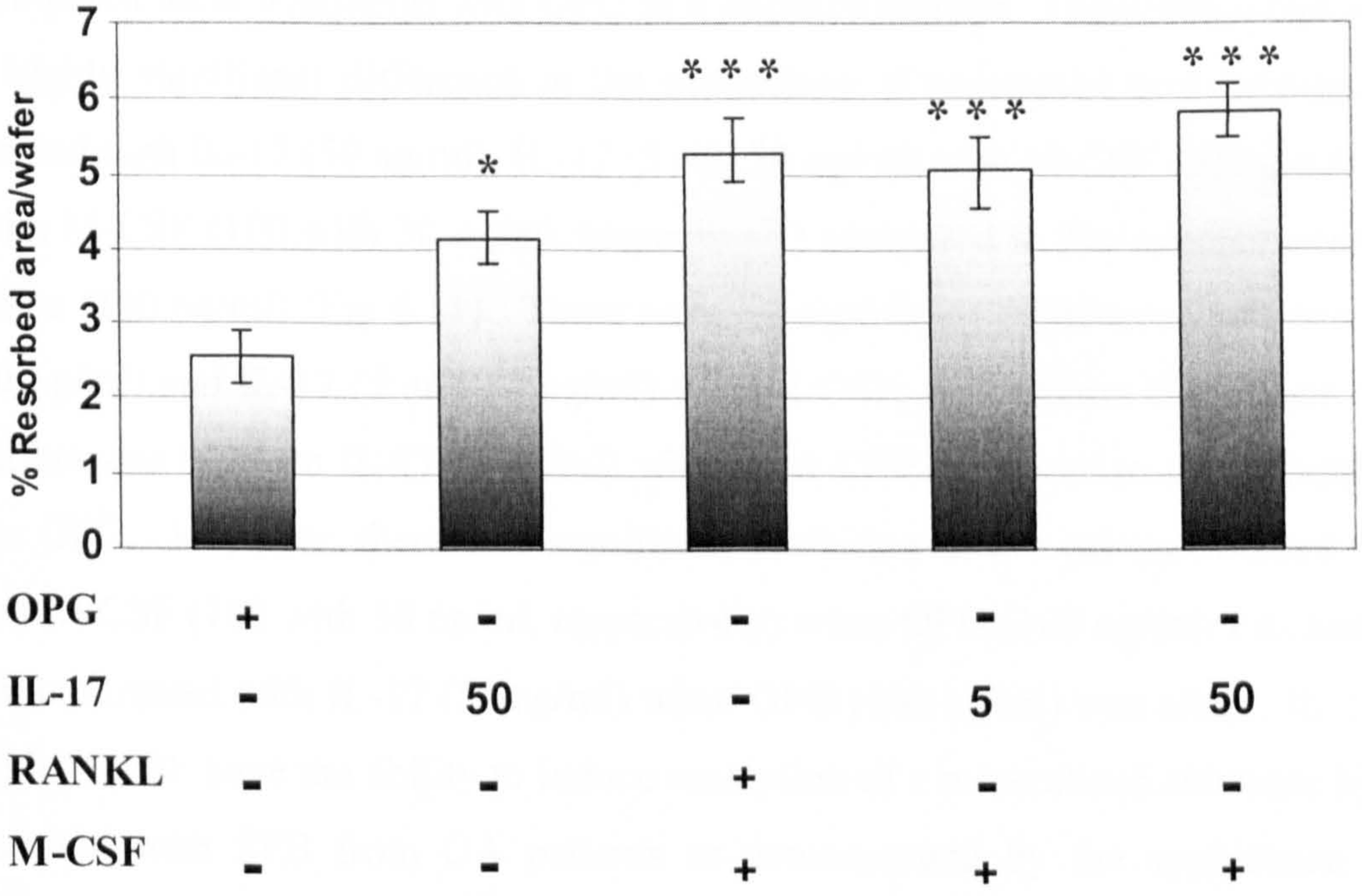


Fig 6.10 Formation of resorption area on commercial dentine slices in the co-cultures of PBMC and Synovial fibroblasts from RA. PBMCs (1 x 10^6) were plated on dentine slices for 2 hr. The dentine slices were then transferred to 48-well plates and incubated for 24 hr in the presence of fresh medium with M-CSF (30 ng/ml). After one day RA synovial fibroblasts (1.5 x 10^4) cells were co-cultured with PBMCs on the dentine slices. The cultures were treated with IL-17 (50 ng/ml), RANKL (100 ng/m), M-CSF (30 ng/ml) and OPG (100 ng/ml). Then the cultures were further incubated for 21 days. Resorption pits formed on dentine slices were visualised under reflected light microscope, The percentage of resorbed area was determined per wafer. Each bar present the mean (n=2) ± SE statistically significant was determined by one way analysis of variance and Tukey and Duncan post test . Where * indicates comparisons with the OPG -ve control.

* ($P \leq 0.05$), *** ($P \leq 0.001$).

6.3.5 The effects of IL-17 on bone resorption activity of PBMC co-cultured with SFB from OA patients.

To examine the potential role of IL-17 in OA, PBMCs were co-cultured with SFB (OA) treated with IL-17 with and without M-CSF, RANKL with M-CSF as a positive control and compared these treatments with OPG as a negative control. The results show that there was a highly significant difference in the percentage of resorption area between the cultures treated with IL-17 (50 ng/ml), IL-17 (5 and 50 ng/ml) with M-CSF (30 ng/ml) and RANKL with M-CSF (100 with 30 ng/ml, respectively) compared to the cultures treated with OPG alone (100 ng/ml) (Fig 6.11). There were no significant differences between IL-17 (5 and 50 ng/ml) and IL-17 (5 and 50 ng/ml) with M-CSF, furthermore there were no significant differences between IL-17 (5 ng/ml) without M-CSF compared to the cultures treated with the OPG. However, there was significant inhibition in the cultures treated with RANKL and M-CSF (100 with 30 ng/ml, respectively) when OPG (100 ng/ml) was added and in the culture treated with IL-17 (50 ng/ml) when OPG (100 ng/ml) was added. IL-17 or RANKL with M-CSF have the ability to induce resorption of a mineralized substrate by PBMCs co-cultured with SFB from OA patients as demonstrated by the appearance of numerous resorption lacunae during co-cultures conducted on ivory slices.

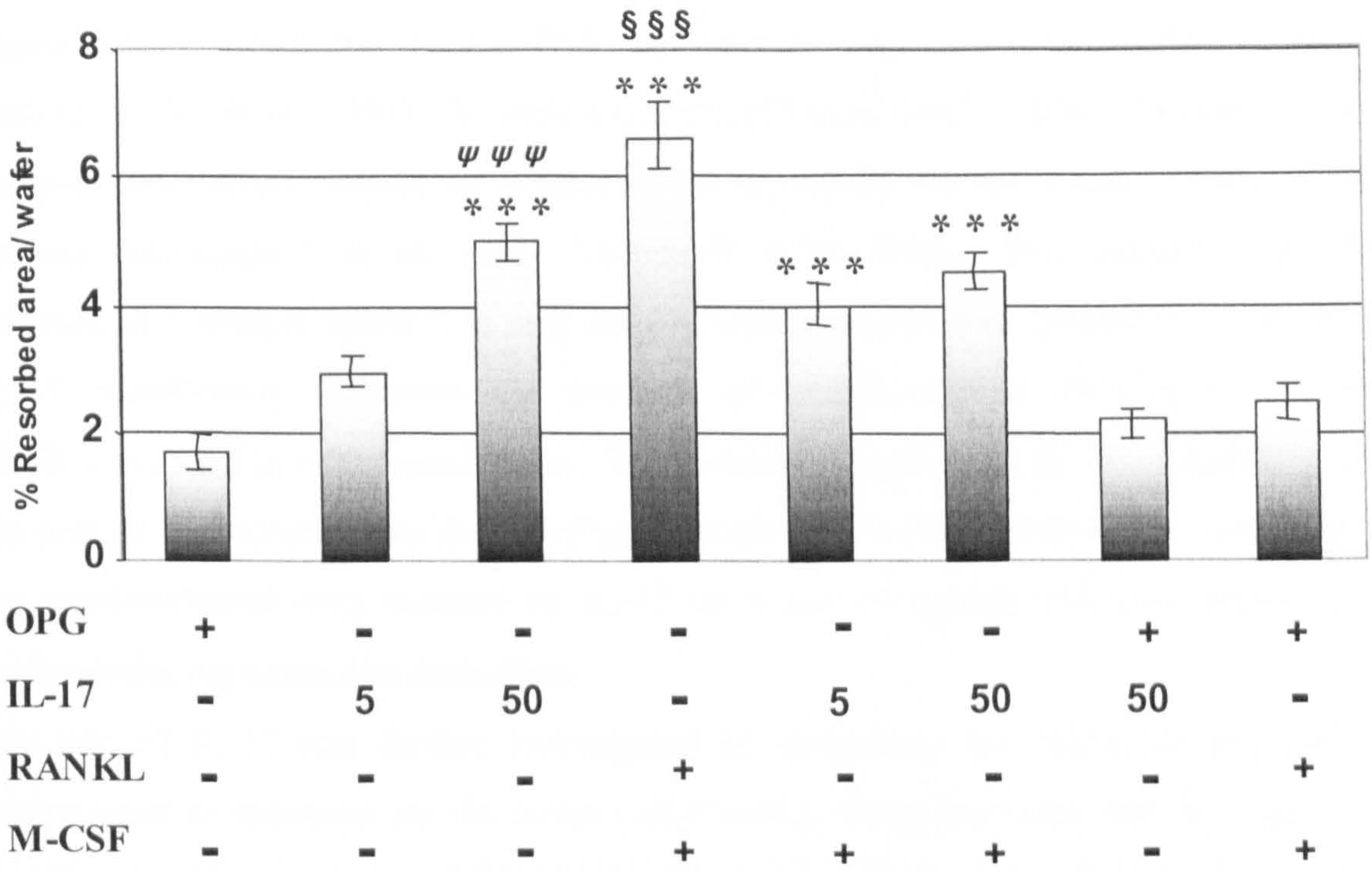


Fig 6.11 Formation of resorption area on ivory slices in the co-cultures of PBMC and OA synovial fibroblasts. PBMCs (1.5×10^6) were plated on ivory slices for 2 hr. The ivory slices were then transferred to 48-well plates and incubated for 24 hr in the presence of fresh medium with M-CSF (30 ng/ml). After one day the OA synovial fibroblasts (2×10^4) cells were co-cultured with the PBMCs. The cultures were treated with IL-17 (5 and 50 ng/ml), RANKL (100 ng/m), M-CSF (30 ng/ml) and OPG (100 ng/ml). Then the cultures were grown for 21 days and the medium were replenished every three days. Resorption pits formed on ivory slices were examined under the reflected light microscopy. The percentage of resorbed area was determined per-ivory slice. Each bar represents the mean (n=5) ± SE. Statistical significance was determined by one way analysis of variance, Tukey and Duncan post test. Where *** indicates comparisons with the OPG -ve control, ***ψ ψ ψ*** with IL-17 + OPG, **§ § §** with RANKL +M-CSF + OPG.

*** ($P \leq 0.001$).

6.4 Discussion

To study the effect of IL-17 on osteoclast formation in vitro, I performed assays using PBMCs as osteoclast precursors. As a positive control, RANKL, a membrane expressed ligand that is a member of the TNF superfamily, was used (Wong BR. et al., 1997; Anderson DM. et al., 1997). In some experiment I used OPG, a novel member of the TNF receptor superfamily, which, in contrast with other family members lacks a trans-membrane domain (Morinaga T. et al., 1998; Yasuda H. et al 1998). This indicates that OPG is secreted as a soluble factor that may act to modulate osteoclast formation. I showed that IL-17 significantly increased the numbers of multinucleated TRAP-positive cells in PBMCs cultured in chambered slides. These results suggest that IL-17 stimulation induces the process that leads to the fusion of preosteoclasts. TRAP-positive stain was detected in the multinucleated cells induced by IL-17 (at 5 and 50 ng/ml) with and without M-CSF further inducing osteoclast formation.

The role of IL-17 was further investigated by measuring the resorption areas on ivory wafers used as substrate for the culture of PBMCs. Treatment with RANKL and M-CSF, and IL-17 with or without M-CSF induced osteoclast formation. This study suggests that may IL-17 have a role in the most aggressive forms of inflammatory bone and cartilage loss by directly stimulating osteoclast precursors to fuse and differentiate to become mature and active osteoclasts.

However, results show that there was a dramatic decrease in the percentage of resorption area when OPG was added to IL-17 treated cultures. These results therefore suggest that IL-17 stimulated bone resorption is RANKL-dependent. sRANKL is released from activated T cells and the membrane form of RANKL is also expressed on the infiltrating T cells in rheumatoid synovial fluid. Kanamaru F et al., (2004) demonstrated that the expression of mRANKL on T cells is strictly limited, and the majority of RANKL protein produced by T cells may be active in the soluble form after shedding. In addition, in vitro studies suggested a role for IL-17 in bone erosion by induction of RANKL expression (Kotake S. et al., 1999). RANKL is expressed by stromal cells and osteoblasts and induces differentiation and maturation of osteoclast progenitors (which are monocyt-macrophage lineage cells), and modulates the survival and function of the resultant giant multinucleated osteoclasts (Suda et al, 1999; Takahashi et al, 1999). IL-17 may promote joint

inflammation as well as tissue destruction during the initial phase of arthritis. After words, RANKL and some other factors such M-CSF continue the osteoclastogenesis process. Bone marrow cells are able to differentiate in vitro into osteoclast-like cells in the presence of RANKL and M-CSF (Blin-Wakkach C. et al., 2004). However, the role of T cell IL-17 during the effector phase of arthritis has still not been identified. M-CSF is shown to be a crucial factor in mouse osteoclast development (Kodama H. et al., 1991). Matsuzaki K. et al., (1998) demonstrated that M-CSF was also essential for inducing OCLs from human PBMCs. Without M-CSF, most of the PBMCs disappeared even in the presence of sRANKL during the 7 day-culture period, suggesting that M-CSF is involved in the proliferation and survival of osteoclast progenitors. IL-17 may be required with M-CSF to prolong the life span of osteoclasts. Therefore, IL-17 may increase the production of RANKL and M-CSF from PBMC in the culture medium which affects differentiation, maturation and activation of multinucleated cells, or perhaps IL-17 has some effect as M-CSF that leads PBMCs to prolong the life span of osteoclasts. In the co-cultures of PBMC with SFB from RA and OA, IL-17 may increase the production of RANKL in the culture medium which increases the differentiation of osteoclasts. In addition, synovial macrophage cells are capable of differentiating to osteoclast-like cells under some conditions, including culturing with M-CSF and RANKL (Itonaga I et al., 2000).

The number of TRAP+ve cells formed and lacunae resorption surface area in the PBMCs co-cultured with SFB from RA and OA are more than the PBMCs alone. However, the number of the osteoclasts and lacunae resorption surface area formed by the PBMC co-cultured with SFB from OA is more than one and half times PBMC alone. The co-culture of PBMCs and SFB from RA produced more resorption than the co-culture of PBMCs with SFB from OA. This resorption is RANKL-dependent and suggests that SFB from RA in response to IL-17 may produce increased levels of RANKL than from OA SFB. This result support the previous suggestion by Kotake, et al, 1999 in mouse as shown in Fig 6.12.

RANKL is an essential factor in osteobiology (Simonet et al, 1997; Lacey et al, 1998), linking osteoblast/stromal cell functions to osteoclast formation and activation. Osteoclast activity is regulated by the differential expression of membrane-RANKL, as well as

sRANKL, its receptor RANK and decoy receptor OPG (Suda et al, 1999; Takahashi et al, 1999). The previous studies may explain the finding of an increase in resorption area in the co-cultures of SFB cells with PBMC which may be mediated by cell-to-cell contact.

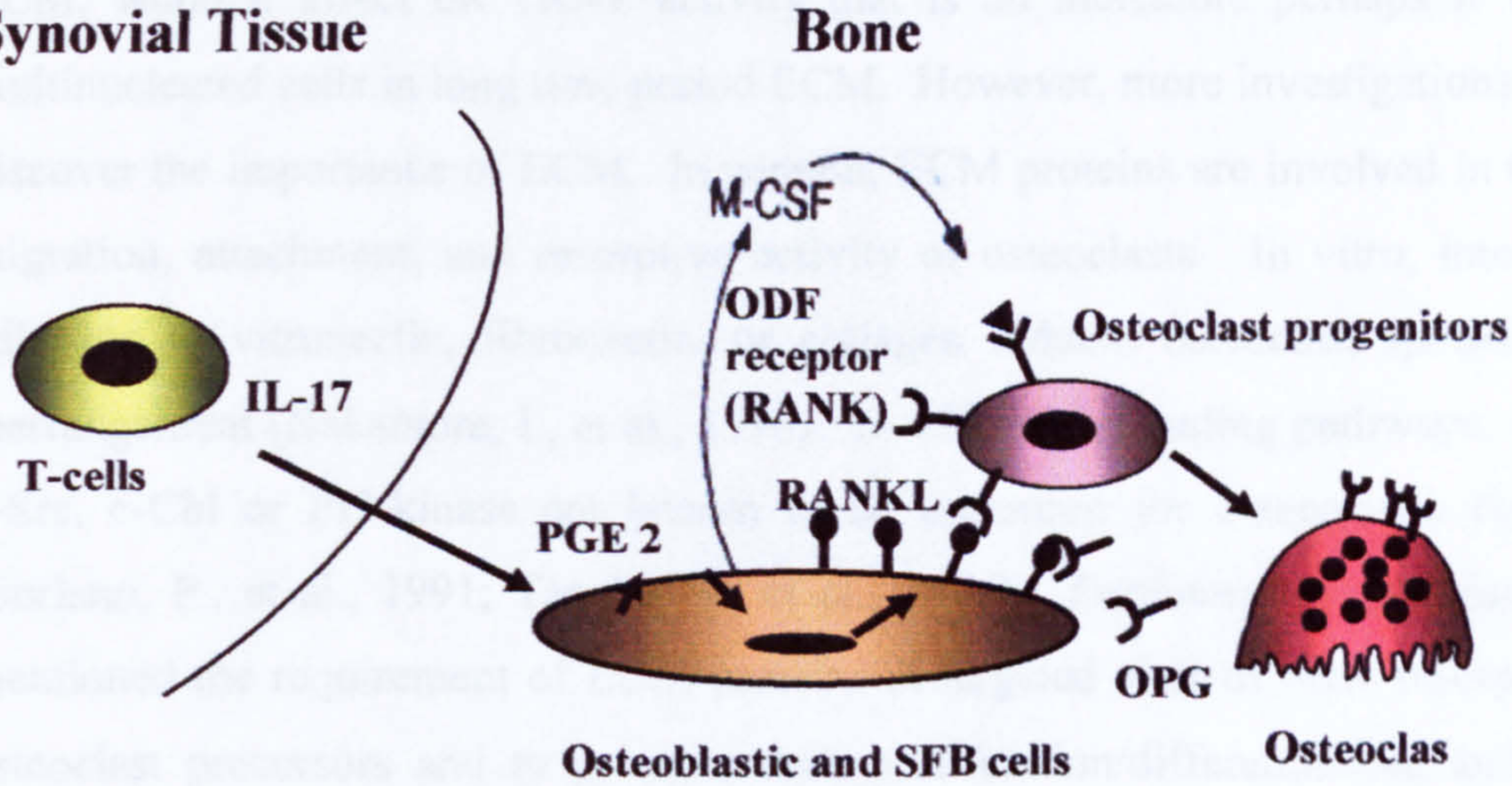


Fig 6.12 osteoclastogenesis mechanism by activated T cells and osteoblasts cytokines. (taken from Kotake, et al, 1999).

In addition, this phenomenon may be mediated by other cytokines, as suggested by Atkins GJ (2000) who found that ST-2 cells in vitro express mRNA encoding a repertoire of many of the reported osteoclastogenic factors (IL-1/IL-1R1, IL-11, IL-6/IL-6R, IL-17 and TGF-β, as well as RANKL). The stromal cells also expressed mRNA encoding two molecules shown to be inhibitory to osteoclastogenesis, OPG and IL-18. OPG, IL-1, IL-1R1, IL-6, IL-6R, IL-11R, IL-17, IL-18, IL-18R, TGF-β and M-CSF were expressed by both the stromal cells and the PBMC. This study showed that the resorption area formed by IL-17 with or without M-CSF and by RANKL with M-CSF was inhibited with OPG suggesting the eroded area was RANKL-dependent.

Osteoclast adhesion to the bone surface induces cytoskeletal reorganization and cell activation. The recognition of ECM components is an important step in initiating

osteoclast function (Reinholt, F.P., et al., 1990; Lakkakorpi, P.T., et al., 1991). My results showed that ECM affects TRAP activity through the observation that TRAP activity was increased in general cell populations and the phenotype of cells became more fibroblastic but did not affect the number of multinucleated cells. This result suggests that period of time used in this experiment to extract ECM may be not long enough to produce mature ECM, while it affect on TRAP activity that is an indicators perhaps it will affect on multinucleated cells in long time period ECM. However, more investigations are needed to discover the importance of ECM. In general, ECM proteins are involved in the formation, migration, attachment, and resorptive activity of osteoclasts. In vitro, integrin-mediated adhesion to vitronectin, fibronectin, or collagen induces osteoclast spreading and actin rearrangement (Nakamura, I., et al., 1996). In addition, signaling pathways, which involve c-Src, c-Cbl or PI3-kinase are known to be important for osteoclastic bone resorption (Soriano, P., et al., 1991; Tanaka, S., et al., 1996). Furthermore, previous studies have mentioned the requirement of ECM proteins at targeted sites of bone resorption to attract osteoclast precursors and to promote their proliferation/differentiation, and/or migration (Lakkakorpi PT and Vaananen HK 1991; Paolo M.et al., 2002; Mareel M. and Leroy A. 2003).

Moreover, Romas E (2002) provided further evidence that OPG protects bone integrity by downregulating osteoclastogenesis and promoting osteoclast apoptosis. Modulation of the RANKL/OPG equilibrium in arthritis may provide additional skeletal benefits, such as chondroprotection. The nexus between T-cell activation, TNF-α overproduction, and the RANKL/OPG/RANK ligand-receptor system points to a unifying paradigm for the entire spectrum of skeletal pathology in RA. Strategies that address osteoclastic bone resorption will represent an important new facet of therapy for RA.

6.4.1 The major findings of this chapter are

- IL-17 enhances the number of multinucleated cells formed from PBMCs in vitro.
- IL-17 stimulates bone resorption activity by PBMCs in vitro.

- IL-17 stimulates bone resorption by PBMCs co-cultured with SFB (RA) in vitro.

- IL-17 stimulates bone resorption activity of PBMCs co-cultured with SFB (OA) on ivory.

- The effects of IL-17 on bone resorption in vitro is mostly RANKL-dependent.

Chapter 7
General discussion

7 General discussion

Arthritis is a chronic inflammatory disease characterized by bone and articular cartilage degradation. The pathogenesis of inflammation and tissue destruction in arthritis results from cell–cell interactions between, among others, T lymphocytes, monocytes and synoviocytes. A number of factors are released that cause osteoclast differentiation, maturation and activation. Recent studies of the pathogenesis of RA have revealed that both synovial fibroblasts and T cells participate in the perpetuation of joint inflammation as dynamic partners in a mutual activation, via secretion of cytokines and chemokines that stimulate each other (Hwang SY et al., 2004).

The main aim of this thesis was to investigate the role of IL-17 (produced by activated T cells) in the activation of RA synovial fibroblasts, and the role of OSM, (a macrophage and activated T cell cytokine). In addition, this thesis examined the synergistic interaction of both cytokines on factors such as RANKL and OPG that affect joint destruction. The early stage of the project involved the examination of cytokine activity in synovial fibroblasts and in osteoblast-like cells. The latter stages of the project involved the examination of the effects of IL-17 on osteoclast formation in vitro by using PBMC as osteoclast precursors. Furthermore, osteoclastogenesis was studied in co-cultures with the SFB from RA and OA patients.

RANKL and OPG may be the target of some factors such as IL-17 and OSM. The molecular mechanisms that underlie this phenomenon have not been well understood. RANKL is a differentiation factor for osteoclasts but not an exclusive osteoclast commitment factor, as RANK is expressed not only in osteoclasts but also in T cells and dendritic cells (Anderson DM. et al., 1997). However, the role of local RANKL and OPG in IL-17 action has been restricted to in vitro studies. To confirm the role of these molecules and to characterize molecular mediators in the physiology of abnormal bone resorption triggered by IL-17, studies that further examine the expression and regulation of these molecules are needed.

The present study demonstrated that the expression of RANKL is rapidly enhanced in response to IL-17. This result indicates that IL-17 may affect the expression of RANKL and lead to an increase in bone resorption. The increase in RANKL expression may provide further evidence that the disturbance of the balance of RANKL and OPG by cytokines such as IL-17 and OSM may be interrupting the mechanism linking osteoblast and osteoclast. The increase in RANKL confirmed that the balance of RANKL and

OPG is altered to assist bone resorption. However, the soluble or membrane bound cytokines, interacting with specific receptors expressed on a variety of cell types, modulate their synthesis, secretion, and expression, thus creating a complex cytokine network that influences immunity and inflammation. Although the balance between the production of pro- and anti-inflammatory cytokines and/or their functional receptors changes during the course of these processes, usually it is self-controlled and eventually reaches equilibrium. However, in certain situations e.g. RA, this balance may be shifted toward the production of proinflammatory cytokines for too long, resulting in catabolic effects. For example, in RA patients the overproduction of TNF-α and IL-1β that contributes to the induction of other proinflammatory mediators (IL-6, IL-8, GM-CSF, and many others), seems to be critical for chronic inflammation, ultimately resulting in joint destruction (Feldmann, M et al., 1996). In addition, Saidenberg Kermanac'h N (2002) explained his data as part of an abnormal phenomena in diseases such as RA characterized by both inflammation and bone destruction. RANKL is expressed in a variety of cell types including osteoblasts and activated T cells. In addition, under some conditions, synovial macrophage cells are believed capable of differentiating into osteoclast-like cells under stimulus from M-CSF and RANKL (Itonaga I et al., 2000).

It has been demonstrated previously that in bone loss there is an elevation of RANKL and a reduction of OPG. In addition, RANKL mRNA is expressed in cells within the RA tissues, and the increases in the ratio of RANKL/OPG in RA tissues significantly correlated with the formation of functional octeoclasts. This identifies the OPG/RANKL/RANK pathway of osteoclast differentiation as a possible target for regulating the bone destruction (Haynes DR. et al., 2001; Crotti T et al., 2003).

However, it remains to be clarified whether RANKL is produced in joint cells, how its expression is regulated by local inflammatory mediators, and whether RANKL produced in the joint microenvironment plays any significant role in pathological conditions such as osteoporosis. Firstly RANKL expression was examined in SFB (RA and OA) and contrasted with osteoblastic like cells MG-63 and SaOS2 cell lines. The results indicate that IL-17 transiently induces RANKL expression in synovial fibroblasts and osteoblastic-like cells. In addition, the early induction of RANKL mRNA at 6 hr and no expression at 24 hr agrees with a previous study in mouse osteoblasts (Okahashi N et al., 2004). A similar finding has shown that histamine modulates RANKL expression in stromal cells in a similar manner (Tasaka K. et al., 1993 and Zheng T. et al., 1994). Histamine markedly increased the expression of

RANKL mRNA in a dose-dependent manner by osteoblastic cells, at the earliest time point, while the production of RANKL protein followed this pattern (Deyama Y. et al., 2002). In my work I found in some OA samples and MG-63 and SaOS-2 cells that RANKL mRNA was enhanced by IL-17 and OSM at 6 hr and 48 hr or at 48 hr only. In addition, Frick KK and Bushinsky DA (2003) reported that cultured mouse osteoblasts in acidosis conditions showed significantly increased expression of RANKL mRNA at both 24 hr and 48 hr, supporting my observation of increased RANKL expression at 48 hr in response to IL-17.

Arai F. et al., (1999) suggested that RANKL– RANK signaling plays several roles in organogenesis. M-CSF stimulates c-Kit^{+} Mac-1^{dull} c-Fms^{+} cells to induce RANK mRNA and protein in 24 hr. These cells can differentiate into osteoclasts in the presence of both M-CSF and sRANKL, whilst with M-CSF only they differentiate into macrophages but not osteoclasts. Pre-cultures of c-Kit^{+} Mac-1^{dull} c-Fms^{+} cells with M-CSF for 24 hr or 72 hr result in different fates for osteoclast precursors. RANK^{+} cells after 24 hr pre-culture differentiate into osteoclasts more capably than after 72 hr pre-culture. RANK^{+} cells may separately differentiate into the macrophage lineage in the absence of sRANKL for 72 hr. By contrast, in the presence of both sRANKL and M-CSF after 24 hr pre-culture with M-CSF, RANK^{+} cells efficiently differentiate into osteoclasts. RANK^{-} cells, after pre-culture with M-CSF, may differentiate into RANK^{+} cells if they are continuously exposed to M-CSF. Once the cells express RANK, existing RANKL binds to the receptors. This is the most productive system for osteoclast differentiation, which may take place in vivo on bone surfaces.

Previous report by Arai F (1999) may explain the results presented here that show RANKL mRNA expression at 6 hr which was followed by RANKL production subsequently at 24 hr. RANKL produced at 24 hr may induce the differentiation of osteoclasts instead of macrophage differentiation. Further expression of RANKL mRNA at 48 hr may also follow with the production of RANKL protein at 72 hr. These results suggest that IL-17 may be directly involved in osteoclastogenesis through RANKL production by synovial fibroblast cells and osteoblasts.

It is therefore possible that the bone erosion seen in arthritis may result from RANKL/RANK activation by activated T cells. RANKL secretion by activated T cells can induce osteoclastogenesis. Consequently these mechanisms are enhanced by IL-17, which promote both inflammation and bone resorption Lubberts E et al., (2001).

Furthermore, the effect of IL-17 and OSM on the expression of OPG was examined by synovial fibroblasts from RA and OA and cell lines MG-63 and SaOS2. OPG mRNA levels in RA and OA samples appear to be enhanced in response to IL-17 and OSM in a variable way at 6 hr and 24 hr or 48 hr compared to the control, and at 48 hr by MG-63 and SaOS2. The mechanism underlying this observation is unknown but previous studies have reported that IL-17 affects the production of PGE_2 from synovial tissue cells and osteoblasts. PGE_2 decreases OPG levels in SFB and human bone marrow stroma cells (hBMSC) (Kotake S et al., 1999; Brandstrom H. et al., 1998a). In addition, PGE_2 inhibited OPG production after 16 hr of incubation and the inhibition was sustained for 72 hr in human bone marrow stromal cells (Brandstrom H et al., (2001). Furthermore, over expression of OPG in ST2 stromal cell line and human bone marrow stromal cells is down-regulated by bone-resorbing factors such as glucocorticoids and upregulated by vitamin D3 [1,25(OH)2D3], Ca_2 ions, or TGF-β (Hofbauer LC, 2000; Kong YY. and Penninger JM 2000; Brandstrom et al., 1998a,b; Huang L. at al., 2001). In contrast to my findings Nagasawa T, et al., (2002) reported that the production of OPG was evident already after 6 hr and increased consistently during the 24 hr while OPG decreased at 48 hr in human gingival fibroblasts in response to LPS.

Generally in this study OPG mRNA expression was detectable in all samples from RA, OA and osteoblast-like cells, but the general pattern of OPG expression in response to IL-17 and OSM between these samples showed some variation in SFB OA. This suggests that there may be considerable heterogeneity between patients. Pulsatelli L et al., (2004) demonstrated that there was an age variation in the expression of OPG in arthritis patients. In addition, a previous study reported that OPG was highly expressed in SFB RA compared to OA and non-inflammatory synovial fibroblasts, and different levels of OPG expression were found among patients with RA (Kubota A. et al., 2004). Alternatively, the induction of OPG at 6 hr and 48 hr may be due to the secretion of IGF-I from the synovial tissue as an initial study reported that IGF-I has an inhibitory effect on OPG mRNA at 24 hr. IGF-I, in a dose- and time-dependent manner, regulates OPG and RANKL in vitro and in vivo (Rubin J. 2002). However, these different modes of regulation may represent an unexpected complexity of the dynamics of IL-17R-mediated transcription. The signaling pathway(s) utilized by IL-17 in various cells still remain unknown. Several studies have provided further details about the physiological and pharmacological relevance of the multiple couplings and their dynamic regulation. In the near future we will know more about the signal transduction of IL-17. The

increase in the production of OPG opens up the possibility that OPG may have therapeutic effects mediated by blockade of the RANKL/RANK system.

IL-17 has been shown to promote bone and cartilage destruction, through PGE_2, RANKL, IL-6, GM-CSF, IL-8 and M-CSF release in vivo and in vitro (Lubberts E et al., 2004; Yamamura Y et al., 2001; Chabaud M et al., 2001; Yao, Z. et al., 1995). Previous studies have shown that IL-17 probably has a role in the presence of other pro-inflammatory cytokines, leading to a synergistic release of cartilage and bone degradation factors (Chabaud M et al., 2001). The present study has investigated the effect of IL-17 combined with OSM on the expression of RANKL and production of OPG in vitro. IL-17 combined with OSM affects on the expression of OPG and RANKL mRNA by RA and OA synovial fibroblast cells. In contrast, Koshy PJ. et al., (2002) reported that IL-17 in combination with other proinflammatory cytokines such as TNF-α, IL-1, OSM and IL-6 promote chondrocyte mediated MMP dependent type II collagen release from cartilage. However, IL-17 may in combination with other proinflammatory cytokines have effects on bone degradation through the release of factors that cause osteoclastogenesis (Kotake S. et al. 1999). In addition, Langdon C. et al., (1997) and Hui W et al., (1997) both demonstrated that OSM was detectable in variable levels in synovial fluids of RA patients but not in OA patients. However, the results lead to the suggestion that OSM may have another role in combination with IL-17. OSM may function in equilibrium with IL-17 to affect the expression of RANKL and OPG. It is also possible that these cytokines are produced in different positions or at different point in time in unhealthy joints. However, a previous study shows that OSM effects the up-regulation of RANKL and its receptor RANK in the periosteum, while osteoclasts were not detected at sites of bone apposition (de Hooge AS. et al., 2002).

Also in this thesis MG-63 and SaOS-2 cells showed some synergistic effects on the expression and production of OPG mRNA in response to the combination of IL-17 and OSM. This combination had no effect on the expression of RANKL mRNA. However, IL-17 activates NF-kB and/or AP-1 while OSM transduces its signal via gp130 and LIF receptor-β or OSM receptor-β (Mosley et al., 1996). In mice, OSM leads to activation of the mitogen activated protein kinase (MAPK) cascades and the Janus kinase-signal transducers and activator of transcription (JAK/STAT) pathway, and modulation of AP-1 (Kasza A et al., 2001; Taga and Kishimoto 1997; Korzus E et al., 1997).

From results from RA and OA synovial fibroblasts, I suggest that the presence of both of these cytokines in the same area together may modulate the effects of each other on the expression of RANKL and OPG and on the production of OPG. OSM may decrease the effect of IL-17 alone. Therefore the combination of IL-17 and OSM together in vivo may decrease expression of RANKL mRNA in arthritis which may lead to a decrease in osteoclast differentiation, formation and activation decreasing osteoclastogenesis and lowering bone and cartilage degradation. OSM has a potentially important function in the modulation of chemokine and MMP production by synovial cells of the joint (Langdon C. et al., (1997). However, the exact proportion of IL-17-producing T cells and OSM and its function remain to be determined. The relative balance of Il-17 and OSM in arthritis might therefore be important in controlling the level of joint destruction. Control of such an effect may lead to a therapeutic anti-inflammatory property.

Osteoclasts are highly specialized multinucleated cells which are uniquely capable of lacunar bone resorption; these cells are formed by fusion of marrow-derived mononuclear phagocytic osteoclast precursors which circulate in the monocyte fraction of peripheral blood (Fujikawa Y., et al., 1996; Massey HM, et al., 1999). Osteoclast differentiation from haematopoietic and circulating osteoclast precursors occurs in the presence of M-CSF and RANKL, which is expressed by bone stromal cells (Lacey DL. et al., 1998; Yasuda H, et al., 1998). RANKL interacts with RANK, which is present on osteoclast precursors (Nakagawa N. et al., 1998). In this study IL-17 induced a significant increase in the numbers of multinucleated TRAP-positive cells in PBMC cultures. These results suggest that IL-17 stimulation leads to the fusion of preosteoclasts. IL-17 induces the expression of TRAP-positive activity in multinucleated cells with and without M-CSF.

Studies further examined the role of IL-17 on osteoclastogenesis by measuring resorption of ivory wafers in culture. IL-17 with and without M-CSF significantly induced osteoclast formation and excavation of resorption lacunae. Co-culture of PBMC and synovial fibroblasts from RA and OA on ivory slices showed increased resorption compared to the cultures of PBMC alone. Therefore, IL-17 may increase the production of RANKL and M-CSF from PBMC and SFB cells. Moreover, the cell to cell contact in the co-culture may increase the response of the cells to each other and the presence of membrane bounded RANKL by synovial fibroblasts may more effectively stimulate PBMC. A previous study demonstrated that membrane-bound RANKL works

more efficiently than sRANKL in osteoclastogenesis in murine bone marrow cell cultures (Nakashisma T. et al., 2000).

TNFα and IL-1α directly induce osteoclastogenesis in mouse marrow cultures, and evidence suggests that in the presence of M-CSF, TNFα is sufficient for inducing human osteoclast differentiation from circulating precursors by a process which is distinct from the RANK/RANKL signaling pathway (Kudo O et al., 2002). However, findings presented here is show that there is a dramatic decrease in the resorption area when OPG is added to IL-17-treated cultures. These finding suggest that IL-17-stimulated bone resorption is RANKL-dependent. The number of TRAP+ve cells and the lacunae resorption surface area in PBMC co-cultured with SFB (RA) and in the PBMC co-cultured with SFB (OA) are higher than the PBMC cultured alone. These results agree with the previous hypothesis by Kotake, et al., (1999) which suggest that IL-17 first acts on osteoblasts or other cell type, which stimulates both COX-2–dependent PGE_2 synthesis and RANKL gene expression, which in turn induces differentiation of osteoclast progenitors into mature osteoclasts. IL-17 is therefore a crucial cytokine for osteoclastic bone resorption in RA patients.

In humans, IL-17 generated by T cell activation may play a major role in bone resorption via RANKL-mediated mechanisms whether or not M-CSF is present. Researchers have suggested that there are two distinct pathways of osteoclast formation, one RANKL-independent and the other RANKL-induced (Fig 7.1). These pathways may operate under different tissue conditions: physiological (e.g. normal bone turnover) and pathological (e.g. RA) with IL-17 enhancing osteoclast formation and bone resorption through RANKL.

Osteoclast adhesion to the bone surface induces cytoskeletal reorganization and cell activation. ECM interaction is an important step in initiating osteoclast function (Reinholt, F.P., et al., 1990; Lakkakorpi, P.T., et al., 1991). Data presented here shows that IL-17 regulates RANKL/OPG production by synovial cells and is capable of mediating increased osteoclastogenesis and bone resorption. Therefore understanding the role of ECM affects on TRAP activity but not multinucleated cell formation although more investigations are need. ECM proteins are involved in the regulation of osteoclast activity. In vitro adhesion molecules are needed to induce osteoclast spreading and rearrangement (Nakamura, I., et al., 1996). In addition, signaling pathways, such as c-Src, c-Cbl, or PI3-kinase, are known to be important for osteoclastic bone resorption (Soriano, P., et al., 1991 and Tanaka, S., et al., 1996).

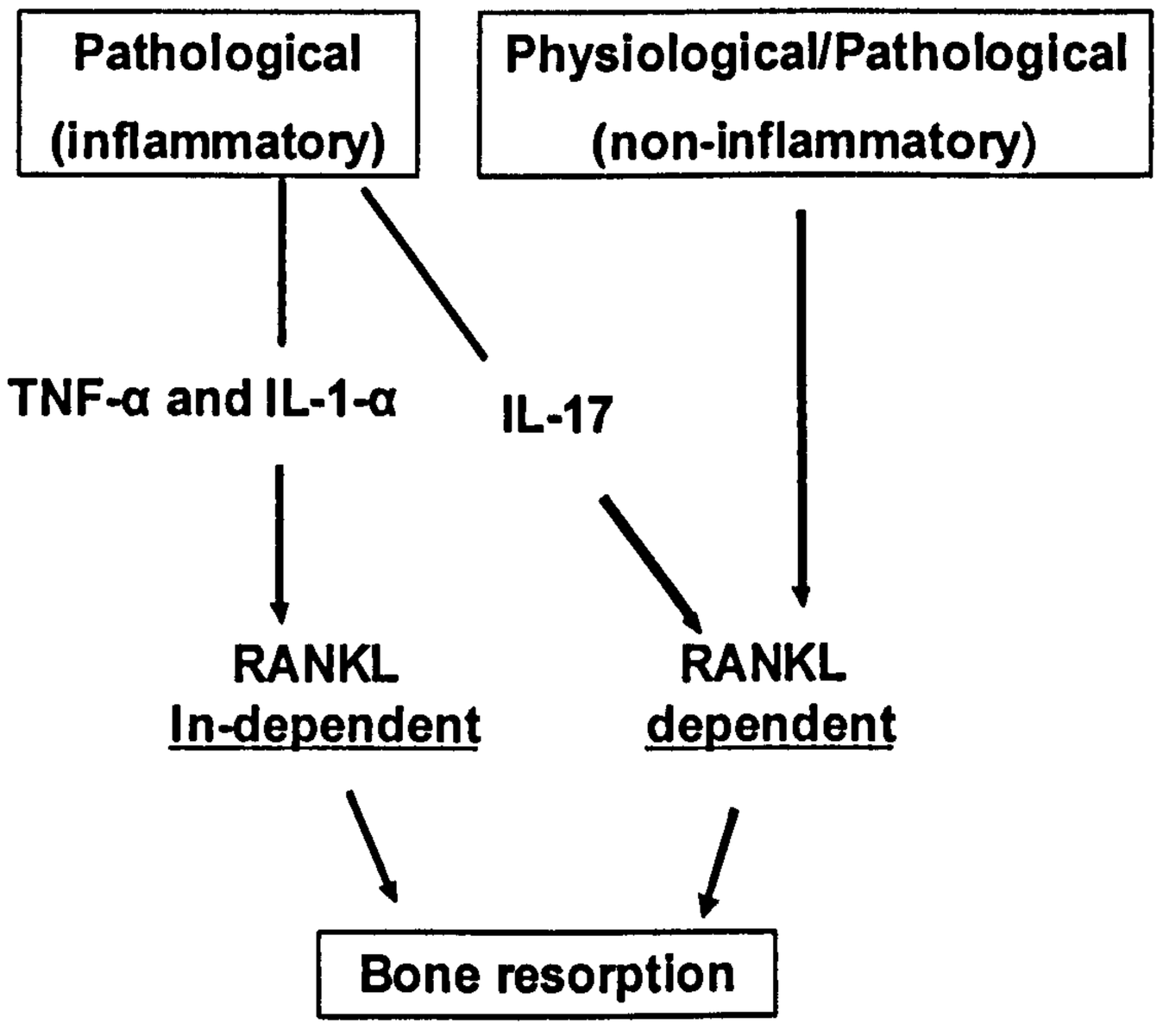


Fig 7.1. Possible role of RANKL-dependent and RANKL-independent (cytokine-induced) osteoclast formation on pathological resorption associated, respectively, with non-inflammatory and inflammatory disorders of bone (Kudo O et al, 2002).

In summary, these finding, support previous data that showed the abnormal phenomena in diseases such as RA are characterized by both inflammation and destruction Saidenberg Kermanac'h N (2002). Activated T cells within the rheumatoid synovium express and produce RANKL and IL-17. IL-17 and RANKL in the pathogenesis of chronic arthritis will contribute to improvement of current therapies. This study highlights the possibility that OPG may have therapeutic effects mediated by blockade of the RANKL/RANK system.

Further studies are needed to elucidate the molecular mechanisms by which IL-17 regulates the expression of RANKL. The affect of IL-17 on the production of RANKL, M-CSF and other osteoclastogenesis factors by connective tissue cells. In addition, study of the signal transduction pathways of IL-17, TNFα, and RANKL, and the cross-

talk between them may lead us to limit their affects on osteoclast formation and diseases exhibiting excessive bone destruction.

Chapter-8

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8 References

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Appendix

9 Appendix

The following data has been included in order to give the reader a full account of the results presented in Chapter 5 and to allow investigation of the statistical analysis of the experiments. However, as previously described in Chapter 5, it is generally easier to follow the descriptives and Analysis of Variance (one way), with Tukey or Duncan post test.

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1=CONT	9	2.1111	2.02759	.67586	.5526	3.6697	.00	6.00
2=IL-17 5 NG/ML	10	7.6000	1.50555	.47610	6.5230	8.6770	5.00	10.00
3= IL-1 50NG/ML	9	11.3333	3.24037	1.08012	8 8426	13.8241	5.00	15.00
4=RANKL+M-CSF 30+25NG/ML	10	16.6000	3.80643	1.20370	13.8770	19.3230	11.00	24.00
5=IL-17+M-CSF 5+25NG/ML	10	14.2000	5.07280	1.60416	10.5711	17.8289	7.00	21.00
6=IL-17+M-CSF 50+25NG/ML	10	13 6000	4.29987	1.35974	10.5241	16.6759	8 00	20.00
7=RANKL 30NG/ML	10	6.2000	1.87380	.59255	4.8596	7.5404	4.00	10.00
8=M-CSF 25NG/ML	10	5.2000	1.93218	.61101	3.8178	6.5822	2 00	8.00
Total	78	9 6795	5 66716	64168	8 4017	10 9572	00	24 00

Table Ape.1a. The descriptive statistical analysis for TRAP+ve multinucleated cells (MNCs) formed in vitro from PBMC as shown in Fig 6.3.

Multiple Comparisons

Dependent Variable: VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1=CONT	2=IL-17 5 NG/ML	-5.4889*	1.48468	.010	-10.9622	-.0156
		3= IL-1 50NG/ML	-9.2222*	1.52325	.000	-14.8377	-3.6067
		4=RANKL+M-CSF 30+25NG/ML	-14.4889*	1.48468	.000	-19.9622	-9.0156
		5=IL-17+M-CSF 5+25NG/ML	-12.0889*	1.48468	.000	-17.5622	-6.6156
		6=IL-17+M-CSF 50+25NG/ML	-11.4889*	1.48468	.000	-16.9622	-6.0156
		7=RANKL 30NG/ML	-4.0889	1.48468	.124	-9.5622	1.3844
		8=M-CSF 25NG/ML	-3.0889	1.48468	.437	-8.5622	2.3844
	2=IL-17 5 NG/ML	1=CONT	5.4889*	1.48468	.010	.0156	10.9622
		3= IL-1 50NG/ML	-3.7333	1.48468	.207	-9.2066	1.7400
		4=RANKL+M-CSF 30+25NG/ML	-9.0000*	1.44508	.000	-14.3273	-3.6727
		5=IL-17+M-CSF 5+25NG/ML	-6.6000*	1.44508	.001	-11.9273	-1.2727
		6=IL-17+M-CSF 50+25NG/ML	-6.0000*	1.44508	.002	-11.3273	-.6727
		7=RANKL 30NG/ML	1.4000	1.44508	.977	-3.9273	6.7273
		8=M-CSF 25NG/ML	2.4000	1.44508	.712	-2.9273	7.7273
	3= IL-1 50NG/ML	1=CONT	9.2222*	1.52325	.000	3.6067	14.8377
		2=IL-17 5 NG/ML	3.7333	1.48468	.207	-1.7400	9.2066
		4=RANKL+M-CSF 30+25NG/ML	-5.2667	1.48468	.015	-10.7400	.2066
		5=IL-17+M-CSF 5+25NG/ML	-2.8667	1.48468	.535	-8.3400	2.6066
		6=IL-17+M-CSF 50+25NG/ML	-2.2667	1.48468	.790	-7.7400	3.2066
		7=RANKL 30NG/ML	5.1333	1.48468	.020	-.3400	10.6066
		8=M-CSF 25NG/ML	6.1333*	1.48468	.002	.6600	11.6066
	4=RANKL+M-CSF 30+25NG/ML	1=CONT	14.4889*	1.48468	.000	9.0156	19.9622
		2=IL-17 5 NG/ML	9.0000*	1.44508	.000	3.6727	14.3273
		3= IL-1 50NG/ML	5.2667	1.48468	.015	-.2066	10.7400
		5=IL-17+M-CSF 5+25NG/ML	2.4000	1.44508	.712	-2.9273	7.7273
		6=IL-17+M-CSF 50+25NG/ML	3.0000	1.44508	.440	-2.3273	8.3273
		7=RANKL 30NG/ML	10.4000*	1.44508	.000	5.0727	15.7273
		8=M-CSF 25NG/ML	11.4000*	1.44508	.000	6.0727	16.7273
	5=IL-17+M-CSF 5+25NG/ML	1=CONT	12.0889*	1.48468	.000	6.6156	17.5622
		2=IL-17 5 NG/ML	6.6000*	1.44508	.001	1.2727	11.9273
		3= IL-1 50NG/ML	2.8667	1.48468	.535	-2.6066	8.3400
		4=RANKL+M-CSF 30+25NG/ML	-2.4000	1.44508	.712	-7.7273	2.9273
		6=IL-17+M-CSF 50+25NG/ML	.6000	1.44508	1.000	-4.7273	5.9273
		7=RANKL 30NG/ML	8.0000*	1.44508	.000	2.6727	13.3273
		8=M-CSF 25NG/ML	9.0000*	1.44508	.000	3.6727	14.3273
	6=IL-17+M-CSF 50+25NG/ML	1=CONT	11.4889*	1.48468	.000	6.0156	16.9622
		2=IL-17 5 NG/ML	6.0000*	1.44508	.002	.6727	11.3273
		3= IL-1 50NG/ML	2.2667	1.48468	.790	-3.2066	7.7400
		4=RANKL+M-CSF 30+25NG/ML	-3.0000	1.44508	.440	-8.3273	2.3273
		5=IL-17+M-CSF 5+25NG/ML	-.6000	1.44508	1.000	-5.9273	4.7273
		7=RANKL 30NG/ML	7.4000*	1.44508	.000	2.0727	12.7273
		8=M-CSF 25NG/ML	8.4000*	1.44508	.000	3.0727	13.7273
	7=RANKL 30NG/ML	1=CONT	4.0889	1.48468	.124	-1.3844	9.5622
		2=IL-17 5 NG/ML	-1.4000	1.44508	.977	-6.7273	3.9273
		3= IL-1 50NG/ML	-5.1333	1.48468	.020	-10.6066	.3400
		4=RANKL+M-CSF 30+25NG/ML	-10.4000*	1.44508	.000	-15.7273	-5.0727
		5=IL-17+M-CSF 5+25NG/ML	-8.0000*	1.44508	.000	-13.3273	-2.6727
		6=IL-17+M-CSF 50+25NG/ML	-7.4000*	1.44508	.000	-12.7273	-2.0727
		8=M-CSF 25NG/ML	1.0000	1.44508	.997	-4.3273	6.3273
	8=M-CSF 25NG/ML	1=CONT	3.0889	1.48468	.437	-2.3844	8.5622
		2=IL-17 5 NG/ML	-2.4000	1.44508	.712	-7.7273	2.9273
		3= IL-1 50NG/ML	-6.1333*	1.48468	.002	-11.6066	-.6600
		4=RANKL+M-CSF 30+25NG/ML	-11.4000*	1.44508	.000	-16.7273	-6.0727
		5=IL-17+M-CSF 5+25NG/ML	-9.0000*	1.44508	.000	-14.3273	-3.6727
		6=IL-17+M-CSF 50+25NG/ML	-8.4000*	1.44508	.000	-13.7273	-3.0727
		7=RANKL 30NG/ML	-1.0000	1.44508	.997	-6.3273	4.3273

*. The mean difference is significant at the .01 level.

Table Ape.1b. the statistical analysis (ANOVA) for TRAP+ve multinucleated cells (MNCs) formed in vitro from PBMC as shown in Fig 6.3.

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1=cont	21	2.0952	1.60950	.35122	1.3626	2.8279	.00	6.00
2=IL-17 5 ng/ml	21	5.1905	1.32737	.28966	4.5863	5.7947	2.00	7.00
3-IL-17 50 mg/ml	19	9.8947	2.86540	.65737	8.5137	11.2758	5.00	14.00
4=RANKL 30 ng/ml + M-CSF 25 ng/ml	20	15.2500	3.65449	.81717	13.5396	16.9604	10.00	24.00
5= IL-17 5 ng/ml + M-CSF 25 ng/ml	20	12.2500	3.59642	.80418	10.5668	13.9332	7.00	21.00
6= IL-17 50 ng/ml +M-CSF 25ng/ml	20	12.5500	3.60519	.80614	10.8627	14.2373	8.00	20.00
7=RANKL 30 ng/ml	20	4.5500	1.31689	.29447	3.9337	5.1663	2.00	7.00
8=M-CSF 25ng/ml	20	4.0000	1.52177	.34028	3.2878	4.7122	2.00	7.00
Total	161	8.1553	5.24709	.41353	7.3386	8.9720	.00	24.00

Table Ape.1a. The descriptive statistical analysis for TRAP+ve multinucleated cells (MNCs) formed in vitro from PBMC as shown in Fig 6.3.

Multiple Comparisons

Dependent Variable: VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig	99% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1=cont	2=IL-17 5 ng/ml	-3.0952*	.81162	.005	-6.0159	-.1746
		3-IL-17 50 mg/ml	-7.7995*	.83270	.000	-10.7960	-4.8030
		4=RANKL 30 ng/ml + M-CSF 25 ng/ml	-13.1548*	.82170	.000	-16.1117	-10.1978
		5= IL-17 5 ng/ml + M-CSF 25 ng/ml	-10.1548*	.82170	.000	-13.1117	-7.1978
		6= IL-17 50 ng/ml +M-CSF 25ng/ml	-10.4548*	.82170	.000	-13.4117	-7.4978
		7=RANKL 30 ng/ml	-2.4548	.82170	.063	-5.4117	.5022
		8=M-CSF 25ng/ml	-1.9048	.82170	.291	-4.8617	1.0522
	2=IL-17 5 ng/ml	1=cont	3.0952*	.81162	.005	.1746	6.0159
		3-IL-17 50 mg/ml	-4.7043*	.83270	.000	-7.7008	-1.7077
		4=RANKL 30 ng/ml + M-CSF 25 ng/ml	-10.0595*	.82170	.000	-13.0165	-7.1026
		5= IL-17 5 ng/ml + M-CSF 25 ng/ml	-7.0595*	.82170	.000	-10.0165	-4.1026
		6= IL-17 50 ng/ml +M-CSF 25ng/ml	-7.3595*	.82170	.000	-10.3165	-4.4026
		7=RANKL 30 ng/ml	.6405	.82170	.994	-2.3165	3.5974
		8=M-CSF 25ng/ml	1.1905	.82170	.833	-1.7665	4.1474
	3-IL-17 50 mg/ml	1=cont	7.7995*	.83270	.000	4.8030	10.7960
		2=IL-17 5 ng/ml	4.7043*	.83270	.000	1.7077	7.7008
		4=RANKL 30 ng/ml + M-CSF 25 ng/ml	-5.3553*	.84253	.000	-8.3872	-2.3234
		5= IL-17 5 ng/ml + M-CSF 25 ng/ml	-2.3553	.84253	.104	-5.3872	.6766
		6= IL-17 50 ng/ml +M-CSF 25ng/ml	-2.6553	.84253	.040	-5.6872	.3766
		7=RANKL 30 ng/ml	5.3447*	.84253	.000	2.3128	8.3766
		8=M-CSF 25ng/ml	5.8947*	.84253	.000	2.8628	8.9266
	4=RANKL 30 ng/ml + M-CSF 25 ng/ml	1=cont	13.1548*	.82170	.000	10.1978	16.1117
		2=IL-17 5 ng/ml	10.0595*	.82170	.000	7.1026	13.0165
		3-IL-17 50 mg/ml	5.3553*	.84253	.000	2.3234	8.3872
		5= IL-17 5 ng/ml + M-CSF 25 ng/ml	3.0000*	.83166	.010	.0072	5.9928
		6= IL-17 50 ng/ml +M-CSF 25ng/ml	2.7000	.83166	.030	-.2928	5.6928
		7=RANKL 30 ng/ml	10.7000*	.83166	.000	7.7072	13.6928
		8=M-CSF 25ng/ml	11.2500*	.83166	.000	8.2572	14.2428
	5= IL-17 5 ng/ml + M-CSF 25 ng/ml	1=cont	10.1548*	.82170	.000	7.1978	13.1117
		2=IL-17 5 ng/ml	7.0595*	.82170	.000	4.1026	10.0165
		3-IL-17 50 mg/ml	2.3553	.84253	.104	-.6766	5.3872
		4=RANKL 30 ng/ml + M-CSF 25 ng/ml	-3.0000*	.83166	.010	-5.9928	-.0072
		6= IL-17 50 ng/ml +M-CSF 25ng/ml	-.3000	.83166	1.000	-3.2928	2.6928
		7=RANKL 30 ng/ml	7.7000*	.83166	.000	4.7072	10.6928
		8=M-CSF 25ng/ml	8.2500*	.83166	.000	5.2572	11.2428
	6= IL-17 50 ng/ml +M-CSF 25ng/ml	1=cont	10.4548*	.82170	.000	7.4978	13.4117
		2=IL-17 5 ng/ml	7.3595*	.82170	.000	4.4026	10.3165
		3-IL-17 50 mg/ml	2.6553	.84253	.040	.3766	5.6872
		4=RANKL 30 ng/ml + M-CSF 25 ng/ml	-2.7000	.83166	.030	-5.6928	.2928
		5= IL-17 5 ng/ml + M-CSF 25 ng/ml	.3000	.83166	1.000	-2.6928	3.2928
		7=RANKL 30 ng/ml	8.0000*	.83166	.000	5.0072	10.9928
		8=M-CSF 25ng/ml	8.5500*	.83166	.000	5.5572	11.5428
	7=RANKL 30 ng/ml	1=cont	2.4548	.82170	.063	-.5022	5.4117
		2=IL-17 5 ng/ml	-.6405	.82170	.994	-3.5974	2.3165
		3-IL-17 50 mg/ml	-5.3447*	.84253	.000	-8.3766	-2.3128
		4=RANKL 30 ng/ml + M-CSF 25 ng/ml	-10.7000*	.83166	.000	-13.6928	-7.7072
		5= IL-17 5 ng/ml + M-CSF 25 ng/ml	-7.7000*	.83166	.000	-10.6928	-4.7072
		6= IL-17 50 ng/ml +M-CSF 25ng/ml	-8.0000*	.83166	.000	-10.9928	-5.0072
		8=M-CSF 25ng/ml	.5500	.83166	.998	-2.4428	3.5428
	8=M-CSF 25ng/ml	1=cont	1.9048	.82170	.291	-1.0522	4.8617
		2=IL-17 5 ng/ml	-1.1905	.82170	.833	-4.1474	1.7665
		3-IL-17 50 mg/ml	-5.8947*	.84253	.000	-8.9266	-2.8628
		4=RANKL 30 ng/ml + M-CSF 25 ng/ml	-11.2500*	.83166	.000	-14.2428	-8.2572
		5= IL-17 5 ng/ml + M-CSF 25 ng/ml	-8.2500*	.83166	.000	-11.2428	-5.2572
		6= IL-17 50 ng/ml +M-CSF 25ng/ml	-8.5500*	.83166	.000	-11.5428	-5.5572
		7=RANKL 30 ng/ml	-.5500	.83166	.998	-3.5428	2.4428

*. The mean difference is significant at the .01 level.

Table Ape.1b. the statistical analysis (ANOVA) for TRAP+ve multinucleated cells (MNCs) formed in vitro from PBMC as shown in Fig 6.3.

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1=OPG	16	1.6000	1.04115	.26029	1.0452	2.1548	.50	4.30
2=IL-17 5	16	3.1938	1.10843	.27711	2.6031	3.7844	1.50	6.00
3=IL-17 50	15	3.7667	1.78152	.45999	2.7801	4.7532	.60	7.90
4=RANKL/M-CSF	16	4.9750	3.24459	.81115	3.2461	6.7039	2.00	12.50
5=IL-17 5/M-CSF	16	4.1875	1.51959	.37990	3.3778	4.9972	.50	6.60
6=IL-17 50/M-CSF	16	4.7688	2.15181	.53795	3.6221	5.9154	1.30	9.90
7=IL-17/OPG	16	1.6562	.93093	.23273	1.1602	2.1523	.40	3.60
8=RANKL/M-CSF/OPG	16	2.3750	2.56502	.64126	1.0082	3.7418	.50	10.80
Total	127	3.3118	2.27375	.20176	2.9125	3.7111	.40	12.50

Table Ape.2a. The statistical description for the resorption area on ivory slices in the cultures of PBMC alone as shown in Fig 6.5.

Multiple Comparisons

Dependent Variable: VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1=OPG	2=IL-17 5	-1.5938	.68967	.297	-3.7218	.5343
		3=IL-17 50	-2.1667*	.70107	.049	-4.3299	-.0035
		4=RANKL/M-CSF	-3.3750*	.68967	.000	-5.5030	-1.2470
		5=IL-17 5/M-CSF	-2.5875*	.68967	.006	-4.7155	-.4595
		6=IL-17 50/M-CSF	-3.1687*	.68967	.000	-5.2968	-1.0407
		7=IL-17/OPG	-.0562	.68967	1.000	-2.1843	2.0718
		8=RANKL/M-CSF/OPG	-.7750	.68967	.950	-2.9030	1.3530
	2=IL-17 5	1=OPG	1.5938	.68967	.297	-.5343	3.7218
		3=IL-17 50	-.5729	.70107	.992	-2.7361	1.5903
		4=RANKL/M-CSF	-1.7812	.68967	.173	-3.9093	.3468
		5=IL-17 5/M-CSF	-.9937	.68967	.836	-3.1218	1.1343
		6=IL-17 50/M-CSF	-1.5750	.68967	.311	-3.7030	.5530
		7=IL-17/OPG	1.5375	.68967	.342	-.5905	3.6655
		8=RANKL/M-CSF/OPG	.8188	.68967	.934	-1.3093	2.9468
	3=IL-17 50	1=OPG	2.1667*	.70107	.049	.0035	4.3299
		2=IL-17 5	.5729	.70107	.992	-1.5903	2.7361
		4=RANKL/M-CSF	-1.2083	.70107	.672	-3.3715	.9549
		5=IL-17 5/M-CSF	-.4208	.70107	.999	-2.5840	1.7424
		6=IL-17 50/M-CSF	-1.0021	.70107	.842	-3.1653	1.1611
		7=IL-17/OPG	2.1104	.70107	.061	-.0528	4.2736
		8=RANKL/M-CSF/OPG	1.3917	.70107	.496	-.7715	3.5549
	4=RANKL/M-CSF	1=OPG	3.3750*	.68967	.000	1.2470	5.5030
		2=IL-17 5	1.7812	.68967	.173	-.3468	3.9093
		3=IL-17 50	1.2083	.70107	.672	-.9549	3.3715
		5=IL-17 5/M-CSF	.7875	.68967	.946	-1.3405	2.9155
		6=IL-17 50/M-CSF	.2062	.68967	1.000	-1.9218	2.3343
		7=IL-17/OPG	3.3188*	.68967	.000	1.1907	5.4468
		8=RANKL/M-CSF/OPG	2.6000*	.68967	.006	.4720	4.7280
	5=IL-17 5/M-CSF	1=OPG	2.5875*	.68967	.006	.4595	4.7155
		2=IL-17 5	.9937	.68967	.836	-1.1343	3.1218
		3=IL-17 50	.4208	.70107	.999	-1.7424	2.5840
		4=RANKL/M-CSF	-.7875	.68967	.946	-2.9155	1.3405
		6=IL-17 50/M-CSF	-.5812	.68967	.990	-2.7093	1.5468
		7=IL-17/OPG	2.5313*	.68967	.008	.4032	4.6593
		8=RANKL/M-CSF/OPG	1.8125	.68967	.156	-.3155	3.9405
	6=IL-17 50/M-CSF	1=OPG	3.1687*	.68967	.000	1.0407	5.2968
		2=IL-17 5	1.5750	.68967	.311	-.5530	3.7030
		3=IL-17 50	1.0021	.70107	.842	-1.1611	3.1653
		4=RANKL/M-CSF	-.2062	.68967	1.000	-2.3343	1.9218
		5=IL-17 5/M-CSF	.5812	.68967	.990	-1.5468	2.7093
		7=IL-17/OPG	3.1125*	.68967	.000	.9845	5.2405
		8=RANKL/M-CSF/OPG	2.3937*	.68967	.016	.2657	4.5218
	7=IL-17/OPG	1=OPG	.0562	.68967	1.000	-2.0718	2.1843
		2=IL-17 5	-1.5375	.68967	.342	-3.6655	.5905
		3=IL-17 50	-2.1104	.70107	.061	-4.2736	.0528
		4=RANKL/M-CSF	-3.3188*	.68967	.000	-5.4468	-1.1907
		5=IL-17 5/M-CSF	-2.5313*	.68967	.008	-4.6593	-.4032
		6=IL-17 50/M-CSF	-3.1125*	.68967	.000	-5.2405	-.9845
		8=RANKL/M-CSF/OPG	-.7188	.68967	.967	-2.8468	1.4093
	8=RANKL/M-CSF/OPG	1=OPG	.7750	.68967	.950	-1.3530	2.9030
		2=IL-17 5	-.8188	.68967	.934	-2.9468	1.3093
		3=IL-17 50	-1.3917	.70107	.496	-3.5549	.7715
		4=RANKL/M-CSF	-2.6000*	.68967	.006	-4.7280	-.4720
		5=IL-17 5/M-CSF	-1.8125	.68967	.156	-3.9405	.3155
		6=IL-17 50/M-CSF	-2.3937*	.68967	.016	-4.5218	-.2657
		7=IL-17/OPG	.7188	.68967	.967	-1.4093	2.8468

*. The mean difference is significant at the .05 level.

Table Ape.2b. The statistical analysis (ANOVA) for the resorption area formation on ivory slices in the cultures of PBMC alone as shown in Fig 6.5.

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1=OPG	38	1.2105	.96136	.15595	.8945	1.5265	.10	4.50
2=IL-17 5	38	3.5132	2.09712	.34020	2.8239	4.2025	.30	10.30
3=IL-17 50	35	5.3229	2.93439	.49600	4.3149	6.3309	.60	13.00
4=RANKLM-CSF	39	6.8590	5.14821	.82437	5.1901	8.5278	.50	18.00
5=IL-17 5/M-CSF	40	3.9725	2.52942	.39994	3.1636	4.7814	.30	12.20
6=IL-17 50 /M-CSF	39	5.6667	2.66294	.42641	4.8034	6.5299	1.60	11.40
7=IL-17 50 / OPG	37	2.9162	1.87328	.30797	2.2916	3.5408	.80	7.30
8=RANKLM-CSF/OPG	40	1.7725	1.36288	.21549	1.3366	2.2084	.10	5.30
9=M-CSF	40	1.9775	1.10789	.17517	1.6232	2.3318	.30	4.70
Total	346	3.6737	3.15045	.16937	3.3406	4.0068	.10	18.00

Table Ape.3a. The statistical description for the resorption area on ivory slices in the co-cultures of PBMC and Synovial fibroblasts from RA as shown in Fig 6.8.

Multiple Comparisons

Dependent Variable: VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1=OPG	2=IL-17 5	-2.3026*	.59443	.004	-4.4571	-.1482
		3=IL-17 50	-4.1123*	.60703	.000	-6.3125	-1.9122
		4=RANKL/MCSF	-5.6484*	.59060	.000	-7.7890	-3.5079
		5=IL-17 5/MCSF	-2.7620*	.58695	.000	-4.8893	-.6346
		6=IL-17 50/M-CSF	-4.4561*	.59060	.000	-6.5967	-2.3155
		7=IL-17 50/ OPG	-1.7057	.59843	.105	-3.8746	.4633
		8=RANKL/MCSF/OPG	-.5620	.58695	.989	-2.6893	1.5654
		9=MCSF	-.7670	.58695	929	-2.8943	1.3604
	2=IL-17 5	1=OPG	2.3026*	.59443	.004	.1482	4.4571
		3=IL-17 50	-1.8097	.60703	.074	-4.0098	.3904
		4=RANKL/MCSF	-3.3458*	.59060	.000	-5.4864	-1.2052
		5=IL-17 5/MCSF	-.4593	.58695	.997	-2.5867	1.6680
		6=IL-17 50/M-CSF	-2.1535*	.59060	.009	-4.2941	-.0129
		7=IL-17 50/ OPG	.5969	.59843	.966	-1.5720	2.7659
		8=RANKL/MCSF/OPG	1.7407	.58695	.078	-.3867	3.8680
		9=MCSF	1.5357	.58695	.184	-.5917	3.6630
	3=IL-17 50	1=OPG	4.1123*	.60703	.000	1.9122	6.3125
		2=IL-17 5	1.8097	.60703	.074	-.3904	4.0098
		4=RANKL/MCSF	-1.5361	.60329	.214	-3.7227	.6505
		5=IL-17 5/MCSF	1.3504	.59971	.375	-.8232	3.5240
		6=IL-17 50/M-CSF	-.3438	.60329	1.000	-2.5304	1.8428
		7=IL-17 50/ OPG	2.4066*	.61095	.003	.1923	4.6210
		8=RANKL/MCSF/OPG	3.5504*	.59971	.000	1.3768	5.7240
		9=MCSF	3.3454*	.59971	.000	1.1716	5.5190
	4=RANKL/MCSF	1=OPG	5.6484*	.59060	.000	3.5079	7.7890
		2=IL-17 5	3.3458*	.59060	.000	1.2052	5.4864
		3=IL-17 50	1.5361	.60329	.214	-.6505	3.7227
		5=IL-17 5/MCSF	2.8865*	.58308	.000	.7732	4.9998
		6=IL-17 50/M-CSF	1.1923	.58676	.522	-.9343	3.3190
		7=IL-17 50/ OPG	3.9428*	.59463	.000	1.7876	6.0980
		8=RANKL/MCSF/OPG	5.0865*	.58308	.000	2.9732	7.1998
		9=MCSF	4.8815*	.58308	.000	2.7682	6.9948
	5=IL-17 5/MCSF	1=OPG	2.7620*	.58695	.000	.6346	4.8893
		2=IL-17 5	.4593	.58695	.997	-1.6680	2.5867
		3=IL-17 50	-1.3504	.59971	.375	-3.5240	.8232
		4=RANKL/MCSF	-2.8865*	.58308	.000	-4.9998	-.7732
		6=IL-17 50/M-CSF	-1.6942	.58308	.091	-3.8075	.4192
		7=IL-17 50/ OPG	1.0563	.59100	.691	-1.0858	3.1983
		8=RANKL/MCSF/OPG	2.2000*	.57938	.005	.1001	4.2999
		9=MCSF	1.9950	.57938	.018	-.1049	4.0949
	6=IL-17 50/MCSF	1=OPG	4.4561*	.59060	.000	2.3155	6.5967
		2=IL-17 5	2.1535*	.59060	.009	.0129	4.2941
		3=IL-17 50	.3438	.60329	1.000	-1.8428	2.5304
		4=RANKL/MCSF	-1.1923	.58676	.522	-3.3190	.9343
		5=IL-17 5/MCSF	1.6942	.58308	.091	-.4192	3.8075
		7=IL-17 50/ OPG	2.7505*	.59463	.000	.5953	4.9058
		8=RANKL/MCSF/OPG	3.8942*	.58308	.000	1.7808	6.0075
		9=MCSF	3.6892*	.58308	.000	1.5758	5.8025
	7=IL-17 50/ OPG	1=OPG	1.7057	.59843	.105	-.4633	3.8746
		2=IL-17 5	-.5969	.59843	.966	-2.7659	1.5720
		3=IL-17 50	-2.4066*	.61095	.003	-4.6210	-.1923
		4=RANKL/MCSF	-3.9428*	.59463	.000	-6.0980	-1.7876
		5=IL-17 5/MCSF	-1.0563	.59100	.691	-3.1983	1.0858
		6=IL-17 50/M-CSF	-2.7505*	.59463	.000	-4.9058	-.5953
		8=RANKL/MCSF/OPG	1.1437	.59100	.590	-.9983	3.2858
		9=MCSF	.9387	.59100	.811	-1.2033	3.0808
	8=RANKL/MCSF/OPG	1=OPG	.5620	.58695	.989	-1.5654	2.6893
		2=IL-17 5	-1.7407	.58695	.078	-3.8680	.3867
		3=IL-17 50	-3.5504*	.59971	.000	-5.7240	-1.3768
		4=RANKL/MCSF	-5.0865*	.58308	.000	-7.1998	-2.9732
		5=IL-17 5/MCSF	-2.2000*	.57938	.005	-4.2999	-.1001
		6=IL-17 50/M-CSF	-3.8942*	.58308	.000	-6.0075	-1.7808
		7=IL-17 50/ OPG	-1.1437	.59100	.590	-3.2858	.9983
		9=MCSF	-.2050	.57938	1.000	-2.3049	1.8949
	9=MCSF	1=OPG	.7670	.58695	.929	-1.3604	2.8943
		2=IL-17 5	-1.5357	.58695	.184	-3.6630	.5917
		3=IL-17 50	-3.3454*	.59971	.000	-5.5190	-1.1718
		4=RANKL/MCSF	-4.8815*	.58308	.000	-6.9948	-2.7682
		5=IL-17 5/MCSF	-1.9950	.57938	.018	-4.0949	.1049
		6=IL-17 50/M-CSF	-3.6892*	.58308	.000	-5.8025	-1.5758
		7=IL-17 50/ OPG	-.9387	.59100	.811	-3.0808	1.2033
		8=RANKL/MCSF/OPG	.2050	.57938	1.000	-1.8949	2.3049

*. The mean difference is significant at the .01 level.

Table Ape.3b. The statistical analysis (ANOVA) for the resorption area on ivory slices in the co-cultures of PBMC and Synovial fibroblasts from RA as shown in Fig 6.8.

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
=OPG	19	2.5579	1.59559	.36605	1.7888	3.3269	.40	7.00
3=IL-17 50ng/ml	17	4.1059	1.45794	.35360	3.3563	4.8555	1.90	7.40
4=RANKL/M-CSF	17	5.3118	1.75103	.42469	4.4115	6.2121	2.20	8.80
5=IL-17 5NG/M-CSF	17	5.0118	1.91764	.46510	4.0258	5.9977	1.20	7.90
6=IL-17 50NG/M-CSF	16	5.8500	1.34858	.33714	5.1314	6.5686	3.80	8.30
Total	86	4.5058	1.98265	.21379	4.0807	4.9309	.40	8.80

Table Ape.4a. The statistical description for the resorption area on dentine slices in the co-cultures of PBMC and Synovial fibroblasts from RA as shown in Fig 6.10.

Multiple Comparisons

Dependent Variable: VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	=OPG	3=IL-17 50ng/ml	-1.5480	.54392	.043	-3.3790	.2830
		4=RANKL/M-CSF	-2.7539*	.54392	.000	-4.5849	-.9229
		5=IL-17 5NG/M-CSF	-2.4539*	.54392	.000	-4.2849	-.6229
		6=IL-17 50NG/M-CSF	-3.2921*	.55282	.000	-5.1531	-1.4311
	3=IL-17 50ng/ml	=OPG	1.5480	.54392	.043	-.2830	3.3790
		4=RANKL/M-CSF	-1.2059	.55883	.206	-3.0871	.6753
		5=IL-17 5NG/M-CSF	-.9059	.55883	.489	-2.7871	.9753
		6=IL-17 50NG/M-CSF	-1.7441	.56749	.023	-3.6545	.1662
	4=RANKL/M-CSF	=OPG	2.7539*	.54392	.000	.9229	4.5849
		3=IL-17 50ng/ml	1.2059	.55883	.206	-.6753	3.0871
		5=IL-17 5NG/M-CSF	.3000	.55883	.983	-1.5812	2.1812
		6=IL-17 50NG/M-CSF	-.5382	.56749	.877	-2.4486	1.3721
	5=IL-17 5NG/M-CSF	=OPG	2.4539*	.54392	.000	.6229	4.2849
		3=IL-17 50ng/ml	.9059	.55883	.489	-.9753	2.7871
		4=RANKL/M-CSF	-.3000	.55883	.983	-2.1812	1.5812
		6=IL-17 50NG/M-CSF	-.8382	.56749	.580	-2.7486	1.0721
	6=IL-17 50NG/M-CSF	=OPG	3.2921*	.55282	.000	1.4311	5.1531
		3=IL-17 50ng/ml	1.7441	.56749	.023	-.1662	3.6545
		4=RANKL/M-CSF	.5382	.56749	.877	-1.3721	2.4486
		5=IL-17 5NG/M-CSF	.8382	.56749	.580	-1.0721	2.7486

*. The mean difference is significant at the .01 level.

Table Ape.4b. The statistical analysis (ANOVA) for the resorption area on dentine slices in the co-cultures of PBMC and Synovial fibroblasts from RA as shown in Fig 6.10.

Descriptives

AREAFRAC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1=OPG	33	1.6848	1.57185	.27362	1.1275	2.2422	.30	8.40
2=IL-17 5ng/ml	34	2.9588	1.26494	.21694	2.5175	3.4002	1.10	5.60
3=IL-17 50ng/ml	34	4.9912	1.86846	.32044	4.3392	5.6431	1.90	9.70
4=RANKL+M-CSF 100+30ng/ml	33	6.6273	2.86108	.49805	5.6128	7.6418	1.30	13.10
5=IL-17+M-CSF 5+30ng/ml	34	3.9794	1.90991	.32755	3.3130	4.6458	.80	9.40
6=IL-17+M-CSF 50+30ng/ml	34	4.5294	1.51068	.25908	4.0023	5.0565	1.70	8.60
7=IL-17+OGP 5+100ng/ml	34	2.1294	1.40853	.24156	1.6380	2.6209	.30	5.20
8=RMO	34	2.4324	1.64993	.28296	1.8567	3.0080	.40	6.30
Total	270	3.6630	2.37422	.14449	3.3785	3.9474	.30	13.10

Table Ape.5a. The statistical description for the resorption area on ivory slices in the co-cultures of PBMC and Synovial fibroblasts OA as shown in Fig 6.11.

Multiple Comparisons

Dependent Variable: AREAFRAC

(I) TREATMEN	(J) TREATMEN	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
Tukey HS 1=OPG	2=IL-17 5ng/ml	-1.2740	.44273	.081	-2.8538	.3058
	3=IL-17 50ng/m	-3.3063*	.44273	.000	-4.8861	-1.7265
	4=RANKL+M-C 100+30ng/ml	-4.9424*	.44602	.000	-6.5340	-3.3509
	5=IL-17+M-CSF 5+30ng/ml	-2.2946*	.44273	.000	-3.8744	-.7147
	6=IL-17+M-CSF 50+30ng/ml	-2.8446*	.44273	.000	-4.4244	-1.2647
	7=IL-17+OGP 5+100ng/ml	-.4446	.44273	.974	-2.0244	1.1353
	8=RMO	-.7475	.44273	.695	-2.3273	.8323
2=IL-17 5ng/ml	1=OPG	1.2740	.44273	.081	-.3058	2.8538
	3=IL-17 50ng/m	-2.0324*	.43942	.000	-3.6003	-.4644
	4=RANKL+M-C 100+30ng/ml	-3.6684*	.44273	.000	-5.2483	-2.0886
	5=IL-17+M-CSF 5+30ng/ml	-1.0206	.43942	.285	-2.5886	.5474
	6=IL-17+M-CSF 50+30ng/ml	-1.5706*	.43942	.010	-3.1386	-.0026
	7=IL-17+OGP 5+100ng/ml	.8294	.43942	.561	-.7386	2.3974
	8=RMO	.5265	.43942	.932	-1.0415	2.0945
3=IL-17 50ng/m	1=OPG	3.3063*	.44273	.000	1.7265	4.8861
	2=IL-17 5ng/ml	2.0324*	.43942	.000	.4644	3.6003
	4=RANKL+M-C 100+30ng/ml	-1.6361*	.44273	.006	-3.2159	-.0563
	5=IL-17+M-CSF 5+30ng/ml	1.0118	.43942	.296	-.5562	2.5797
	6=IL-17+M-CSF 50+30ng/ml	.4618	.43942	.966	-1.1062	2.0297
	7=IL-17+OGP 5+100ng/ml	2.8618*	.43942	.000	1.2938	4.4297
	8=RMO	2.5588*	.43942	.000	.9908	4.1268
4=RANKL+M-C 100+30ng/ml	1=OPG	4.9424*	.44602	.000	3.3509	6.5340
	2=IL-17 5ng/ml	3.6684*	.44273	.000	2.0886	5.2483
	3=IL-17 50ng/m	1.6361*	.44273	.006	.0563	3.2159
	5=IL-17+M-CSF 5+30ng/ml	2.6479*	.44273	.000	1.0680	4.2277
	6=IL-17+M-CSF 50+30ng/ml	2.0979*	.44273	.000	.5180	3.6777
	7=IL-17+OGP 5+100ng/ml	4.4979*	.44273	.000	2.9180	6.0777
	8=RMO	4.1949*	.44273	.000	2.6151	5.7747

5=IL-17+M-CSF 5+30ng/ml	1=OPG	2.2946*	.44273	.000	.7147	3.8744
	2=IL-17 5ng/ml	1.0206	.43942	.285	-.5474	2.5886
	3=IL-17 50ng/ml	-1.0118	.43942	.296	-2.5797	.5562
	4=RANKL+M-CSF 100+30ng/ml	-2.6479*	.44273	.000	-4.2277	-1.0680
	6=IL-17+M-CSF 50+30ng/ml	-.5500	.43942	.915	-2.1180	1.0180
	7=IL-17+OGP 5+100ng/ml	1.8500*	.43942	.001	.2820	3.4180
	8=RMO	1.5471	.43942	.012	-.0209	3.1150
6=IL-17+M-CSF 50+30ng/ml	1=OPG	2.8446*	.44273	.000	1.2647	4.4244
	2=IL-17 5ng/ml	1.5706*	.43942	.010	.0026	3.1386
	3=IL-17 50ng/ml	-.4618	.43942	.966	-2.0297	1.1062
	4=RANKL+M-CSF 100+30ng/ml	-2.0979*	.44273	.000	-3.6777	-.5180
	5=IL-17+M-CSF 5+30ng/ml	.5500	.43942	.915	-1.0180	2.1180
	7=IL-17+OGP 5+100ng/ml	2.4000*	.43942	.000	.8320	3.9680
	8=RMO	2.0971*	.43942	.000	.5291	3.6650
7=IL-17+OGP 5+100ng/ml	1=OPG	.4446	.44273	.974	-1.1353	2.0244
	2=IL-17 5ng/ml	-.8294	.43942	.561	-2.3974	.7386
	3=IL-17 50ng/ml	-2.8618*	.43942	.000	-4.4297	-1.2938
	4=RANKL+M-CSF 100+30ng/ml	-4.4979*	.44273	.000	-6.0777	-2.9180
	5=IL-17+M-CSF 5+30ng/ml	-1.8500*	.43942	.001	-3.4180	-.2820
	6=IL-17+M-CSF 50+30ng/ml	-2.4000*	.43942	.000	-3.9680	-.8320
	8=RMO	-.3029	.43942	.997	-1.8709	1.2650
8=RMO	1=OPG	.7475	.44273	.695	-.8323	2.3273
	2=IL-17 5ng/ml	-.5265	.43942	.932	-2.0945	1.0415
	3=IL-17 50ng/ml	-2.5588*	.43942	.000	-4.1268	-.9908
	4=RANKL+M-CSF 100+30ng/ml	-4.1949*	.44273	.000	-5.7747	-2.6151
	5=IL-17+M-CSF 5+30ng/ml	-1.5471	.43942	.012	-3.1150	.0209
	6=IL-17+M-CSF 50+30ng/ml	-2.0971*	.43942	.000	-3.6650	-.5291
	7=IL-17+OGP 5+100ng/ml	.3029	.43942	.997	-1.2650	1.8709

*. The mean difference is significant at the .01 level.

Table Ape.5b. The statistical analysis (ANOVA) for the resorption area on ivory slices in the co-cultures of PBMC and Synovial fibroblasts OA as shown in Fig 6.11.