UMEÅ UNIVERSITY ODONTOLOGICAL DISSERTATIONS

New Series No. 95
ISSN 0345-7532
ISBN 91-7264-195-9

Kinins – Important Regulators in Inflammation Induced Bone Resorption

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2006

Cover Picture: The picture on the front page illustrates the human osteosarcoma cell line, MG-63, in culture. The cells were fixed with methanol before the photograph was taken. MG-63 cells were used in a lot of different experiments in the present thesis.

ISBN 91-7264-195-9
Printed in Sweden by Print & Media
Umeå 2006

To Magnus, Erik and Lisa – My wonderful family

Tardi ingenii east rivulos consectari, fontes rerum non videre –
Liten i anden är den som bara följer bäckarna och inte ser till tingens källor.

Cicero 106-43 f.Kr.

ABSTRACT

Kinins – Important Regulators in Inflammation Induced Bone Resorption

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Inflammatory processes in, or in close vicinity of, the skeleton often lead to loss of bone tissue. Different cytokines have been shown to be involved as stimulators of inflammatory induced osteoclastic bone resorption. During inflammatory processes also the kallikrein-kinin system is activated, leading to production of kinins that can cause pain, vasodilation and increased permeability of vessels. Kinins can also induce bone resorption *in vitro*. All cytokines and kinins that stimulate bone resorption stimulate in parallell prostaglandin synthesis, and prostaglandins, per se, have also been shown to induce bone resorption.

The aim of this project was to increase the knowledge about the mechanisms involved in the interactions between different inflammatory mediators (i.e. kinins, cytokines and prostaglandins) suggested to be involved in the pathogenesis of inflammatory bone resorbing diseases.

Human osteoblasts (MG-63) are equipped with both kinin B1 and B2 receptors linked to prostaglandin release and the stimulation of prostaglandin release are likely mediated via separate molecular mechanisms (Paper I). Activation of B1 or B2 receptors causes synergistic stimulation of PGE_2 synthesis induced by either interleukin-1β (IL-1β) or tumour necrosis factor-α (TNF-α) (Paper II). The molecular mechanism involves increased expression of cyclooxygenase-2 (COX-2) and results in synergistic potentiation of receptor activator of NF-κB ligand (RANKL) protein expression. The synergistic interaction is dependent on the activation of NF-κB and the mitogen-activated protein kinases (MAPK) p38 and JNK (Paper II). The synergistic increase in RANKL expression might be an explanation why kinins potentiate IL-1β induced bone resorption, a mechanism likely to be important in inflammation induced bone resorption in diseases such as periodontal disease and rheumatoid arthritis.

The synergism between kinins and IL-1β or TNF-α might also be dependent on regulation of kinin receptors, since both IL-1β and TNF-α markedly upregulated B1 and B2 receptors, both at the mRNA level and protein level (Paper III). This upregulation is not further potentiated by the kinins, and different kinin receptor agonists do not regulate the receptors for IL-1β or TNF-α, in MG-63 cells. No other cytokines known to stimulate bone resorption regulates the expressions of B1 and B2 receptors. The IL-1β- or TNF-α-induced enhancements of B1 and B2 receptor expressions involve activation of NF-κB and MAPK. The enhancement of kinin receptors may also be an important mechanism in the synergistic interactions between the two pro-inflammatory cytokines and kinins (paper III). IL-4 and IL-13 are two cytokines that have been shown to inhibit bone resorption. We have shown that COX-2 and both B1 and B2 receptors are down-regulated by IL-4 and IL-13, via a 'signal transducer and activator of transcription-6' (STAT6) dependent pathway, which might be an important regulatory mechanism in inflammation induced bone resorption (paper IV).

In conclusion, the mechanisms behind the synergistic potentiation of prostaglandin formation and increased bone resorption caused by co-stimulation with kinins and IL-1β or TNF-α seem to involve both potentiation of COX-2 and subsequently increased levels of RANKL, as well as upregulation of B1 and B2 kinin receptors. Interestingly, IL-4 and IL-13 decreased the expressions of COX-2 and both B1 and B2 receptors. These events might be important in the regulation of inflammation induced bone resorption in diseases such as periodontitis and rheumatoid arthritis.

Key words: Bone resorption, Osteoblasts, Kinins, B1 and B2 receptors, IL-1β, TNF-α, Prostaglandin, COX-2, RANKL, Transcription factors, IL-4, IL-13

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PREFACE

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I Brechter A.B. and Lerner U.H.
Characterization of bradykinin receptors in a human osteoblastic cell line.
Regulatory Peptides 2002;103:39-51

II Brechter A.B. and Lerner U.H.
Bradykinin potentiates cytokine induced prostaglandin biosynthesis in osteoblasts by enhanced expression of COX-2 resulting in increased RANKL.
Arthritis and Rheumatism, in press

III Brechter A.B., Persson E., Lundgren I. and Lerner U.H.
Kinin B1 and B2 receptor expression in osteoblasts and fibroblasts is enhanced by interleukin-1β and tumour necrosis factor-α – Effects dependent on activation of NF-κB and MAP kinases.
Submitted

IV Brechter A.B. and Lerner U.H.
IL-4 and IL-13 inhibit cytokine-induced enhancements of COX-2 and kinin receptor expression – Effects important for their inhibition of the synergistic stimulation of PGE_2 formation caused by co-treatment with cytokines and kinins.
Manuscript

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ABBREVATIONS

α-MEM	α-modification of Minimum Essential Medium
AP-1	activating protein-1
BCA	bicinchoninic acid
BK	bradykinin
B1	bradykinin receptor type 1
B2	bradykinin receptor type 2
BSA	bovine serum albumin
BSP	bone sialoprotein
cAMP	cyclic 3', 5' adenosine monophosphate
Cbfa1	core binding factor 1
cDNA	complementary deoxyribonucleic acid
COX	cyclooxygenase
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
DABK	des-Arg^{9}-bradykinin
DALBK	des-Arg^{10}-Lys-bradykinin
DAP12	DNAX-activating protein 12
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated protein kinase
FcRγ	Fc receptor common γ subunit
FCS	foetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCF	gingival crevicular fluid
G protein	guanine nucleotide-binding protein
GPCR	G-protein coupled receptor
Hoe 140	D-Arg-[Hyp^{3}, Thi^{5}, D-Tic^{7},Oic^{8}]
Hyp	4-Hydroxyproline
IFN	interferon
IGF	insulin-like growth factor
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin
IL-1R	interleukin-1 receptor
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
mPGES	membrane associated prostaglandin E synthase
NF-κB	nuclear factor κB
NSAID	nonsteroidal anti-inflammatory drugs
Oic	L-(3a*S*,7a*S*)-Octahydroindol-2-yl-carbonyl
OPG	osteoprotegerin
OSM	oncostatin M

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDTC	pyrrolidine dithiocarbamate
PG	prostaglandin
PGE_2	prostaglandin E_2
PGES	prostaglandin E synthase
PGI_2	prostacyclin
6-keto-$PGF_{1\alpha}$	6-keto-prostaglandin $F_{1\alpha}$
PLA_2	phospholipase A_2
PTH	parathyroid hormone
RANK	receptor activator of nuclear factor κB
RANKL	receptor activator of nuclear factor κB ligand
RIA	radioimmunoasssay
RPL13A	60S ribosomal protein L13A
Sar	sarcosine (N-methylglycine)
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
STAT	signal transducer and activator of transcription
TBS	tris buffered saline
Thi	β-(2-Thienyl)alanine
Tic	1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid
TTBS	TBS with Tween-20 solution
TGF-β	transforming growth factor-β
TNF	tumour necrosis factor
TNF-R	TNF receptor
TRAF	TNF receptor-associated factor
TRAP	tartrate-resistant acid phosphatase
Vit D_3	1α,25-$(OH)_2$ vitamin D_3

INTRODUCTION

Bone structure and composition

Bone is a very specialized form of connective tissue, in which the extracellular matrix is mineralized. It is a complex tissue, and the composition contributes to the skeletons ridigity and strength, and also giving the structure some level of elasticity. About 70% of the bone tissue is mineralized, and this inorganic part is mainly composed by calcium and phosphate in the form of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] dispersed in the matrix. 5% is water and the remaining 25% is the organic matrix in which the main component (90-95%) is type I collagen. The collagen fibrils/fibers create networks that give the bone (and also skin, tendons and ligaments) a higher degree of tissue strength and elasticity (Rossert and de Crombrugghe, 2002). The remaining 5-10% of the organic matrix is composed of a number of noncollagenous proteins, as well as bone cells. The variety of noncollagenous proteins that are present in the bone tissue may influence the organization of the matrix, the mineralization of bone and the behaviour of bone cells. These proteins include proteoglycans (e.g. decorin, biglycan, osteoglycin, osteoadherin), glycoproteins (e.g. alkaline phosphatase, osteonectin, vitronectin, osteopontin, bone sialoprotein) and glutamic acid (GLA)-containing proteins (e.g. matrix Gla protein, osteocalcin) (Robey, 2002). There have also been a number of growth factors identified in bone matrix including transforming growth factor-β (TGF- β) (Bonewald, 2002), insulin-like growth factor I and II (IGF-I, IGF-II) (Conover, 2000) and bone morphogenic proteins (BMP:s) (Rosen and Wozney, 2002). The inorganic hydroxyapatite crystals coat the fibrils in the collagen network, and thereby improving the rigidity of the tissue (Weiner and Traub, 1992). The deposition of crystals in the organic matrix of bone is under cellular control and it serves as an ion reservoir (Buckwalter *et al*., 1996a). In addition to its supportive and protective actions, where the skeleton protects vital organs and bone marrow from physical damage, the bone tissue serves as an ion reservoir, participating in the calcium homeostasis in the body. The skeleton also has a mechanical function, where it provides sites for muscles to attach.

The skeleton consists of two morphologically different types of bone tissue, the cortical (compact) bone and the trabecular (cancellous, spongy) bone. In cortical bone, the collagen fibrils are densely packed forming concentric lamellae, while the trabecular bone has a more loosly-organized matrix. The external dense layer of most bones is composed of cortical bone, which forms approximately 80% of the mature skeleton. The outside of the cortical bone is covered by the periosteum, which separates the bone from the surrounding tissues. The periosteum is composed of two layers, an outer denser layer of collagen containing fibroblastic cells, and networks of nerves and vessles, and an inner layer with a higher density of cells including bone cells, fibroblasts and nerve cells (Allen *et al*., 2004). The inside of the cortical bones and the trabecular bone surfaces are covered by the endosteum which separates the bone surface from the bone marrow. Cortical and trabecular bone consist of the same type of cells and the same matrix, but they have structural and functional differences. The more loose structure of trabecular bone, results in a higher surface area per bone unit than cortical bone, which contributes to an enhanced rate of remodeling, since the metabolic activities are dependent on the surface (Buckwalter *et al*., 1996a). Thus, the cortical bone provides the mechanical and protective functions while trabecular bone provides the metabolic functions.

The embryonic development of the skeleton includes two different bone forming processes, endochondral or intramembranous ossification. Most of the bones, including long

bones, develop through endochondral ossification. During this process, the embryonic mesenchyme is condensed and transformed by chondrocytes into a cartilage template with the shape of the forming bone. The chondrocytes undergo a strictly regulated life cycle of proliferation, maturation and apoptosis. Cells in the middle of the shaft differentiate into hypertrophic chondrocytes and secrete a matrix. This matrix contains molecules that make the tissue more susceptible to angiogenesis. As capillaries form, osteoblastic cells are carried from the blood stream and invade the cartilage tissue. The chondrocytes closest to the osteoblast zone mineralize their matrix before they undergo apoptosis. Differentiating osteoblasts replace the matrix with a collagen I-rich bone matrix (osteoid) that eventually mineralizes. Osteoclast-mediated resorption of the ossified matrix in the center of the shaft (trabecular bone) gives rise to the bone marrow cavity where bone marrow stromal cells reside (Kronenberg, 2003; Provot and Schipani, 2005). In contrast, the flat bones (i.e. the cranial vault, facial bones and parts of the mandible and clavicle), are formed using intramembraneous ossification. In this process, the embryonic mesenchyme is condensed at skeletogenic sites, and the mesechymal cells are directly transformed into bone-forming osteoblasts in the connective tissue. The osteoblasts produce an extracellular matrix that results in formation of bone islands, which increase in size and eventually develop into flat bones without the formation of a cartilagenous template (Karaplis, 2002).

Bone cells

The bone is a very dynamic tissue and the cells responsible for the remodelling of the bone, to meet the different demands of physiological changes, are the osteoblasts that produce new bone, the osteocytes entrapped in the bone and the osteoclasts, which are the cells responsible for bone resorption. The fourth type is the bone lining cells, which are the inactive osteoblasts covering the surface of the bone. These different cell types can be distinguished by there specific morphology, location and function (Fig. 1).

Osteoblasts

The osteoblasts are the cells responsible for the formation of bone tissue. This process requires two steps. First, the osteoblasts form a non-mineralized extracellular matrix (osteoid) consisting mainly of collagen fibers type I. The matrix then gets impregnated with hydroxyapatite crystals, to form the mineralized bone tissue. Importantly, the mineralization is never complete, since there always is a non-mineralized zone of osteoid between the mineralized bone tissue and the osteoblasts on the surface. Osteoblasts originate from multipotent mesenchymal stem cells and they are closely related to odontoblasts, cementoblasts and fibroblasts. However, one fundamental difference between osteoblasts and fibroblasts is that osteoblasts release matrix in a polarized direction towards the bone surface, while the fibroblasts release the matrix all around them, in a pericellular way. The mesenchymal stem cells can also differentiate into chondrocytes, myoblasts, adipocytes and tendon cells. In which direction the mesenchymal stem cells develop is due to which cell type-specific factors that are activated. The cell type-specific factors that have been identified in osteoblasts are the transcription factor runt-related transcription factor 2 / core binding factor 1 (Runx2/cbfa1), and the secreted protein osteocalcin (Ducy *et al*., 2000; Harada and Rodan, 2003). Other factors that seem to be essential for bone formation are the transcription factors osterix (Osx) and β-catenin. β-catenin is coupled to the Lrp5/Wnt/Frizzled-system (Komori, 2006), and recently, Nishio *et al*., found that Osx seems to regulate the transcription of Runx2 (Nishio *et al*., 2006). Also the enzyme Tissue-Nonspecific Alkaline Phosphatase

(TNAP) seems to be important for bone formation, especially the mineralization of the bone tissue. The active osteoblasts are easily distinguished from inactive osteoblasts (lining cells) due to there cuboidal structure and the obvious endoplasmatic reticulum (ER). The bone lining cells on the other hand are flat and show only a little amount of ER. In addition to the osteoblasts ability to form bone, they are also able to control the formation and activation of the bone-resorbing osteoclasts.

Osteocytes

The osteocyte is the most abundant cell type in mature bone tissue and approximately 90% of the bone cells are osteocytes. They are more numerous in trabecular bone than in cortical bone (Noble and Reeve, 2000). During bone formation, some of the osteoblasts are incorporated into the osteoid and eventually these cells will be trapped in lacunae and transformed into more dendritic-shaped cells. Long processes extend from the osteocytes into canaliculi in the mineralized bone. These processes communicate with similar processes from other osteocytes and with processes from the osteoblasts on the surface, and thereby enhancing nutrition (Knothe Tate *et al*., 2004). There functions are not fully understood, but they seem to have the ability to form bone matrix (Buckwalter *et al*., 1996a) and it has been suggested that these cells act as mechanosensors responding to mechanical loading and thereby regulating the metabolism of bone tissue together with osteoblasts and osteoclasts (Knothe Tate, 2003).

Osteoclasts

Osteoclasts are multinucleated, non-dividing, motile giant cells with the specific capacity to resorb mineralized bone tissue. This is a unique feature for the osteoclasts. The multinucleated, terminally differentiated osteoclasts are formed through the fusion of mononuclear precursor cells, originated from the monocyte/macrophage hematopoietic stem cell linage. The osteoclast progenitors proliferate and differentiate into mononuclear preosteoclasts. They are then recruited from bone marrow or other hematopoietic sites, via the circulation. The preosteoclasts are guided to sites on the bone surface where resorption of bone tissue is going to take place, and then fusioned to multinucleated osteoclasts. These multinucleated osteoclasts are only found at or near the bone surface (Lerner 2000; Boyle *et al*., 2003). Both late preosteoclasts and mature multinucleated osteoclasts express receptors for calcitonin (calcium regulating hormone) and show positive staining for tartrate resistant acid phosphatase enzyme (TRAP) (Granholm *et al*., submitted). Active osteoclasts show characteristic nuclei polarity, meaning that their nuclei are typically located in the cytoplasm, away from the bone surface.

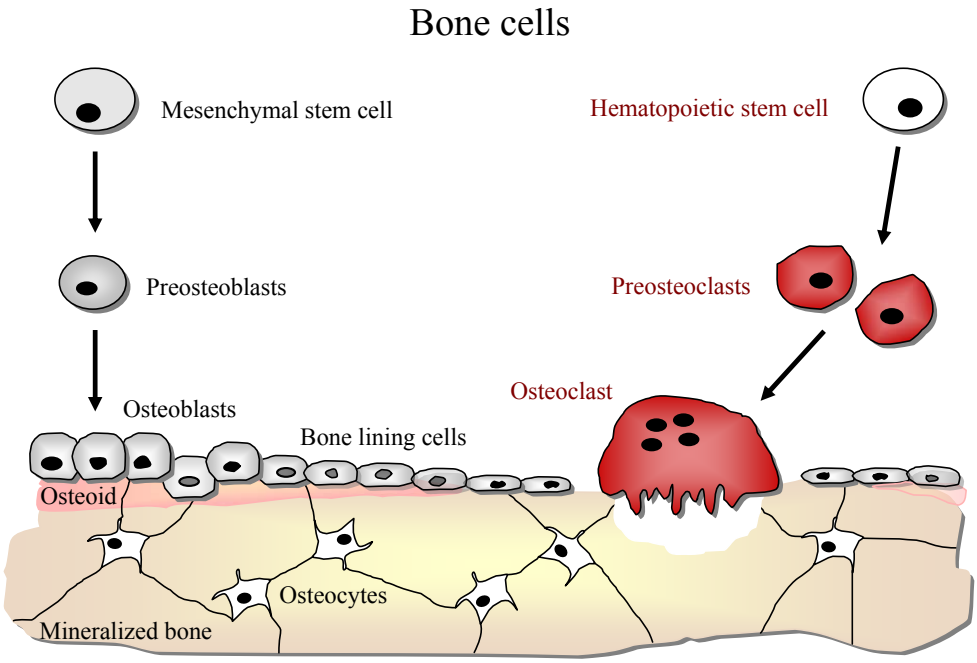

Figure 1. Schematic illustration of the cell types in the bone.

Bone metabolism

The activities of the cells in the skeleton vary substantially over the life span of an organism, and this reflects the temporally variations in bone metabolism. Throughout life, there are interactions between different cell types that are essential for bone metabolism. These interactions are strictly regulated by biochemical and mechanical factors. The balance between osteoclastic bone resorption and osteoblastic bone formation must be tightly controlled to maintain the normal bone homeostasis, both during *de novo* bone formation during post-natal growth, and to meet the demands that are placed upon the mature skeleton. The first two decades in life when the skeleton develops, and bone formation necessarily must precede and exceed bone resorption, is called modelling. After that, in the adult skeleton, there need to be a balance between bone formation and resorption, and this is called coupling. This is very important in the remodelling processes which dominate the adult skeleton's capacity to respond and adapt to different up-coming situations (Marks and Odgren, 2002). It has been estimated that about 10% of the adult skeleton is remodelled per year, and this is of importance not only to maintain the skeletal structure and strength but also to regulate calcium homeostasis (Parfitt, 1994).

The remodelling cycle

Bone tissue is continuosly replaced to respond to the changing needs of the body. This process, called bone remodelling, occurs in restricted areas and includes recruitment and activation of both osteoblasts and osteoclasts, and involves bone formation, as well as bone resorption. The events and signalling behind the determination of location and initiation of the remodelling process are still unknown. The physiological bone remodelling takes place in so called 'bone multi-cellular units' (BMUs), which is initiated by recruitment, formation and activation of osteoclasts. Old bone is resorbed and subsequently new bone is synthesized by activated osteoblasts. The new bone fills up the Howships resorption lacunae. Such BMUs are present both at the surfaces of cortical and trabecular bone and in the Haversian canals of cortical bone, but are more frequent in the trabecular bone. Finally, the BMUs dissolve and leave inactive cells lying on the newly formed bone. The length of the resorption phase is very short (2-4 weeks) compared to the bone formation phase (4-6 months), and the lifetime of an osteoclast is much shorter than that of an osteoblast (Manolagas, 2000). 10-15% of the bone surfaces undergo remodelling, at any time, while the remaining surfaces are covered with inactivated bone lining cells (Ott, 2002).

Initiation

The events and signalling responsible for the determination of location and initiation of a BMU remains still unknown. What is known though, is that the remodelling process begins with activation of the inactive osteoblasts (lining cells), a process that is regulated by various factors including both systemic circulating hormones (e.g. parathyroid hormone (PTH) and $1\alpha,25(OH)_2$ vitamin D_3 (Vit D_3), growth factors, cytokines, as well as signals from osteocytes, in response to mechanical loading. The activated osteoblasts change their apperance to more rounded cells and start secreting proteolytic enzymes which degrade the osteoid (the non-mineralized matrix) that covers all minerlized bone surfaces, to enable for the osteoclasts to reach the mineralized surface of the bone (Vaes, 1988).

When bone resorption is about to occure, osteoclast progenitor cells are attracted to the resorptive site, from the circulation, through a 'homing process', which is still not understood. However, the following events in which osteoclasts differentiate and become activated have been, to some extent, elucidated. The activated osteoblasts stimulate mononucleated osteoclast progenitors in the periosteum and endosteum to differentiate to preosteoclasts and subsequently fuse to generate multinuclear osteoclasts. Activated stromal cells/osteoblasts play a key role in differentiation, fusion and activation of multinucleated osteoclasts, and it was first reported by Takahashi *et al*., that the presence of bone marrow stromal cells (multipotent cells that can differentiate into cells with an osteoblastic phenotype) was essential for osteoclastogenesis in cultures of hematopoietic cells (Takahashi *et al*., 1988). Soon thereafter it was discovered that cell-to-cell contact with stromal cells/osteoblasts is crucial for osteoclast differentiation (Udugawa *et al*., 1989). Evidently, there have been two proteins produced by stromal cells/osteoblasts that have been proven to be both necessary and sufficient for osteoclastogenesis, namely the cytokine macrophage colony-stimulating factor (M-CSF) and the tumour necrosis factor (TNF)-related protein receptor activator of nuclear factor κB ligand (RANKL) (Fig. 2). M-CSF is secreted from osteoblasts (and their precursors), and binds to its receptor c-Fms on osteoclast progenitor cells, and this leads to proliferation and survival of these cells (Tanaka *et al*., 1993; Felix *et al*., 1994). Cell-to cell contact with stromal cells/osteoblasts is essential

for further differentiation and activation of the osteoclast precursors. Remarkable progress in research has occurred during the last ten years on the molecular mechanism of osteoclast differentiation and activation, especially by the findings of RANKL. The cell-to-cell contact is mediated by binding of RANKL (which is expressed on the surface of osteoblasts) to its receptor, receptor activator of nuclear factor κB (RANK), situated on osteoclast precursors and multinuclear osteoclasts. The RANK-RANKL interaction has been shown to promote the differentiation of mononuclear osteoclast progenitor cells, fusion of preosteoclasts and activation of multinuclear osteoclasts, resulting in a mature terminally differentiated osteoclast that can resorb bone (Lacey *et al*., 1998; Hsu *et al*., 1999) (Fig. 2). These important steps can be inhibited by osteoprotegerin (OPG), a secreted glucoprotein produced by osteoblasts/stromal cells, and which, like RANK, is a member of the TNF receptor superfamily. OPG binds to RANKL, and thereby function as a decoy-receptor in the interaction between RANK and RANKL, resulting in inhibition of the development of osteoclasts and bone resorption (Simonet *et al*., 1997; Yasuda *et al*., 1998b). The importance of the RANK/RANKL/OPG system has been nicely demonstrated in mice with target deletions of these factors. So, *rank -/-* and *rankl -/-* mice have no osteoclasts and develop severe osteopetrosis, and in contrast *opg -/-* mice have enhanced numbers of osteoclasts leading to osteoporosis (Suda *et al*., 1999; Lerner, 2004).

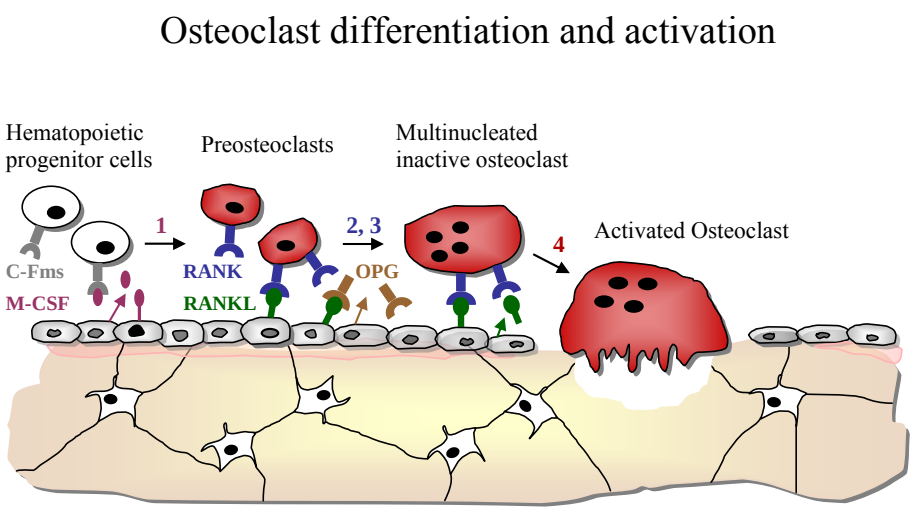

Figure 2. Schematic illustration of osteoclast formation and activation. This process include 1) proliferation of progenitor cells induced by M-CSF, 2) RANKL-induced differentiation to preosteoclasts, 3) fusion of the preosteoclasts and finally 4) activation of the multinucleated latent osteoclast to a mature bone-resorbing osteoclast.

Resorption

During the process of bone resorption the osteoclasts undergo major cellular alterations. After osteoid degradation, the activated osteoclasts establish contact with the mineralized bone surface through interactions between the $\alpha_v\beta_3$-integrin in the osteoclastic cell membrane and several bone matrix proteins, including osteopontin and bone sialoprotein (Reinholt *et al*., 1990). The attachement results in the formation of a tight ring-like zone of adhesion, called the "sealing zone", in the periphery of the cell membrane facing the bone surface (Baron, 1989). The sealing zone isolates the resorption site, known as "Howship's lacuna", beneath the osteoclast, from the surrounding space. Cellular attachement to the bone also induces intracellular signalling that promotes the transport of acidifying vesicles, containing vacuolar type H^+-adenosine triphosphatase (H^+ATPase) proton pumps, towards the apical membrane. The following fusion of the proton pump-containing vesicles with the apical membrane, results in formation of a characteristic structure with numerous folds, called the "ruffled border".

The first event in bone resorption is to dissolve the hydroxyapatite crystals and after that there is the degradation of the organic matrix proteins (Lerner *et al*., 1997). Mineral dissolution starts with the acidification caused by the proton pumps present in the ruffled border. These pumps deliver protons (H^+) generated intracellularly, by the osteoclast specific enzyme carbonic anhydrase II, to the Howship's lacunae. In addition to the transport of H^+ through the proton pumps, chloride ions (Cl^-) are released into the Howship's lacuna through chloride channels also situated in the ruffled border. This is a way to maintain the electroneutrality in the cell. Production of hydrochloride acid (HCl) in the lacuna results in a decrease of the pH to about 3-4, and this contributes to the dissolution of the inorganic components in the bone tissue. Osteoclasts also synthesize several proteolytic enzymes, including cysteine proteinases such as Cathepsin K and matrix metalloproteinases (MMPs), which are transported toward the apical side of the cells and released into Howship's lacuna and play important roles in the degradation of proteins in the bone matrix (Teitelbaum, 2000a,b; Lerner, 2000). The degradation products seem to be ingested by the osteoclast through endocytosis and secreted on the basolateral surface of the cell, to prevent accumulation of degradation products in the lacuna (Rouselle and Heymann, 2002; Väänänen and Zhao, 2002)

Formation of new bone

Following the resorption phase, a not completely elucidated process called coupling, guides the osteoblasts to the resorption site and activates the cells to produce new bone. It seems like that non-collagenous proteins, including growth factors (such as IGF-I, IGF-II and TGF-β), which have been deposited by osteoblasts during bone formation, are released from the degraded matrix during bone resorption, and these factors might function as autocrine coupling-factors (Rodan, 1991). When the osteoclasts have detached from the bone surface, these proteins are released from the resorption site, and activate the osteoblasts near the resorption pit, causing these cells to invade the lacuna and start to form new bone. The avtivated osteoblasts start to synthesize type I collagen, which together with the non-collagenous proteins, form the osteoid. The osteoid is then subsequently mineralized by the osteoblasts (Buckwalter *et al*., 1996b). The length of the bone formation phase is much longer (4-6 months), than the resorption phase that only takes about 2-4 weeks (Manolagas, 2000).

Mineralization

The hydroxyapatite crystals found in mineralized bone are smaller in size (100-400 Å) and less perfect in atomic structure than natural hydroxyapatite, resulting in a more reactive and soluble mineral (Posner *et al*., 1969). Osteoblasts produce matrix vesicles (MVs) that arise from the basal plasma membrane areas near newly formed osteoid (Morris *et al*., 1992). It is generally accepted that MVs are the initial site of calcification in cartilage, bone and dentin, and these vesicles contain many different enzymes, including alkaline phosphatase (ALP), and calcium and phosphorous sources. MMPs have also been identified in association with MVs and are probably involved in MV membrane degradation which permits crystals to grow out into the extracellular matrix (D'Angelo *et al*., 2001). In the initial phase of bone mineralization high intra-vesicular concentrations of calcium and inorganic phosphate ions induce hydroxyapatite precipitation. Then, after accumulation and growth, the crystals become exposed to the extracellular surroundings by protruding through the membrane of the MV. The extracellular exposure of the crystals enables further growth and proliferation, and ultimately saturating the matrix with hydroxyapatite in the final stages of complete bone formation (Andersson, 1995). It is known that osteoblasts express the tissue-nonspecific alkaline phosphatase (TNAP or ALP), which is a ubiquitous plasma membrane bound enzyme and is synthesized by many cell types (Henthorn *et al*., 1999). An increase in the expression of ALP is associated with osteoblastic differentiation and positively correlates with ossification. Some evidence for ALP's role in mineralization is the findings that osteoblasts from TNAP knock-out (*tnap*$^{-/-}$) mice failed to produce mineralized bone nodules *in vitro* (Wennberg *et al*, 2000).

Regulators of bone metabolism

The development and homeostasis of the skeleton depends on dynamic balancing of the activities of osteoblastic bone formation and osteoclastic bone resorption (Karsenty and Wagner, 2002). In pathological conditions in the skeleton, there are often an imbalance between bone formation and bone resorption. To prevent this imbalance, the metabolism of the bone tissue is tightly controlled by a complex network of different factors, including both systemic factors and local factors, generated in the bone. The systemic factors include Vit D_3, PTH, calcitonin, glucocorticoids, sex steroids and thyroid hormones. Also various local factors are important for the regulation of bone metabolism, including osteotropic cytokines, prostaglandins, kinins, neuropeptides and growth factors. I will here present an introduction to some of the osteotropic factors that have been investigated in this project. Though I haven't studied M-CSF in this thesis, I think that a short presentation of this factor still is in place.

M-CSF

Macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) belong to the colony-stimulating factor (CSF) family. M-CSF was first isolated from fetal mouse yolk sac (Johnsson and Metcalf, 1978), and was found to trigger the stimulation of macrophage colony formation of mouse bone marrow cells (Johnsson and Burgess, 1978). M-CSF is a growth factor for monocytes/macrophages and GM-CSF for granulocytes and monocytes/macrophages. Both can be synthezised by osteoblasts and bone marrow stromal cells, but also by other cell types, including monocytes, granulocytes, endothelial cells and fibroblasts. M-CSF promotes the survival, proliferation and differentiation of monocytes and macrophages, and plays an important role in the survival

and proliferation of osteoclast progenitor cells (Tanaka *et al*., 1993; Felix *et al*., 1994). There are several forms of M-CSF, due to differential splicing and posttranslational modifications, including a secreted soluble glycoprotein, a membrane-bound glycoprotein and a secreted glycoprotein, which can be attached to an extracellular matrix (Stanley *et al*., 1997). The importance of M-CSF in osteoclastogenesis was demonstrated by studies on the osteopetrotic *op/op* mouse, which are defective in the production of functional M-CSF. This defect is due to a single base pair insertion in the coding region of the M-CSF gene that generates a stop codon (TGA) 21 base pairs downstream, causing lack of full-length M-CSF protein, although the levels of M-CSF mRNA were normal (Yoshida *et al*., 1990). The lack of functional M-CSF leads to systemic osteopetrosis, due to a severe deficiency in mature osteoclasts (Wiktor-Jedrzejczak *et al*., 1990). However, a progressive expression of the related cytokine GM-CSF seems to compensate for this effect over time (Myint *et al*., 1999). In contrast, there are other evidence indicating that the secreted forms of M-CSF is crucial for osteoclastogenesis (Dai *et al*., 2004).

M-CSF binds to its receptor c-Fms, to mediate its biological activities. This receptor is a 165 kDa glycoprotein with a single transmembraneous domain that connects the extracellular ligand-binding domain with the intracellular domain (Fixe and Praloran, 1998). When ligand binding occurs, c-Fms is homodimerized and the tyrosine kinase domain induces autophosphorylation of the receptor. The phosphorylation promotes interaction of the receptor with different adapter proteins (Src, Grb2, p85) that induce intracellular signalling, including the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein (MAP) kinase cascades (Bourette and Rohrschneider, 2000). In osteoclast progenitor cells the intracellular signalling that results from the binding of M-CSF to c-Fms on the cell surface, leads to induction of cell proliferation and survival. Similar to lacking functional M-CSF, mice deficient in c-Fms develop severe osteopetrosis, due to the absence of c-Fms on osteoclast progenitor cells (Dai *et al*., 2002).

RANKL/RANK/OPG

It has been known for some time that cell-to-cell contact is essential for osteoclast formation and activation, but it was not until the observations of the crucial role of RANKL (expressed on osteoblasts/stromal cells) binding to its cognate receptor RANK (expressed on osteoclast precursor cells and mature osteoclasts) that the molecular mechanism involved in this cell-to-cell contact was more clarified. The binding of RANK to RANKL is inhibited by OPG, which serves as a decoy receptor in the system. The RANKL-RANK signalling is crucial both for the differentiation and fusion of osteoclast progenitor cells to multinucleated preosteoclasts, as well as for the activation of mature multinucleated osteoclasts to start to resorb bone (Fuller *et al*., 1998; Horowitz *et al*., 2001). There were several research groups that simultanously discovered these important proteins, and many different names were given. Therefore, to avoid confusion, the American Society for Bone and Mineral Research, has suggested RANKL, RANK and OPG as standard nomenclature for these proteins (ASBMR Committee on Nomenclature, 2000).

RANKL

The receptor activator of nuclear factor κB ligand (RANKL) is a member of the TNF ligand superfamily of cytokines. It was discovered by several research groups. Yasoda and co-workers cloned a protein, expressed by osteoblasts/stromal cells, which enhanced osteoclast differentiation, so they called it osteoclast differentiating factor (ODF) (Yasoda *et al*., 1998b). The protein was shown to be identical to the earlier found cytokine TNF-related activation-induced cytokine (TRANCE) that bound to TNF-

receptors on T-cells (Wong *et al*., 1997) and RANKL, a protein that stimulated T-cell growth (Anderson *et al*., 1997). The same molecule was also cloned by another group, at this time, and they named the protein osteoprotegerin ligand (OPGL) (Lacey *et al*., 1998).

The RANKL gene is present on the human chromosome 13q14 and on the mouse chromosome 14. The human and mouse RANKL contains 317 and 316 amino acids, respectively. Like several other TNF-like proteins, RANKL is biologically active both in a membrane-bound form and soluble cleaved form. The membrane-bound form is a type II membrane-embedded protein, with a large extracellular, receptor-binding domain, a membrane-anchoring domain, and a small connecting stem. The different forms of RANKL are produced by proteolytic shedding of the RANKL ectodomain by the metalloprotease-disintegrin TNF-α convertase (TACE) or other related metalloproteases (Lum *et al*., 1999). The ectodomain of murine RANKL has been crystallized, and shown to be homotrimeric. This trimeric protein contains four surface loops that promote the specificty in the interactions with its receptor RANK (Lam *et al*., 2001; Ito *et al*., 2002). Although both the soluble and the membrane-bound forms are functionally active, it has been indicated that the membrane-bound form is more efficient in inducing osteoclastogenesis in vitro (Nakashima *et al*., 2000).

RANKL is abundantly expressed in bone and lymphoid tissues (spleen, thymus, lymph nodes, intestinal lymphoid patches), but can also be seen in other extraskeletal tissues (Kartsogiannis *et al*., 1999). The expression of RANKL in osteoblasts/stromal cells is regulated by several cytokines and hormones, which stimulate osteoclast formation and bone resorption (Lerner, 2004). The important role of RANKL in osteoclast development has been shown in mice with a targeted deletion of the RANKL gene. These *rankl -/-* mice have an osteopetrotic phenotype due to the absence of osteoclasts. The lack of RANKL also results in growth retardation, impaired tooth eruption, disturbed T and B lymphocyte differentiation and absence of lymph nodes (Kong *et al*., 1999). On the other hand, mice overexpressing soluble RANKL developed an osteoporotic phenotype, with increased numbers of osteoclasts and decreased bone mineral density (Mizuno *et al*., 2002).

RANK

Receptor activator of nuclear factor κB (RANK) was initially discovered in dendritic cells, and shown to be a regulator of the interactions between dendritic cells and T-cells (Anderson *et al*., 1997). It was later demonstrated that RANK, expressed on osteoclasts, was the receptor for RANKL (Hsu *et al*., 1999). RANK belongs to the TNF-R superfamily, a family of proteins containing four cysteine-rich domains, in the amino-terminal extracellular region, that are involved in ligand binding (Locksley *et al*., 2001). The RANK of human and mouse contains 616 and 625 amino acids respectively. In humans, RANK is situated on chromosome 18q22.1. The transmembrane part of the receptor connects the extracellular region with a long intracellular cytoplasmatic tail. The intracellular tails of the receptor interacts with a number of signalling pathways to mediate the biological responses. The adapter proteins, TNF-receptor-associated factors (TRAFs) that bind to the cytoplasmic tail of all receptors in the TNF-R family, mediate the signalling pathways downstream RANK (Arch *et al*., 1998). The main adapter protein involved in RANK signalling is TRAF6, which binds to the membrane-proximal domain of RANK (Galibert *et al*., 1998; Darnay *et al*., 1999). The activation of TRAF6

results in stimulation of a network of intracellular signalling pathways including c-Src, nuclear factor κB (NF-κB) and several mitogen-activated protein (MAP) kinases, such as p38, extracellular signal-regulated protein kinases (ERKs) and c-Jun N-terminal kinase (JNK). This activation subsequently results in nuclear translocation of a number of transcription factors, including NF-κB, activator protein-1 (AP-1) and nuclear factor of activated T-cells 2 (NFAT2) (Lerner, 2004). The transcription factors regulate the transcription on genes responsible for osteoclastogenesis.

Similar to RANKL-deficient mice, targeted deletion of RANK gives rise to severe osteopetrosis, due to the absence of multinucleated osteoclasts (Dougall *et al*., 1999; Li *et al*., 2000a). However, the osteopetrotic phenotype of *rank-/-* mice could be prevented by bone marrow transplantation, in contrast to the *rankl-/-* mice, demonstrating the defect in the osteoclastic linage in *rank-/-* mice (Li *et al*., 2000a). The *rank-/-* mice also exhibit a deficiency in B-cells, lack of peripherial lymph nodes, hypocalcemia, hypophosphatemia and defective tooth eruption. The latter being a typical finding in mice with decreased osteoclastogenesis. In addition, TRAF6-deficient mice also develop an osteopetrotic phenotype, and exhibit defective tooth eruption, impaired B-cell-differentiation and lack of lymph nodes, similar to the RANK and RANKL knockout mice (Lomaga *et al*., 1999; Naito *et al*., 1999). These facts show that there can be no doubt about the importance of RANK in osteoclastogengesis and bone resorption.

OPG

Osteoprotegerin (OPG) was initially found as a secreted protein, from human skin fibroblasts, which was shown to inhibit osteoclast formation *in vitro*, and the protein was called osteoclast inhibitory factor (OCIF) (Tsuda *et al*., 1997). At the same time, another group reported about a protein cloned from fetal rat intestinal cDNA that reminded of OCIF in its actions, and they named the protein osteoprotegerin (Simonet *et al*., 1997). OCIF was later cloned and shown to be identical to OPG (Yasoda *et al*., 1998a). Another protein that inhibited osteoclastogenesis was identified in a sequence tag database and denoted TNF receptor-like molecule 1 (TR-1), because of its similarity to the TNF receptor superfamily members (Tan *et al*., 1997). TR-1 is also identical with OPG (Kwon *et al*., 1998). In addition, also follicular dendritic cell-derived receptor-1 (FDCR-1) was discovered, as a TNF-related receptor in lymphoid cells, and shown to be identical to OPG (Yun *et al*., 1998).

In humans, the OPG gene is situated on chromosome 8q23-24. The synthesized product of the OPG gene, in humans, rats and mice, is a 401 amino acid pro-peptide, and after cleavage of a signal peptide (21 amino acids), the protein becomes a biologically active protein of 380 amino acids. The OPG of human, mouse and rat share 85-94% sequence homology. The binding to its ligand requires involvement of several domains, including four cysteine-rich domains in the amino-terminal of the OPG-protein. OPG lacks both a transmembrane domain and a cytoplasmic tail and therefore, unlike the other members of the TNF receptor (TNF-R) superfamily, OPG exists only as a soluble receptor. Secreted OPG has affinity to both membrane-bound and soluble RANKL, and acts as a 'decoy-receptor', to prevent activation of RANK. OPG inhibits osteoclast formation in bone marrow cultures, organ-cultured fetal long bones, as well as in neonatal mouse calvariae stimulated by severel different cytokines and hormones (Kwon *et al*., 1998; Palmqvist *et al*., 2002).

OPG is expressed in a variety of different cells, including osteoblasts, stromal cells, endothelial cells, aortic smooth-muscle cells, fibroblasts, dendritic cells and lymphoid cell lines. To show the importance of OPG, in the development of osteoclasts and bone resorption, both OPG-deficient mice and transgenic mice overexpressing OPG have been studied. Targeted deletion of the OPG gene results in mice that exhibit a substantial loss of bone density in both the cortical and trabecular bone (Bucay *et al*., 1998; Mizuno *et al*., 1998), showing that the *opg-/-* mice develop early-onset osteoporosis (Yasoda *et al*., 1998c). The decresed density of the bone is due to both increased numbers of osteoclasts and increased activity of the mature osteoclasts. In contrast, transgenic mice overexpressing OPG, have a normal appearance, but the skeleton is osteopetrotic with increased bone mineral density and fewer trabecular osteoclasts (Simonet *et al*., 1997). Compared to RANK and RANKL deficient mice, the OPG knockout mice have normal shapes and sizes of the bones, no defects in tooth eruption, lymphocyte development or lymph node formation. It is clear that osteoclastogenesis is very much regulated by the RANKL/RANK/OPG system, and that the ratios between these molecules will determine how many osteoclasts that are formed and activated and subsequently also determine the bone mineral density.

DAP12 and FcRγ

Recently, it has been demonstrated that activation of two adapter proteins, DNAX-activating protein 12 (DAP12) and Fc receptor common γ subunit (FcRγ), also is critical for the differentiation of osteoclasts (Takayanagi, 2005a,b). FcRγ preferentially associates with two different immunoreceptors, called paired immunoglobulin-like receptor A (PIR-A) and osteoclast-associated receptor (OSCAR) whereas DAP12 associates with a number of different ligand-recognizing immunoreceptors called DAP12-associated receptors (DARs), including 'triggering receptor expressed by myeloid cells 2' (TREM2), TREM3, 'natural killer cells group 2D' (NKG2D), 'myeloid DAP12-associated lectin-1' (MDL-1) and 'signal-regulatory protein β1' (SIRPβ1). The activation of DAP12 and FcRγ subsequently leads to activation of the immunoreceptor tyrosine-based activation motifs (ITAM) that are situated in the cytoplasmic tails of both DAP12 and FcRγ. The importance of DAP12 and FcRγ in osteoclastogenesis has been demonstated in mice deficient in these genes. DAP12 knockout mice only exhibit a mild osteopetrosis, whereas the double knockout (*dap12-/-*, *fcrγ-/-*) mice exhibit severe osteopetrosis due to a defect in the differentiation of osteoclasts (Koga *et al*., 2004; Mocsai *et al*., 2004). These data indicate that DAP12 and FcRγ are compensating for each other.

Cytokines

Cytokines are small secreted proteins that mediate and regulate immunity, inflammation, and hematopoiesis. They are produced *de novo* in response to different immune stimuli and are used for intercellular communication. Cytokines are produced by a variety of cell types, but the predominant producers are T helper cells (Th) and macrophages. They may act on the cells that secrete them (autocrine action), on cells in the vincinity (paracrine action), or in some situations on more distant cells (endocrine action).The cytokines consist mainly of smaller water-soluble proteins and glycoproteins with a mass of 8-30 kDa. The cytokines bind to specific cell-surface receptors. Subsequent pathways of intracellular signalling then alter the cell functions, including upregulation and/or downregulation of several genes and their transcription factors, resulting in the formation of other cytokines, increasing numbers of surface receptors, or suppression of their own effects. Different cell types can secrete the

same cytokine and one cytokine may act on several different cell types (pleiotropism). They are redundant in their activity, meaning that similar functions can be stimulated by different cytokines. Their main functions are initiation and maintenance of immune and inflammatory responses, hematopoiesis, wound healing and regulation of cell growth and differentiation, as well as stimulation of other cytokines. They have a short half-life, leading to low plasma concentrations, to ensure limitation of their activities. There are different types of cytokines including lymphokines (cytokines made by lymphocytes), interleukins (cytokines made by one leukocyte and acting on other leukocytes or other cells) and chemokines (cytokines with chemotactic activities), based on their presumed function, cell of secretion or target of action. In this project we used a variety of osteotropic cytokines to stimulate/inhibit different cell responses. I here present a short introduction of the main cytokines used in this project.

Interleukin-1 (IL-1)

IL-1 is primarily an inflammatory cytokine, and was one of the first cytokines ever described. It was a factor that induced fever, controlled lymphocytes, enhanced the number of bone marrow cells and caused degeneration in the joints. In 1984 it was confirmed that there were two distinct genes for IL-1, called IL-1α and IL-1β (Dinarello, 1994a). Both forms of IL-1 seem to have similar activities and potencies (Dinarello, 1991). The IL-1 family consists of IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1Ra) (Eisenberg *et al*., 1991).

IL-1α and IL-1β

Both IL-1α and IL-1β are produced by a variety of different cell types, including macrophages, monocytes, dendritic cells, osteoblasts, gingival and periodontal ligament fibroblasts, epithelial and endothelial cells. Thus, it appears that both hematopoietic and mesenchymal/stromal/osteoblastic cells can produce IL-1 and the production increases when both cell types are co-cultured together (Haynes *et al*., 1999). Observations in osteoblast-like cells from human adult bone have shown that these cells also can produce IL-1 *in vitro* (Keeting *et al*., 1991). Each gene is situated on chromosome 2q14, and codes for the IL-1α and IL-1β proteins, respectively (Webb *et al*., 1986). IL-1α and IL-1β are produced as precusor peptides, and for example, mature IL-1β, is released from pro-IL-1β following cleavage by an enzyme called caspase-1 or the interleukin-1 converting enzyme (ICE) (Cerretti *et al*., 1994). ICE is a member of the cysteine protease family. ICE does not cleave the IL-1α precursor. The mature sizes of both IL-1α and IL-1β are 17 kDa. They have different amino acid sequences, with only 22% homology. IL-1 plays an important role in the inflammatory response of the body against infection, and is biologically active in the low picomolar and femtomolar range. These cytokines enhance the expression of adhesion molecules on endothelial cells to enable transmigration of leukocytes, to sites of infection and also re-set the thermoregulatory center of hypothalamus, leading to an increased body temperature (fever), which helps the body's immune system to fight infection. Il-1 is a potent inducer of hypotension and shock. Humans are particularly sensitive to the pyrogenic and hypotensive properties of IL-1 (Smith *et al*., 1992). IL-1 is also an important factor in the regulation of hematopoiesis, and has been shown to be a potent stimulator of bone resorption *in vitro* (Lorenzo *et al*., 1987) and *in vivo* (Sabatini *et al*., 1988) as well.

IL-1Ra

The third member of the IL-1 gene family is the IL-1Ra. IL-1Ra was initially called the IL-1 inhibitor and was discovered separately in 1984 by two independent laboratories (Lomedico *et al*., 1984; Auron *et al*., 1984). IL-1Ra, is a secreted protein that binds to the same receptor on the cell surface as IL-1α and IL-1β, and thus prevents the signal transduction in that cell. It is used in the treatment of the autoimmmune disease rheumatoid arthritis, in which IL-1 plays a key role. IL-1Ra inhibits the ability of IL-1 to stimulate bone resorption and PGE_2 formation in bone organ cultures (Seckinger *et al*., 1990). IL-1β is more closely related to IL-1Ra than to IL- 1α. The primary amino acid sequence that is identical between mature human IL-1α and mature IL-1β is 22% while it is 26% when comparing IL-1β to IL-1Ra and only 18% when comparing IL-1α to IL-1Ra (Dinarello 1994a).

IL-1 receptors (IL-1R)

Two distinct forms of receptors for IL-1 have been cloned in mammalian cells, IL-1R1 and IL-1R2 (Sims *et al*., 1988; McMahan *et al*., 1991; Chizzonite *et al*., 1989; Dinarello, 1993a,b). The type 1 receptor is an 80 kDa glycoprotein found on a variety of cells, but predominantly on smooth muscle cells, endothelial cells, hepatocytes, fibroblasts, keratinocytes and T-lymphocytes. It is a member of the Toll receptor family (Means *et al*., 2000). The IL-1R type 2 is a 68 kDa glycoprotein prominently found on monocytes, B-lymphocytes and neutrophils. The extracellular domains of IL-1R1 and IL-1R2 share only 28% amino acid homology. The IL-1R1 is the primary signal transducing receptor (Sims *et al*., 1993), and the post-receptor signalling involves breakdown of sphingomyelin and ceramide production (Kolesnick and Golde, 1994), activation of NF-κB (Jimi *et al*., 1996), mitogen-activated protein kinases (MAPK), as well as activating protein-1 (AP-1) (Suzuki *et al*., 2001; Rannou *et al*., 2006). IL-1R2 also binds IL-1 but does not transduce signals. It appears to function as a decoy receptor by preventing the binding of IL-1 to IL-1R1, and therefore inhibiting its activity (Colotta *et al*., 1993). Additionally, IL-1R2 can be released as a soluble protein inhibiting the interactions between IL-1 and IL-1R1 (Dinarello, 1993a,b). IL-1R2 may also synergize with IL-1Ra to inhibit IL-1's activation of IL-1R1 (Burger *et al*., 1995). However, only 2% of the IL-1 receptors need to be occupied for the development of a biological response.

The members of the TRAF family are cytoplasmic adapter proteins that are recruited by receptors of the TNF-R family, as well as the IL-1 receptor family. In mice and humans, there are six members of the TRAF family (i.e. TRAF1 to TRAF6), and these proteins have a conserved sequence of amino acids near the carboxy-terminal end, called the TRAF domain. This domain is essential for the binding of these signal-transducing adaptor proteins to the receptors. Additionally, there are at least two other functional domains, the RING finger domain and the zinc finger domain, located on the amino-terminal end of the TRAF protein, and these seem to be required for the activation of the downstream signalling (Cha *et al*., 2003). TRAF6 is distinct from the other TRAFs, since it is the only one involved in Toll/IL-1 receptor signalling. TRAF2 and TRAF6 have been found to mediate the transcription of downstream target genes through the activation of two different intracellular signalling pathways, namely JNK and NF-κB (Cha *et al*., 2003). There are two groups that have generated TRAF6-deficient mice, and these are found to be osteopetrotic and exhibit defective tooth eruption, B-cell differentiation, lymph node organogenesis and IL-1 signalling (Lomaga *et al*., 1999; Naito *et al*., 1999). Transfection of *traf6-/-* spleen cells with TRAF6 from

wild type mice restores the osteoclastogenic response to RANKL/M-CSF (Kobayashi *et al*., 2001).

Effects of IL-1 on bone

Horton *et al*., demonstrated the first proof of that immune-competent cells could influence bone cells, when peripheral blood leukocytes, stimulated with either phytohemagglutinin or dental plaque, released factors into the culture supernatants that could stimulate bone resorption (Horton *et al*., 1972). This activity was called osteoclast-activating factor (OAF) and this factor was then found to be the cytokine IL-1β (Dewhirst *et al*., 1985). Thus, IL-1 was the first protein mediator of immune cell functions which was proven to regulate bone resorption (Gowen *et al*., 1983) and bone formation (Canalis, 1986), and IL-1 is one of the most potent stimulators of bone resorption known today (Lorenzo *et al*., 1987). IL-1 has also been shown to increase prostaglandin biosynthesis in bone (Lorenzo *et al*., 1987), an important effect that might be responsible for some of the resorptive activity caused by IL-1, since prostaglandins themselves can stimulate bone resorption (Klein and Raisz, 1970). IL-1 has been shown to increase RANKL formation in stromal/osteoblastic cells (Hofbauer *et al*., 1999), and stimulate OPG formation in a human osteosarcoma cell line (Vidal *et al*., 1998), as well as directly stimulate the resorptive effect of mature osteoclasts, through binding to IL-1R1 (Jimi *et al*., 1999), by a mechanism involving activation of NF-κB (Miyazaki *et al*., 2000). IL-1 has also been shown to promote osteoclast survival through prevention of apoptosis (Jimi *et al*., 1996). Additionally, IL-1 seems to be involved in the differentiation of osteoclasts from hematopoietic progenitor cells (Akatsu *et al*., 1991), and has been shown to be a potent stimulator of bone resorption *in vivo* (Sabatini *et al*., 1988). Regarding bone formation IL-1 appear to be mainly inhibitory (Canalis, 1986), but it does stimulate DNA synthesis in bone organ cultures, as well as primary cultures of human bone cells (Canalis, 1986; Gowen *et al*., 1985). The influence of IL-1 on bone resorption will be further described in the section about inflammation induced bone remodelling.

Tumour necrosis factor-α (TNF-α)

The TNF family consists of two related polypeptides, TNF-α and TNF-β, which are two separate gene products, similar to IL-1 (Beutler and Cerami, 1989). TNF-α is also a pleiotropic inflammatory cytokine involved in systemic inflammation. It was first isolated in 1975 in an attempt to identify tumour necrosis factors responsible for necrosis of the sarcoma Meth A (Carswell *et al*., 1975). It seems like the cytokine possesses both growth stimulatory and growth inhibitory properties. The cytokine is produced by a variety of cell types, including monocytes/macrophages, neutrophils, osteoblasts, gingival and periodontal ligament fibroblasts, epithelial and endothelial cells. It consists of 185 amino acids, cleaved from a 212 amino acid-long propeptide on the surface of the cells. The gene is situated on chromosome 6p21.3 in humans. Its release is stimulated by several other mediators, such as IL-1 and bacterial endotoxin (Taniguchi and Yamamoto, 2005).

TNF-α acts as a key mediator in the local inflammatory immune response. It is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophages and neutrophils to a site of infection. High levels of TNF-α correlate with increased risk of mortality (Rink & Kirchner, 1996). Lipopolysaccharide from from bacteria cell walls is an especially potent stimulus

for TNF-α biosynthesis (Tracey *et al*., 1988). TNF-α exhibits chronic effects, as well as resulting in acute pathologies. A locally increasing concentration of TNF-α will cause the cardinal signs of inflammation (i.e. heat, swelling, redness and pain). TNF-α promotes the inflammatory response, which in turn causes many of the clinical problems associated with autoimmune disorders such as rheumatoid arthritis, Crohn's disease, psoriasis and asthma.

TNF-α is a trimeric protein and it is produced as a noncleaved 27 kDa precursor peptide (Perez, *et al*., 1990). Stimulated macrophages produce TNF-α, which can either bind directly to TNFR-I (p55) or TNFR-II (p75) receptors through cell-to-cell contact, or undergo cleavage and bind in its soluble form. TNF-α shares only 36% amino acid sequence homology with TNF-β, but the tertiary structures of the two proteins are very similar so both bind to the same TNF receptors. These receptors are expressed on nearly all somatic cells.

TNF receptors (TNFR)

TNF exerts its effect by binding to two cell surface receptors called TNFRI (p55) and TNFRII (p75) (Brouckaert *et al*., 1993). Both receptors are present on several cell types including bone marrow hematopoietic cells (Rusten and Jacobsen, 1995; Sato *et al*., 1997), and both receptors transmit biological responses, although TNFRI seems to mediate most of the biological properties of TNF-α (i.e. apoptosis and activation of NF-κB) (Wiegmann *et al*., 1992; Hsu *et al*., 1995). There are occasions when both receptors appear to interact with each other (Tartaglia *et al*., 1993), and for full biologic effect to occur, activation of both receptors is sometimes necessary (Vandenabeele *et al*., 1995). However, some activities can be induced by selective activation of either receptor (Sheehan *et al*., 1995). Mice deficient in the TNFRI and TNFRII breed normally and appear rather healthy, but they lack normal immune response and mechanisms regarding apoptosis (Rothe *et al*., 1993; Erickson *et al*., 1994). The TNF receptors can undergo proteolytic cleavage and release the extracellular fragment (soluble TNF-receptors). Soluble TNF-receptors are able to bind to TNF and inhibit its actions (Björnberg *et al*.,1994). The members of the TNF-R superfamily can mediate a variety of cellular responses, including cell proliferation, differentiation and apoptosis. Mainly, these functions are mediated by a family of intracellular TNFR-binding proteins, called the TNFR-associated factors (TRAFs). TRAF2 has been shown to be the common signal transducer of TNFRI and TNFRII (Wajant *et al*., 2003; Liu, 2005). TRAF2 has also been found to mediate the transcription of downstream target genes through the activation JNK and NF-κB (Cha *et al*., 2003).

Effects of TNF-α on bone

The biologic activities of TNF-α and TNF-β are similar, and they have potent stimulatory effects on bone resorption (Bertolini *et al*., 1986, Lorenzo *et al*., 1987) and inhibitory effects on collagen formation in bone (Bertolini *et al*., 1986, Canalis *et al*., 1987). The effects of TNF on bone resorption seem to be mediated by its effects on osteoclasts, since the number of osteoclasts was enhanced after TNF treatment of bones (Johnson *et al*., 1989), and since bone resorption stimulated by TNF was inhibited by calcitonin (Stashenko *et al*., 1987). Similar to IL-1, TNF-induced formation of osteoclast-like cells in bone marrow culture (Pfeilschifter *et al*., 1989) is mediated by an enhancement of RANKL expression (Hofbauer *et al*., 1999). Additionally, TNF also increases the expresion of OPG in osteoblastic cell models (Hofbauer *et al*., 1998).

Since the RANK/RANKL signalling system was discovered, the differentiation of osteoclasts has been regarded to be exclusively induced by RANKL. At the moment, there are discussions about whether or not TNF can stimulate formation of osteoclasts from osteoclast precursors in the absence of RANKL. In 2000, two research-groups independently found TNF-α to stimulate differentiation of osteoclasts without RANKL-RANK interaction (Kobayashi *et al*., 2000; Azuma *et al*., 2000). In contrast, other authors have found that TNF stimulate the differentiation of osteoclasts precursors into osteoclasts solely in the presence of RANKL (Lam *et al*., 2000). Similar to IL-1, TNF stimulates bone resorption, inhibits bone formation and seem to mimic the *in vivo* response of bone to haematological malignancies (Bertolini *et al*., 1986).

The stimulatory effects of TNF on RANKL-induced osteoclastogenesis are mediated by activation of TNFRI (p55) (Zhang *et al*., 2001). Basal osteoclast formation by RANKL was decreased in TNFRI knockout mice. Further stimulation with TNF-α, of the cells derived from TNFRI-deficient mice, failed to increase osteoclastogenesis, and in these mice there was also reduced DNA-binding of the transcription factors AP-1 and NF-κB, compared to wt mice (Zhang et al., 2001). When it comes to the osteoblasts, *in vitro* studies have shown that TNF directly inhibits the differentiation of osteoblast precursors into mature osteoblasts (Gilbert *et al*., 2000). The apoptosis of osteoblasts can also be induced by TNF (Jilka *et al*., 1998). Human osteoblast-like cells are able to produce TNF-α (Gowen *et al*., 1990), and this production is stimulated by IL-1, LPS and GM-CSF. The effects of TNF-α on osteoblastic cells seem to be mediated by stimulation of NF-κB (Ali *et al*., 1999; Yao *et al*., 2000). There are a lot of data indicating that TNF-α plays an important role in the event of bone resorption in metabolic diseases such as rheumatoid arthtritis and periodontitis. This aspect will be further described in the section about inflammation induced bone resorption.

Interleukin 4 and Interleukin 13

IL-4 (19 kDa) and IL-13 (10 kDa) are multifunctional immunoregulatory cytokines secreted mainly by activated T helper type 2 (T_H2) cells, mast cells and basophils. These cytokines can cause similar responses, and many of these are associated with the regulation of immune responses, such as allergy, asthma and inhibition of autoimmunity (Nelms *et al*., 1999; Hershey, 2003). Some of the functions of IL-4 are the growth and activation of B-cells and the inhibition of macrophage function (Hart *et al*., 1989). IL-4 inhibits the ability, of cells from a macrophage linage, to produce a number of inflammatory cytokines (e.g. IL-1, TNF-α and IL-6), and has therefore been considered as an anti-inflammatory cytokine (Hart *et al*., 1989, 1991; Suzuki *et al*., 1993; Sugiyama *et al*.,1996; Lacey *et al*., 1995). Inhibitory effects on bone resorption have been seen by both IL-4 and IL-13 (Horowitz and Lorenzo, 2002). IL-4 also seems to inhibit osteoclast formation and bone resorption both *in vitro* and *ex vivo* (Miossec *et al*., 1994; Riancho *et al*.,1993; Palmqvist *et al*., 2006). Both IL-4 and IL-13 can inhibit bone resorption, partially by decreasing prostaglandin biosynthesis (Kawaguchi *et al*., 1996; Onoe *et al*., 1996; Palmqvist *et al*., 2006), but both cytokines can also inhibit bone resorption by mechanisms independent on COX-2- inhibition (Palmqvist *et al*., 2006).

The inhibition of bone resorption by IL-4, *in vitro*, seems to involve decreased osteoclastogenesis, and target the RANK/RANKL pathway (Watanabe *et al*., 1990,

Abu-Amer, 2001). Several mechanisms have been proposed for the inhibitory actions of IL-4 on osteoclastogenesis including inhibition of NF-κB (Abu-Amer, 2001), MAPK signalling (Wei *et al*., 2002), and induction of peroxisome proliferator-activated receptor-γ1 (PPAR-γ1) (Bendixen *et al*., 2001). IL-4 has also been reported to downregulate RANK expression in osteoclast progenitor cells (Moreno *et al*., 2003, Palmqvist *et* al., 2006). The process seems to be dependent on activation of the IL-4-responsive STAT (signal transducers and activators of transcription), namely the transcription factor STAT6 (Abu-Amer, 2001; Palmqvist *et al*., 2006). Obsevations indicate that IL-4 and IL-13 exhibit their effects not only on osteoclasts, but also on osteoblasts since both cytokines downregulate RANKL and upregulate OPG in osteoblasts isolated from mouse cavarial bones (Palmqvist *et al*., 2006).

The gene coding for IL-13 is located 12 kb upstream of the gene encoding IL-4, on the chromosome 5q31. Although IL-4 and IL-13 only have 25% homology in their amino acid sequences, they seem to share many properties including a receptor subunit (the α subunit of the IL-4 receptor (IL-4Rα)). There are at least 2 different types of IL-4 receptors, called type 1 and 2. IL4R type 1 is formed by heterodimerization of IL-4Rα chain and the common γ chain (γc), a receptor component that also is found in many other receptors (i.e. IL-2, IL-7, IL-15 and IL-21). The IL-4 receptor type 2, however, is composed of IL-4Rα and the IL-13 receptor α1 (IL-13Rα1) protein (Callard *et al*., 1996; Murata *et al*., 1998; Zurawski *et al*.,1995; Kelly-Welch *et al*., 2003; Hershey, 2003). IL-13 can also bind to its other receptor, the IL-13 receptor subtype α2 (IL-13Rα2), but it seems that this binding is not resulting in any biological response, which indicates that IL-13Rα2 may function as a form of decoy receptor.

Binding of either IL-4 to IL-4Rα in IL-4R type 1 or 2, or IL-13 to IL-13Rα1 in IL-13 receptors results in an activation of Janus tyrosine kinase 1 (JAK1). This activation leads to phosphorylation of tyrosine residues of IL-4Rα, which subsequently binds to the transcriptionfactor STAT6. STAT6 then homodimerizes and translocates into the nucleus (Hebenstreit *et al*., 2006). Activation of IL-4 and IL-13 receptors also results in an activation of other members in the JAK family and several signalling molecules including insulin receptor substrate 1 and 2 (IRS 1 and 2). Two pathways have been proposed to be involved in signalling down-stream IRS-1 and 2: the phosphatidylinositol 3 (PI3) kinase and the Ras/mitogen-activating protein kinase pathways (Jiang *et al*., 2000). The IRS cascade has shown to be involved in cellular proliferation whereas an activation of STAT6 seems to be important for the regulation of gene expression, immunoglobulin E (IgE)-production from B-cells and the development of T-helper Type 2-cells (Nelms *et al*., 1999). As mentioned earlier, IL-4 and IL-13 share many structural characteristics, but they also have some important differences. One difference is their ability to act across species. IL-4 is absolutely species specific (i.e. human IL-4 acts only on human cells) (Park *et al*., 1987), whereas IL-13 is not species specific, although it appears to be species selective (i.e. human IL-13 has greater activity on human cells than on mouse cells) (de Vries, 1996).

The kallikrein-kinin system

The kallikrein-kinin system was discovered nearly 60 years ago when Rocha e Silva *et al*., (1949) showed that ”bradykinin” was released by snake venom, and the first attempts to purify bradykinin (BK) was made by Prado *et al*., 1950. Not until 1960, BK was identified as a nonapeptide, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Boissonnas *et al*., 1960). At the same time another group also solved the primary structure of BK (Elliott *et al*., 1960, 1961). A year later also the structure of Lys-BK (Kallidin) was identified (Werle *et al*. 1961). Both BK and Lys-BK are rapidly inactivated by different enzymes called Kininase I and II. Kininase I is similar to Carboxypeptidase N and M, and Kininase II was later shown to be identical with the angiotensin I-converting enzyme (Erdös, 1979a,b). This study led to the opportunity to develop synthetic inhibitors against Kininase II called ACE inhibitors. Kinins are short peptides released from precursors (kininogens) through clevage by proteolytic enzymes (kallikreins) present in several body tissues and fluids. Kinins are the molecules responsible for the biologial effects caused by the kallikrein-kinin system (Fig 3).

Kallikreins

The plasma kallikrein-kinin system is triggered following activation of the Hageman Factor (coagulation Factor XII), by endotoxin (Kalter *et al*., 1983) and microbial proteases (Molla *et al*., 1989) yielding Factor XIIa, or autoactivation initiated by injury to the endothelium. The Hageman Factor also activates the coagulation cascade. Plasma kallikrein is synthesized by hepatocytes in the liver, and released as an inactive proenzyme (prekallikrein). Factor XIIa cleaves plasma prekallikrein, to its active form (kallikrein) which subsequently acts on high molecular weight (HMW) kiningen, to release BK, a nonapeptide with arginine at both ends. Plasma kallikrein and Factor XIIa can be rapidly inactivated by the C1-inhibitor (complement system), α_2-macroglobulin (α_2-M) and antithrombin III (AT-III) (Schreiber, 1976; Shapira et al., 1981; de Agostini *et al*., 1984; Davis, 2004).

Tissue kallikrein is a member of a large multigene (KLK) family of enzymes, and is expressed in a variety of tissues, though at different levels. Tissue kallikrein acts primarly to generate Lys-BK (kallidin) from both HMW and low molecular weight (LMW) kininogens, but since LMW kininogen is the most abundant substrate, tissue kallikrein mainly uses LMW kininogen for its purpose. Tissue kallikrein is not as sensitive to inhibition as plasma kallikrein.

Kininogens

Kininogens are defined as circulating proteins that contain the BK sequence. HMW (88-120 kDa) and LMW (50-68 kDa) kininogens are products from a single gene (Takagaki *et al*., 1985), of 11 exons, and the different forms are due to alternative splicing of the transcript. The kininogens are synthetizised by hepatocytes in the liver, as glycoproteins with an amino-terminal heavy chain and a carboxyterminal light chain. The kininogens have multiple protein domains, with different activities associated with each domain. The heavy chain consists of domain 1-3. Domain 4 is BK, and domain 5-6 build up the light chain. Domains 2 and 3 contain an amino acid sequence found in cysteine protease inhibitors (Salvesen *et al*., 1986), suggesting the possibility that kininogens may act as both pro- and anti-inflammatory proteins.

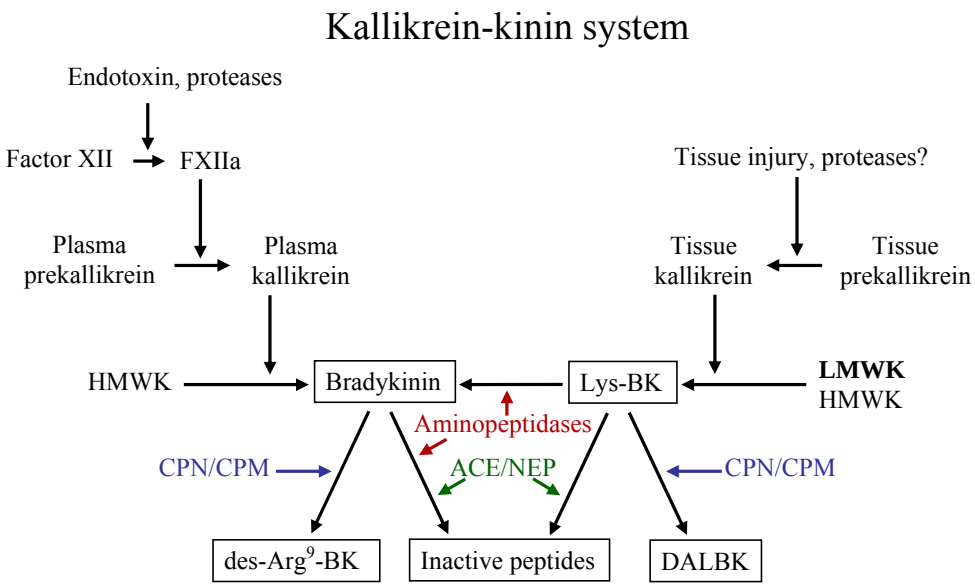

Figure 3. Schematic presentation of the two primary pathways of the kallikrein-kinin system, leading to formation of kinins.

Kinins

Kinins are short bioactive peptides related to the nonapeptide bradykinin. The term kinin originates from the Greek word kineo (= to move). Originally the word kinin was used for substances having effects on smooth musculature. The kinins are effector molecules of the kallikrein-kinin system, and are not released from cells. They are instead potent peptides cleaved from circulating kininogens (HMW or LMW), an action catalysed by either plasma- or tissue kallikrein. The HMW kininogen is cleaved by either plasma- or tissue kallikrein yielding BK or Lys-BK respectively, whereas LMW kininogen only can be cleaved by tissue kallikrein yielding Lys-BK (Margolius, 1989). The amino acid sequences for some of the natural kinins and kinin analogues with antagonistic properties are shown in Table 1. Kinins are very rapidly degraded in plasma, due to the enzymatic actions by proteases, mainly Kininase I and II. Kininase I cleaves the carboxy-terminal arginine from the kinins and des-Arg^9-BK (DABK) or des-Arg^{10}-Lys-BK (DALBK) are produced. These peptides are also bioactive in several cell types, and they are therefore also included in the group of natural kinins (Table 1).

The physiological and pathophysiological functions of BK include vasodilation and constriction (Regoli and Barabé, 1980; Hall, 1992), increasing of microvascular permeability and promote plasma extravasation (Campos and Calixto, 1995), releasing of histamine from mast cells (Bueb *et al*., 1990, 1993) and stimulation of pain and hyperalgesia (Dray and Perkins, 1993). The kallikrein-kinin system plays an important role in the inflammatory response.

Table 1. Amino Acid Sequences for some of the Natural Kinins and Kinin Analogues with Antagonistic Properties

B2 receptor agonists											
Bradykinin			Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
Lys-BK		Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
Met-Lys-BK	Met-	Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
B1 receptor agonists											
des-Arg^{9}-BK			Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe	
des-Arg^{10}-Lys-BK		Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe	
B2 receptor antagonists											
D-Arg-[Hyp^{3},Thi^{5,8}, D-Phe^{7}]-BK		Arg-	Arg-	Pro-	Hyp-	Gly-	Thi-	Ser-	Phe-	Thi-	Arg
Hoe 140 (Icabitant)		Arg-	Arg-	Pro-	Hyp-	Gly-	Thi-	Ser-	Tic-	Oic-	Arg
B1 receptor antagonists											
des-Arg^{9}-[Leu^{8}]-BK			Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Leu	
des-Arg^{10}-[Leu^{9}]-Lys-BK		Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Leu	
des-Arg^{10}-Hoe 140		Arg-	Arg-	Pro-	Hyp-	Gly-	Thi-	Ser-	Tic-	Oic	

Kininases

Released kinins have a very short half-life, because of the fast degradation by the enzymatic action of proteases, mainly Kininase I and II. These proteases are existing both as circulating and as membrane-bound enzymes. Several different kininases have been described, including Carboxypeptidase N and M (CPN, CPM), which together is called Kininase I. Kininase II consists of two different enzymes called angiotensin I-converting enzyme (ACE) and neutral endopeptidase (NEP). Other types of kininases are prolidase and aminopeptidases. CPN/CPM circulates in plasma and cleaves the carboxy-terminal Arg from kinins. As a result des-Arg^{9}-BK or des-Arg^{10}-Lys-BK are produced, and these peptides are also biologically active. However, ACE and NEP cleaves the the carboxyterminal Phe-Arg, or even Ser-Pro-Phe-Arg from kinins and thereby inactivating their effects (Regoli and Barabé, 1980) (Fig 4). Aminopeptidases catalyze the removal of one amino acid at a time from the amino-terminal of peptides and proteins. Prolidase and aminopeptidase P specifically cleaves peptides with a Pro in the second position and, therefore, they cleave the amino-terminal Arg, from BK and thereby inactivating its effect (Griswold *et al*., 1996; Koch *et al*., 2003). Aminopeptidases can also participate in the release of BK, since they can cleave Lys from Lys-BK yielding BK (Erdös, 1979a). Kininases have been shown in a variety of cells and in body fluids, but there is no available information of these enzymes in bone tissue so far, though there are some indications that some of the Kininase II inhibitors potentiate the bone-resorbing effect of BK (Lerner *et al*., 1987a).

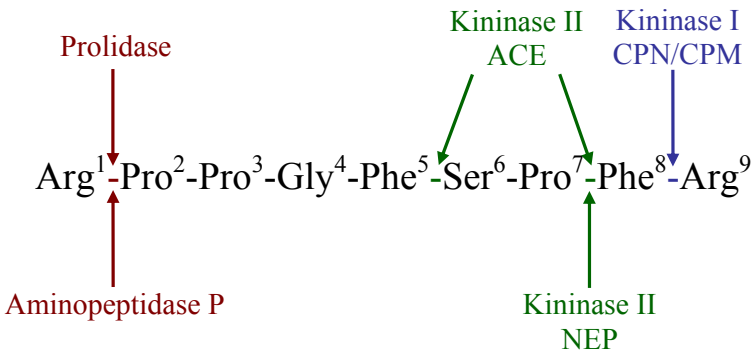

Figure 4. Different kininases cleave the bradykinin molecule at different sites.

Bradykinin receptors

In the late 1970's, Regoli and co-workers started the molecular characterization of kinin receptors by showing the existence of two types of kinin receptors, B1 and B2. These differ in their pharmacological profiles and in their expression patterns (Regoli *et al*., 1977, 1978; Drouin *et al*., 1979). 1985, there was a breakthrough when the first BK analogues with antagonistic properties for the B2 receptors were developed (Vavrek and Stewart, 1985). Another important discovery, during this time, was the identification of the B2 receptor as a G protein-coupled receptor (GPCR), which signals through the phospholipase C pathway leading to inositol 3-phosphate (IP_3) formation and intracellular increase of Ca^{2+}, as well as through the phospholipase A_2 pathway resulting in the release of arachidonic acid (Burch and Axelrod, 1987). The culmination of the search for the kinin receptors came when Jarnagin and co-workers cloned the rat B2 receptor cDNA in 1991 (McEachern *et al*,. 1991) and soon thereafter the human B1 receptor was cloned (Menke *et al*,. 1994). More recently the targeted ablation of the genes for the B2 (Borkowski *et al*,. 1995) and B1 receptor (Pesquero *et al*,. 2000) in mice has started to reveale the pathophysiological functions of the kinin receptors.

There are two pharmacologically distinct subtypes of kinin receptors, called B1 and B2, which are linked to the effects of kinins (Prado *et al*., 2003; Leeb-Lundberg *et al*., 2005). They belong to the rhodopsin family of GPCR. Both receptors seem to signal through $G\alpha_q$ to stimulate phospholipase Cβ yielding phosphoinositide (PI) hydrolysis and an increase in intracellular free Ca^{2+}, as well as through $G\alpha_i$ to downregulate adenylate cyclase and stimulate the MAPK cascades. Also stimulation of phospholipase A_2 seems to occur via G protein-coupled mechanisms (Burch and Axelrod, 1987; Leeb-Lundberg *et al*., 2005). The distribution of BK receptors in different cells and tissues and the relative expressions of B1 and B2 receptors have been extensively studied, preferentially by using pharmacological methods, such as rank order potencies for different agonists, sensitivity to receptor antagonists, and radioligand-binding studies. Selective antagonists for the B1 receptor are for example des-Arg^9[Leu^8]-BK, des-Arg^{10}[Leu^9]-LysBK or des-Arg^{10}-Hoe140 (Wirth *et al*., 1992), and for the B2 receptor has Hoe140 (Icatibant; D-Arg-[Hyp^3, Thi^5, D-Tic^7,Oic^8] been shown to be a very selective antagonist (Regoli *et al*., 1998; Simpson *et al*., 2000). Some non-peptide BK receptor antagonists have also been developed including WIN64338 (Salvino *et al*,. 1993) and FR173657 (Aramori *et al*, 1997; Abe *et al*,. 1998) for the B2 receptors, and SSR240612 (Gougat *et al*., 2004) for the B1 receptors.

The natural kinins with high affinity to the B2 receptor are BK and Lys-BK. Those are generated in response to injury, from kininogen precursors, cleaved by either plasma or tissue kallikreins, in all mammalian species. The B2 receptor require the hole nonapeptide to be activated, and is constitutively expressed in a variety of different cell types including endothelial cells, synovial cells, sensory fibers, dermal and gingival fibroblasts, smooth muscle cells and epithelial cells. The expression of the B2 receptors can be enhanced by cytokines, cyclic AMP, estrogen and glucocorticoids (Schmidlin *et al*., 1998; Haddad *et al*., 2000; Pesquero *et al*., 1996., Madeddu *et al*., 1997; Scherrer *et al*., 1999) and the promoter contain binding elements for AP-1, cyclic AMP responsive element (CRE), NF-κB, estrogen receptor (ER) and glucocorticoid receptor (GR), as well as a silencer element (Baptista *et al*., 2002).

The most important structural determinant for high affinity to B1 receptors is the removal of the carboxy-terminal arginine from BK or Lys-BK, by carboxypeptidase N and M, yielding des-Arg^{9}-BK or des-Arg^{10}-Lys-BK (Regoli and Barabe, 1980; Marceau, 1995). The B2 receptors are constitutively expressed in many cell types, whereas the B1 receptors are believed to be induced during inflammation, by lipopolysaccharides, pro-inflammatory cytokines (Marceau *et al*., 1998), as well as by kinins themselves (Schanstra *et al*., 1998; Phagoo *et al*., 1999), although this auto-regulation model is not universally applicable or may be species-specific (Sabourin *et al*., 2001; Marceau *et al*., 2002). The B1 promoter has numerous putative binding sequences for several transcription factors including NF-κB and AP-1 (Bachvarov *et al*., 1996), and c-Jun has been shown to be an important mediator of B1 receptor regulation (Yang *et al*., 2001). It has also been shown that pharmacological inhibitors of different MAPKs and of NF-κB can reduce increased expression of B1 receptors (Larrivée *et al*., 1998; Sabourin *et al*., 2002; Ganju *et al*., 2001; Medeiros *et al*., 2004).

The signalling patterns of the B1 and B2 receptors seem to differ with respect to their duration. B2 receptor-mediated signalling is transient, whereas B1 receptor-mediated signalling is sustained (Mathis *et al*., 1996; Faussner *et al*., 1998). At the cellular level, BK stimulation of the B2 receptor leads to rapid desensitization of the receptor response determined by both PI hydrolysis and the increase in intracellular Ca^{2+}, in a variety of native systems including vascular endothelial cells (Smith *et al*., 1995), vascular smooth muscle cells (Mathis *et al*., 1996), as well as cell systems transfected with the B2 receptor (Blaukat *et al*., 1996; Fathy *et al*., 1999). The B2 receptor desensitization involves phosphorylation of specific serines and threonines in the receptor carboxy-terminal end. The B1 receptor differs from the B2 receptor in this aspect since it is only desensitizised to a very limited degree (Bascands *et al*., 1993; Smith *et al*., 1995; Mathis *et al*., 1996), it lacks serines and threonines in the carboxy-terminal tail. Moreover, human B1 receptors are not phosphorylated to a significant degree in either the presence or absence of agonist (Blaukat *et al*., 1999). The difference in the amount of desensitization of B2 and B1 receptors might contribute to the distinct pattern of receptor signalling (Bascands *et al*., 1993; Mathis *et al*., 1996).

The relative importance of signalling through B1 and B2 receptors *in vivo* is not known, but it has been proposed that B1 receptors may be important in inflammatory conditions, not only because of their induction, but also due to the fact that des-Arg metabolites of BK and Lys-BK, acting preferentially on B1 receptors, are produced in inflammatory processes by carboxypeptidases.

Cloning of the kinin receptors

The B2 receptor was first cloned from rat uterus (McEachern *et al*., 1991). Subsequently the B2 receptor has been molecularly cloned in several mammalian species including human (Hess *et al*., 1992). The structure of the B2 receptor protein is typical of that of a GPCR, containing seven transmembrane-spanning domains, with the amino-terminal end being extracellular and the carboxy-terminal end being intracellular, and with three extracellular loops and three intracellular loops. Three consensus sites for amino-terminal linked glycosylation are found in extracellular domains and the carboxy-terminal tail contains serines and threonines that are putative sites for acylation (Fredriksson *et al*., 2003). The three-exon structure of the gene (*BDKRB2*) has been determined, and the gene is located on the human chromosome 14q32 (Powell *et al*., 1993; Kammerer *et al*., 1995). The coding sequence of the human B2 receptor was first believed to be intronless in the exon 3, but an initiation codon has also been found in exon 2 (extends the amino-terminal with 27 residues).

The cloning of the human B1 receptor was achieved later (Menke *et al*., 1994). Thereafter, the B1 receptor has also been cloned in several mammalian species. The B1 receptor also possesses three consensus sites for amino-terminal linked glycosylation in extracellular domains and putative sites for phosphorylation and acylation. The three-exon structure of the human B1 receptor gene (*BDKRB1*) has also been determined with the protein sequence being encoded only by exon 3 (Bachvarov *et al*., 1996; Yang and Polgar, 1996). The gene corresponding with the B1 receptor is also located on the chromosome 14q32 in close vincinity to the B2 receptor gene, with the B2 receptor gene being proximal to the B1 receptor gene, separated by only 12 kb (Cayla *et al*., 2002). The close proximity of the two genes suggests that they evolved from a common ancestor by a gene duplication event. Both B1 and B2 receptors, from several species, have been found to be highly conserved (70-80% homology). In humans, the predicted sequences for the B1 and B2 receptor proteins are 353 and 364/391 amino acids respectively. The mRNA coding for the B2 receptor is large (~4 kb) compared to that of the B1 receptor (~1.4 kb), and the large 3'-untranslated region of the B2 receptor accounts for most of the difference.

Targeted disruption ("knockout") of both kinin receptor genes, by homologous recombination has been reported. The B2 receptor knockout mice fail to respond to BK in assays such as smooth muscle contraction and afferent nerve stimulation (Borkowski *et al*., 1995). These animals seems apparently healthy, are fertile, but they may not develop and age in an entirely normal manner. Interestingly, B2 receptors might be important in senescence-associated bone loss since mice double mutant for $Ins2^{Akita/+}$ and $Bdkrb2^{-/-}$ genes exhibit a complex phenotype including kyphosis and osteoporosis (Kakoki *et al*., 2006). Targeted deletion of the B2 receptor gene has also resulted in mice with severe hypertension, with end-organ damage, when they are challenged to excess dietary sodium chloride (Alfie *et al*., 1996).

More recently the B1 receptor knockout mice have been produced (Pesquero *et al*., 2000). These mice develop normally with normotension, but fail to respond to des-Arg^9-BK in assays measuring contractility of the mouse-isolated stomach. However, lipopolysaccharide (LPS)-induced hypotension is blunted and the number of polymorphonuclear (PMN) leukocytes is decreased in inflamed tissues. Additionally, B1 receptor knockout mice are analgesic in behavioral experiments of nociception (Pesquero *et al*., 2000).

The use of mice deficient in each receptor gene, and a number of different specific B1 and B2 receptor antagonists, have suggested that both B1 and B2 receptors are potential

therapeutic targets in several pathophysiological events related to inflammation (e.g. pain, sepsis, allergic asthma, rhinitis, oedema), as well as diabetes and cancer (Leeb-Lundberg *et al*., 2005).

Kinins and Bone Metabolism

Observations of the effects of kinins on bone metabolism have been made *in vitro*, mainly in bone organ cultures, and most of these investigations have been done using a mouse calvarial bone culture model, in which bones, prelabelled with ^{45}Ca or [^{3}H]proline *in vivo* are used (Lerner *et al*., 1987a; Ljunggren *et al*., 1991a). Treatment of mouse calvarial bones with BK for 72-96 hours resulted in increased bone resorption, as assessed by the release of ^{45}Ca or by the mobilization of stable calcium and inorganic phosphate (Gustafson and Lerner, 1984; Lerner *et al*., 1987a). BK also increases the bone matrix degradation, as assessed by the release of ^{3}H from [^{3}H]proline-labelled bones (Lerner *et al*., 1987a). The minimal concentration for action of BK in mouse calvarial bones is 3nM, and half-maximal stimulation (EC_{50}) is achieved at 100 nM (Lerner *et al*., 1987a). The action of BK on bone resorption is delayed, with no effect seen until after 24 hours (Lerner *et al*., 1987a), and this might indicate that BK stimulates bone resorption due to enhanced osteoclast recruitment. .

BK and Lys-BK have been shown to stimulate bone resorption in mouse calvariae, indicating that there are B2 receptors present (Gustafson *et al*., 1986; Lerner *et al*., 1987a; Ljunggren and Lerner, 1988). This observation is further supported by the fact that the B1 receptor antagonist des-Arg^{9}-[Leu^{8}]-BK does not inhibit the bone resorptive effect caused by BK (Lerner *et al*., 1987a), an observation that opens the possibility that the effect of BK is not due to the conversion of BK by carboxypetidases to the B1 receptor agonist des-Arg^{9}-BK. The addition of des-Arg^{9}-BK to mouse calvarial bones results in increased release of ^{45}Ca (Lerner *et al*., 1987a; Ljunggren and Lerner, 1990), an effect that is inhibited by the B1 receptor antagonist des-Arg^{9}-[Leu^{8}]-BK (Ljunggren and Lerner, 1990), suggesting that bone cells also are equipped with B1 receptors.

Inflammatory induced bone loss may not only be due to increased bone resorption, but also decreased bone formation. Very little is known about the possible effects of BK regarding osteoblast cell proliferation, biosynthesis of bone matrix proteins, and the activity of alkaline phosphatase. In the human osteosarcoma cell line MG-63, BK does not stimulate cell proliferation or the biosynthesis of type I collagen or osteocalcin (Rosenqvist *et al*., 1996). In agreement with the findings in the MG-63 cells, BK has no effect on the proliferation of osteoblast-like cells isolated from human bone (Frost *et al*., 1999). However, there are observations showing that HMW kininogens might be involved in the growth-promoting activity of milk (Yamamura *et al*., 2000), and these observations suggest that kininogens may not only be important for BK formation, but also for bone growth.

Prostaglandins – Mediators of BK induced Bone Resorption

It is well known that bone resorption induced by BK is inhibited by indomethacin, a potent inhibitor of prostaglandin formation (Gustafson and Lerner, 1984). It has also been shown that a lot of inhibitors of the cyclooxygenase pathway, in the arachidonic acid cascade, including indomethacin, flurbiprofen, naproxen and meclofenamic acid, all abolish BK-induced mineral mobilization and bone matrix degradation (Lerner *et al*., 1987a). These nonsteroidal anti-inflammatory drugs (NSAID) also clearly inhibit the bone resorptive effects

of Lys-BK and Met-Lys-BK (Gustafson *et al*., 1986; Ljunggren and Lerner, 1988). Thus, these observations indicate that the bone resorptive effect caused by BK is totally dependent on its capacity to activate prostaglandin biosynthesis. Moreover, most stimulators of bone resorption *in vitro* also stimulate prostaglandin biosynthesis in bone tissue and bone cells, although the level of the prostaglandin response varies between different stimulators. However, there are stimulators of bone resorption, including PTH, vit D_3 and transforming growth factor-β (TGF-β) that are totally independent of prostaglandin formation, meaning that they neither stimulate prostaglandin production nor are inhibited by NSAIDs (Ljunggren and Lerner, 1989; Lerner, 1996). In addition, IL-1β, another stimulator of bone resorption, can stimulate bone resorption both dependent and independent of prostaglandin formation (Lerner *et al*., 1991), and then there are the kinins, which are unable to stimulate bone resorption in the absence of prostanoid production.

In primary cultures of mouse calvarial osteoblasts, BK causes a rapid burst of PGE_2 and 6-keto-$PGF_{1\alpha}$ (the stable breakdown product of PGI_2) that is maximal after only 5-10 minutes (Lerner *et al*., 1989). A similar effect has been found in the mouse calvarial osteoblastic cell line MC3T3-E1 (Lerner *et al*., 1989) and in isolated human osteoblast-like cells (Ljunggren *et al*., 1990; Rahman *et al*., 1992). By removal of the carboxyterminal arginine from BK, by CPN/CPM, des-Arg^9-BK is formed, and this peptide has also been found to stimulate bone resorption and prostaglandin formation, but the effect is very much delayed compared to the effect caused by BK (Lerner *et al*., 1987a; Ljunggren and Lerner, 1990).

Results achieved with mouse calvarial bones demonstrate the presence of both B1 and B2 receptors linked to bone resorption, by a process depending on the stimulation of prostaglandin formation. Differences in the kinetics for the prostanoid response indicate different molecular mechanisms of action in B1 and B2 receptor stimulation of bone resorption.

Biosynthesis of prostaglandins

Eicosanoids

Eicosanoids are a group of polyunsaturated hydrophobic molecules, with both autocrine and paracrine functions. They are not found preformed in the tissues but are generated *de novo* from phospholipids in response to a wide range of different stimuli. They are involved in the control of many physiological processes and are one of the most important mediators in an inflammatory reaction. The term ”eicosanoid” is originally from *eicosa*, meaning that there are 20 carbon atoms, and *enoic* means that it contains ’double bonds’. The main source of the eicosanoids is arachidonic acid (5,8,11,14-eicosatetraenoic acid), a 20-carbon unsaturated fatty acid, containing four double bonds. Arachidonic acid (AA) is esterfied, mainly in the sn-2 position of membrane phospholipids (Irvine, 1982). The amount of free AA under normal physiological conditions is very low. However, different stimuli can trigger the release of AA by phospholipases. The eicosanoids are usually referring to prostaglandins, thromboxanes, leukotrienes and also lipoxins. The term prostanoid is used to encompass both prostaglandins and thromboxanes.

Prostaglandins

Prostaglandins are potent biologically active lipid mediators derived from arachidonic acid in the plasma membrane. They were first extracted from semen, prostate and seminal

vesicles and proven to lower blood pressure and cause smooth muscle contraction (Goldblatt, 1933; von Euler, 1935). In 1936, von Euler named the substance ”prostaglandin”, deriving from prostate gland, since it was first believed to be part of the prostatic secretions (von Euler, 1936). Prostaglandin was first purified during the early 1960’s, and two research groups indepentently identified AA, a 20-carbon polyunsaturated fatty acid (C20:4,ω6) as the precursor to prostaglandins in 1964 (van Dorp *et al*., 1964; Bergström *et al*., 1964). Prostaglandins can be produced by a variety of different cell types, and act as local mediators. Different stimulatory agents such as cytokines, hormones, neurotransmitters, antigens, endotoxin or mechanical stress, trigger the release of AA by phospholipase A_2 enzymes. In activated cells, phospholipase A_2-derived AA is oxidized by different cyclooxygenases (COXs) to form PGG_2 and then subsequently PGH_2. PGH_2 is a common substrate for several downstream enzymes involved in the biosynthesis of different prostanoids, i.e. PGE_2, PGD_2, $PGF_{2\alpha}$, TXA_2 or PGI_2 (Fig 5). One of these downstream enzymes is the membrane-associated prostaglandin E synthase-1 (mPGES-1), which is an enzyme specifically catalyzing the conversion of PGH_2 to PGE_2 (Jakobsson *et al*., 1999; Murakami *et al*., 2000), and is expressed especially in cells activated by inflammation. After the prostanoids are biosynthesized, they are released from the cells and bind to specific receptors, to exert their biological effects. Prostaglandins are potent but have a short half-life before they will be inactivated and excreted. Therefore, they only exert a paracrine or autocrine function.

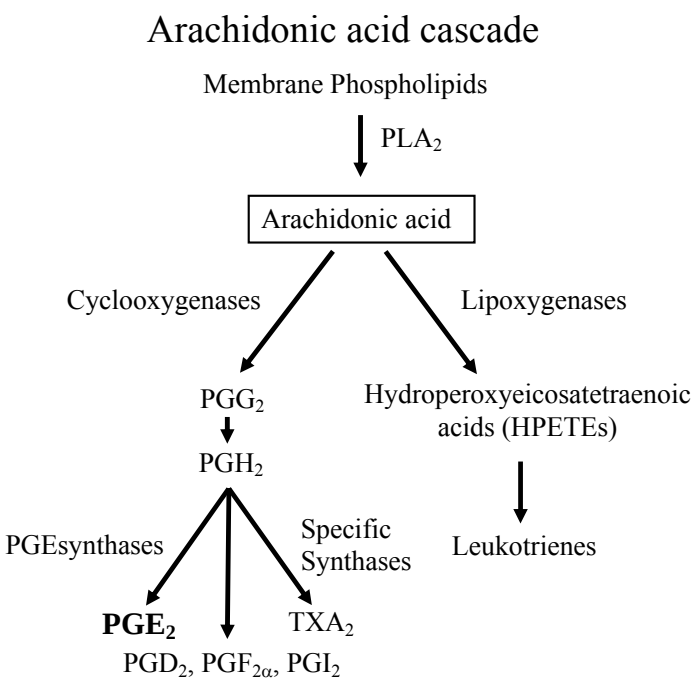

Figure 5. Schematic diagram over two of the major families of eicosanoids derived from arachidinic acid: the prostanoids and the leukotriens.

Phospholipase A_2 enzymes release arachidonic acid

Phospholipase A_2 enzymes (PLA_2) catalyze the hydrolysis of membrane phospholipids, releasing free fatty acids, and arachicidonic acid is the most common fatty acid in human cells. A number of stimulatory mediators trigger the release of AA by PLA_2 enzymes. The release of free AA is tightly regulated step in the biosynthesis of eicosanoids. The family of PLA_2 enzymes are subdivided into several classes: secretory PLA_2 ($sPLA_2$), cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), cytosolic Ca^{2+}-independent PLA_2 ($iPLA_2$) and platelet-activating factor (PAF) acetyl hydrolase (Kudo and Murakami, 2002). At least two classes of PLA_2 enzymes have important functions in prostanoid production – the group II $sPLA_2$ enzymes and $cPLA_2$ (Murakami *et al*., 1997).

$sPLA_2$ enzymes, with a molecular mass of 14–19 kDa, nonselectively release fatty acids from the phospholipids, and need a mM concentration of Ca^{2+} to be activated. $sPLA_2$ enzymes can be induced by proinflammatory stimuli (Oka and Arita, 1991). Two sPLA2 enzymes, type IIA and V, have been proven to be involved in prostaglandin production (Bingham and Austen, 1999; Sawada *et al*., 1999). Secretory PLA_2-IIA is the most common isozyme in human.

$cPLA_2$ is consitutively expressed in the cytoplasm of a large variety of cells. There are at least four different subgroups of $cPLA_2$, namely $cPLA_2\alpha$, $cPLA_2\beta$, $cPLA_2\gamma$ and $cPLA_2\delta$, and $cPLA_2\alpha$ has been shown to be highly selective for phospholipids containing AA in the sn-2 position (Leslie *et al*., 1988, Clark *et al*., 1991; Hirabayashi *et al*., 2004). $cPLA_2\alpha$ is activated at sub-μM concentrations of Ca^{2+}, and this leeds to translocation of $cPLA_2\alpha$ from the cytosol to the nuclear membrane, which is essential for the release of AA (Reynolds *et al*., 1993). Cells and tissues from $cPLA_2\alpha$–deficient mice fail to produce prostaglandins, leukotrienes or PAF (Uozumi *et al*., 1997; Bonventre *et al*., 1997).

Cyclooxygenases

Cyclooxygenase (COX), also known as prostaglandin endoperoxide H synthase or prostaglandin G/H synthase (PGHS), is membrane-bound, and catalyses the formation of cyclic endoperoxides, PGG_2, and subsequently PGH_2 by oxidation of arachidonic acid. There are two isoforms of cyclooxygenase, called COX-1 and COX-2, encoded by separate genes. COX-1 is expressed at relatively constant levels in most tissues and is therefore considered to be constitutively expressed, whereas COX-2 is generally expresssed at very low levels, but can be induced to much higher levels by different inflammatory mediators (Herschman, 1994). PGH_2 is then converted into different bioactive prostanoids like PGE_2, PGD_2, $PGF_{2\alpha}$, TXA_2 or PGI_2 by specific downstream enzymes in a cell-specific manner. Both COX-1 and COX-2 are situated similarly on the inner and outer membranes of the nuclear envelope and on the lumenal surface of the endoplasmic reticulum (Morita *el al*., 1995; Smith *et al*., 1996; Spencer *et al*., 1998), and share 60% identity in the amino acid sequence (Simmons *et al*., 2004).

COX-1

The genes for COX-1 and COX-2 from mouse or human origin were isolated by different laboratories (Fletcher *et al*., 1992; Kraemer *et al*., 1992; Appleby *et al*., 1994). These results show that the COX-1 gene contains 11 exons and is transcribed as 2.8 kb mRNA, which then is translated to form a protein with a molecular mass of ~68 kDa (Kraemer *et al*., 1992). The gene is located on the human chromosome 9q32-q33.3 and

is approximately 25 kb in size. In the promoter region of this gene, there are a number of transcriptional regulatory elements (SP1, AP-1 and NF-IL-6), but there is not so much known about the promoter elements that control the transcription (Kraemer *et al*., 1992; Smith and Dewitt, 1996). The expression of the COX-1 gene is often constitutive and ubiquitous, and COX-1 is preferentially utilized in the immediate PG-biosynthesis, which takes place within minutes after stimulation with Ca^{2+}-mobilizers.

COX-2

COX-2 was discovered independently by two research groups in 1991 (Xie *et al*., 1991; Kujubu *et al*., 1991). The COX-2 gene is approximately 8 kb in size, contains 10 exons and is situated on the human chromosome 1q25.2-25.3. The mRNA transcript is about 4.1-4.5 kb and this encodes the COX-protein with a molecular weight of about 72 kDa. The promoter region of the COX-2 gene has been more extensively characterized. The cAMP response element (CRE) seems to be important in COX-2 transcription (Xie *et al*., 1994), and activation of different MAP kinase pathways, results in the activation of transcription factors binding to the CRE element (Herschman *et al*., 1997; Mestre *et al*., 2001). Other researchers have found that CREB/NF-IL-6 elements or NF-κB sites, either together or alone, are responsible for maximal induction of transcription by cytokines and growth factors (Crofford *et al*., 1997; Herschman *et al*., 1997). Not only the activation of transcription seems to be important, but also the stability of the mRNA. It has been shown that pro-inflammatory agents have increased the stability of COX-2 mRNA and proteins (Ristimäki *et al*., 1996; Sheng *et al*., 2000; Cok and Morrison, 2001). NF-κB-dependent transcription of COX-2 has been shown to be inhibited by high concentrations of NSAIDs, that block IκB kinase activation (Yan and Polk, 1999). The induction of COX-2 is also downregulated by anti-inflammatory glucocorticoids. This downregulation of COX-2 induction seems to be dependent on AP-1 and NF-κB transcription (Auphan *et al*., 1995; Scheinman *et al*., 1995), as well as destabilization and degradation of COX-2 mRNA and protein (Dixon *et al*., 2000; Newton *et al*., 1998; Cok and Morrison, 2001). Finally, the regulation of transport of the COX-2 mRNA, from the nucleus to the rough endoplasmic reticulum (ER), may also have functional significance (Jang *et al*., 2003). The inducible COX-2 is absolutely essential for the delayed prostaglandin biosynthesis, which lasts for several hours following various stimuli.

COX-3

The exon/intron organization is very similar between the COX-1 and COX-2 genes, however, the COX-1 gene contains an extra intron (intron-1), that participates in the alternative splicing, yielding COX-3 and PCOX-1 (partial COX-1) proteins (Chandrasekharan *et al*., 2002). COX-3 consists of the COX-1 mRNA that retains intron-1. Intron-1 is a small intron, and in dogs it contains 90 nucleotides. The COX-3 variant generates a protein containing the encoded intron-1 sequence (also called COX-1b). The protein does not have as good effect on prostaglandin biosynthesis as COX-1, but analgesic/antipyretic drugs such as acetaminophen (Paracetamol) preferentially inhibit its activity. In human, COX-3 mRNA is transcribed as a 5.2 kb transcript and is abundantly expressed in cerebral cortex and heart.

Prostaglandin E synthases

Prostaglandin E synthases are the enzymes involved in the conversion of cyclooxygenase derived PGH_2 into PGE_2. Until now, three proteins have been identified in this conversion, namely membrane-associated PGES-1 (mPGES-1), mPGES-2 and cytosolic PGES (cPGES) (Jakobsson *et al*., 1999; Murakami *et al*., 2000; Tanikawa *et al*., 2002; Tanioka *et al*., 2000).

Membrane-associated PGE synthase-1

Membrane-associated PGE synthase-1 is a gluthathion (GSH)-requiring perinuclear protein belonging to the MAPEG (for Membrane-Associated Proteins involved in Eicosanoid and GSH metabolism) family (Jakobsson *et al*., 1999; Murakami *et al*., 2000; Mancini *et al*., 2001). The mPGES-1 gene is located on chromosome 9q34.3 (Forsberg *et al*., 2000), and is about 15 kb in size, devided into three exons. The promoter of the human mPGES-1 gene contains binding sites for C/EBPα and β, AP-1, progesterone receptors, GRE, CRE and serum response elements (SRE) (Naraba *et al*., 2002). mPGES-1 is markedly induced by pro-inflammatory agents, downregulated by anti-inflammatory glucocorticoids, and is functionally coupled with COX-2 in marked preference to COX-1 (Murakami *et al*., 2000; Kamei *et al*., 2004). Induction of mPGES-1 expression has also been observed in several systems in which COX-2 derived PGE_2 has been implicated to play an important role, such as inflammation, fever, pain, female reproduction, tissue repair and cancer (Kudo and Murakami, 2005). Co-localization of COX-2 and mPGES-1 in the same perinuclear membrane may contribute to their efficient functional coupling. Inducible expression of mPGES-1 is partially regulated by the MAP kinase pathways (Han *et al*., 2002). Very recently Degousee *et al*., demonstrated the role of JNK in mPGES-1 expression, in rat neonatal cardiomyocytes. There was no evidence about increased transcriptional activity, but the induction of mPGES-1 mRNA was found to be dependent on mRNA stability (Degousee *et al*., 2006).

Microsomal PGE synthase-2

The second membrane-bound form of PGES is mPGES-2 (Tanikawa *et al*., 2002). This enzyme is initially produced as a Golgi membrane-associated protein followed by proteolytic removal of the amino-terminal hydrodophic domain, leading to the formation of a mature cytosolic enzyme (Tanikawa *et al*., 2002; Murakami *et al*., 2003). The mPGES-2 gene is localized on human chromosome 9q33-34 in the vicinity to the genes for mPGES-1 and COX-1 (Tanikawa *et al*., 2002). Co-transfection of mPGES-2 with either COX-1 or COX-2 (in a human embryonic kidney cell line, HEK293), demonstrated that mPGES-2 is functionally coupled with both COX isoforms (Murakami *et al*., 2003). The expression of mPGES-2 seems to be constitutive in a variety of cells and tissues, and is not increased during inflammation or tissue damage. However, a marked elevation of mPGES-2 expression has been observed in human colorectal cancer (Murakami *et al*., 2003).

Cytosolic PGE synthase

Cytosolic PGE synthase (cPGES) is a glutathione-requiring enzyme that is constitutively expressed in a wide variety of cells. cPGES is a protein of 23 kDa, and identical to p23, a binding protein for heat shock protein 90 (Hsp90) (Tanioka *et al*,. 2000). Cotransfection and antisense experiments indicated that cPGES is functionally linked to COX-1, and not COX-2, to promote immediate PGE_2 biosynthesis, elicited by

Ca^{2+}-evoked stimuli (Tanioka *et al*., 2000). In another study by Tanioka *et al*, it was shown that Hsp90 significantly affects the activity of PGE_2-production of cPGES (Tanioka *et al*., 2003). Although cPGES expression is constitutive and not affected by pro-inflammatory stimuli in most cases, some exceptions have been reported. For example, Moore *et al*., have shown that administration of IL-1 into the mouse cortex, led to an elevation of PGE_2, which was accompanied by increased expression of cPGES, as well as that of COX-2 and mPGES-1, with differences in the kinetics (Moore *et al*., 2004).

Prostaglandin receptors

There are currently nine known receptors of prostaglandins on various cell types. The prostaglandin receptors are seven-transmembrane G-protein-coupled receptors that are designated by the letter ”P” and a prefix of ”D, E, F, I or T” to suggest preference for prostaglandin D, E, F, I or thromboxane, respectively. Up to date there are four different subtypes of EP receptors identified, called EP1-EP4. PGE_2 contracts gastrointestinal smooth muscle through stimulation of EP1 receptors. The EP2 receptors generally mediate arterial dilatation, but are also involved in the salt-sensitive hypertension (Kennedy *et al*., 1999). The pyretic action of PGE_2 is mediated by the EP3 receptor, since mutant mice lacking this receptor, do not develop fever after administration of either PGE_2, IL-1 or LPS (Uschikubi *et al*., 1998). The EP4, as well as EP2 receptors might be important for angiogenesis induced by PGE_2 (Harada *et al*., 1994; Ben-Av *et al*., 1995). Stimulation of the TP receptor on platelets leads to their aggregation, and TP-deficient mice have very prolonged bleeding times, demonstrating the importance of TXA_2 in hemostasis (Thomas *et al*., 1998). Normal production of PGI_2 is crucial for vessel tone control and inhibition of thrombosis. This is because the PGI_2/TXA_2 balance is critical. PGI_2 is mainly secreted by endothelial cells. It binds to the IP receptors on vascular smooth muscle cells and reduces vascular contraction (FitzGerald *et al*., 1983; Murata *et al*., 1997). Prostaglandin receptors in bone cells are presented below.

Prostaglandins and Bone Metabolism

Prostaglandins are involved in a wide variety of actions, including muscular constriction, inflammation, pain, fever, vascular homeostasis and thrombosis; they are “cytoprotective” in the gastrointestinal tract and have effects on bone tissue. They are potent but have a short half-life before being inactivated. Therefore, they exert mainly a paracrine (locally active) or autocrine function. As earlier mentioned, prostaglandins are metabolites from arachidonic acid, and PGE_2 is a very potent stimulator of bone resorption in organ cultures and osteoclastogenesis in bone marrow and spleen cell cultures (Pilbeam *et al*., 2002).

Prostaglandin receptors

There are a lot of complexity around the prostaglandins effects on bone tissues, and this can be partially explained by the fact that there are several transmembrane G-protein-coupled receptors that prostaglandins can bind to, and that may produce altered effects trough different signalling pathways. There are four different types of receptors for PGE_2. The EP1 receptor acts mainly by enhancing the calcium influx and phosphatidyl inositol turnover and has been suggested to be involved in mineralized nodule formation (Fujieda *et al*., 1999), as well as the stimulation of *c-fos* and *c-jun* expression (Suda *et al*., 2000). Additionally, EP1 has been

shown to be involved in the induction of IL-6 and COX-2 (Kozawa *et al*., 1998; Suda *et al*., 1998). EP2 and EP4 receptors activate adenylyl cyclase, and both receptors seem to be involved in the regulation of osteoblast function, and they have also been reported to be important for the effects of PGE_2 on osteoclastogenesis and bone resorption (Li *et al*., 2000b; Suzawa *et al*., 2000). At least two different mechanisms are involved in PGE_2-induced bone resorption. The first is due to activation of prostaglandin receptors in osteoblasts/stromal cells, resulting in an increased RANKL expression (Yasuda *et al*., 1998b). The other mechanism involves potentiation of RANK signaling, caused by activation of prostaglandin receptors on osteoclast progenitor cells (Ono *et al*., 2005). The EP3 receptor acts mainly by inhibiting cyclic AMP formation and has not been shown to play a specific role in bone. There is also evidence for the existence of receptors for PGI_2 on bone, and one study demonstrated IP-receptors in mouse osteoblastic MC3T3-E1 cells induced by TNF-α (Wang *et al*., 1999).

Regulation of prostaglandin production in osteoblasts

Prostanoids produced by bone cells include PGE_2, 6-keto-$PGF_{1\alpha}$ (the stable breakdown product of PGI_2), $PGF_{2\alpha}$ and some PGD_2 and thromboxane (Raisz *et al*., 1979; Feyen *et al*., 1984). There are a lot of factors that stimulate prostaglandin production in bone, including inflammatory mediators such as IL-1 and TNF-α (Sato *et al*., 1986; Tashjian *et al*., 1987); regulators of cell growth and differentiation, such as transforming growth factor (TGF)-α and β (Tashjian *et al*., 1985, Hurley *et al*., 1989; Sumitani *et al*., 1989), systemic calcium-regulating hormones, such as PTH and Vit D_3 (Ljunggren and Lerner, 1989; Pilbeam *et al*., 1989; Klein-Nulend *et al*., 1991a,b), as well as, mechanical loading of bone (Rawlinson *et al*., 1991, Cheng *et al*., 1997). Also bradykinin (Ljunggren *et al*., 1991b) and thrombin (Ljunggren *et al*., 1991c) can stimulate prostaglandin production in osteoblasts.

The most commonly used osteoblastic cell models constitutively express COX-1 and can be induced to express COX-2. Induction of COX-2 in osteoblastic cells seems to be required for most stimulated prostaglandin responses. Several different agonists are capable of inducing COX-2 in osteoblastic cells, including IL-1, TNF-α, IL-6, TGF-α, TGF-β, PTH, $VitD_3$ and mechanical loading (Harrison *et al*., 1994; Kawaguchi *et al*., 1994, 1996; Pilbeam *et al*., 1997b; Min *et al*., 1998; Okada *et al*., 2000a; Klein-Nulend *et al*., 1997). It has been shown that prostaglandins induce COX-2 expression and therefore can amplify prostaglandin responses from other agonists (Kawaguchi *et al*., 1994; Pilbeam *et al*., 1995). This mechanism of amplifying may be important in sustaining the production of prostaglandins in cell, as well as organ cultures.

Inhibition of prostaglandin production

Glucocorticosteroids have been reported to be potent inhibitors of prostaglandin formation in many studies. They can inhibit phospholipase A_2 production, as well as inhibit the inducible COX-2 mRNA and protein expression, in bone and other tissues (Kawaguchi *et al*., 1994). The cytokines IL-4 and IL-13 have also been shown to inhibit COX-2 expression and prostaglandin formation in bone cell and organ cultures (Kawaguchi *et al*., 1996; Onoe *et al*., 1996). Nonsteroidal antiinflammatory drugs (NSAIDs) reduce prostaglandin production by competing directly with arachidinic acid for binding to the COX catalytic site (Pilbeam *et al*., 1997a). Therefore, NSAIDs will not inhibit leukotriene production. Most available NSAIDs inhibit both COX-1 and COX-2 activity, but the development of the selective COX-2 inhihitors (coxibs; e.g. celecoxib (Celebra) *etc*.) have made it possible to avoid some of the negative gastrointestinal effects while still effectively reducing inflammation (Silverstein *et al*., 2000). COX-2 is the enzyme responsible for the prostaglandin enhancement of stimulated osteoclastogegensis. In bone marrow cultures from COX-2-deficient mice, osteoclast

formation stimulated by PTH or Vit D3 was reduced by 60-70% compared to the wild type mice cultures (Okada *et al*., 2000a). The PGE_2 formation was also markedly decreased, and the inhibited osteoclast formation was completely reversed by addition of PGE_2 to the cultures. Prostaglandins produced by COX-2 seems to be essential for *in vitro* osteoclastogenesis in response to several agents.

Prostaglandins in bone resorption

The effects of prostaglandins on bone resorption is predominantly caused by PGE_2 (Raisz and Martin, 1983), but PGI_2 and $PGF_{2\alpha}$ can also induce bone resorption, though to a lesser extent. The ability of $PGF_{2\alpha}$ to stimulate bone resorption is partially dependent on PGE_2-induction (Raisz *et al*., 1990). PGE_2, but not $PGF_{2\alpha}$, can stimulate osteoclast formation in bone marrow cultures (Collins and Chambers, 1991; Kaji *et al*., 1996). There are many different agonists reported to stimulate prostaglandin-dependent osteoclastogenesis in bone marrow cultures, including IL-1, TNF-α, PTH, Vit D_3, IL-11, IL-6 and IL-17. The formation of mature active bone-resorbing osteoclasts requires an interaction with cells from the osteoblastic linage (Suda *et al*., 1999). The molecules mediating this interaction is RANKL (Anderson *et al*., 1997; Wong *et al*., 1997; Yasoda *et al*., 1998b), binding to its cognate receptor RANK on the osteoclast precursor. OPG works as a decoy receptor for RANKL as mentioned earlier. Osteoblastic cells produce both RANKL and OPG, as well as M-CSF, also essential for osteoclastogenesis, whereas osteoclastic cells express RANK. The bone resorption caused by PGE_2 seems to be dependent on the induction of RANKL (Tsukii *et al*., 1998). Additionally, prostaglandins are not only able to act on osteoblastic cells to enhance the expression of RANKL, but they can also act directly on osteoclastic progenitor cells to induce formation of preosteoclasts. PGE_2 has been reported to increase the combined effects caused by RANKL and M-CSF, to induce osteoclastogenesis in spleen cultures (Wani *et al*., 1999). In contrast, when PGE_2 was added to isolated osteoclasts *in vitro*, it transiently inhibited bone resorption (Fuller and Chambers, 1989). Such transient inhibition of bone resorption, by prostaglandins, has also been observed in mouse calvarial bone cultures (Lerner *et al*., 1987b). There is not much known about the role of prostaglandins in bone resorption *in vivo*, but one important role might be the ability to generate new osteoclasts from osteoclastic precursors, since this becomes the factor limiting the rate of resorption. The prolonged bone resorption *in vivo*, caused by IL-1, was found to depend on prostaglandins (Boyce *et al*., 1989). Thus, prostaglandins increase the formation and differentiation of osteoclast precursors, as well as act on the osteoblasts to increase their endogenous prostaglandin production. This leads to an enhanced osteoclast formation. When fully differentiated mature osteoclasts are formed, prostaglandins are inhibitory. These effects seem to be cAMP-mediated (Lerner *et al*., 1987b).

Prostaglandins in bone formation

Studies *in vitro* have shown that prostaglandins seem to have both stimulatory and inhibitory effects on bone formation, although *in vivo* studies have more prominently shown a potent anabolic effect (Jee and Ma, 1997). PGE_2 has been shown to stimulate both cell replication and differentiation, in rat calvarial organ cultures (Woodiel *et al*., 1996). In addition, marrow stromal cells or primary calvarial cells from COX-2-deficient mice seem to have delayed osteoblastic differentiation compared to wt mice (Okada et al., 2000b). At high concentrations though, prostaglandins seem to inhibit collagen formation in cell and organ cultures (Fall *et al*., 1994). Prostaglandins can be produced by osteoblasts, osteocytes and adjacent hematopoietic cells. They seem to increase both the replication and the differentiation of the osteoblastic precursors, but inhibit collagen synthesis in mature osteoblasts as well.

Inflammation induced bone remodelling

In general, inflammation is the first response of the immune system to infection or other exogenous or endogenous stimuli and may be referred to as the acute/innate response. Inflammation is characterised by: redness (*rubor*), heat (*calor*), swelling (*tumor*), pain (*dolor*) and dysfunction of the organs involved (*functio laesa*). Inflammation consists of two main events including a cellular and an exudative event. The cellular component involves the movement of the leukocytes from blood vessels into the inflamed tissue. The leukocytes extravasate from the capillaries into tissue, and act as phagocytes. The exudative events lead to accumulation of fluid and leukocytes in extravascular tissues. Various leukocytes are involved in the initiation and maintenance of the inflammatory process.

Acute inflammation is predominantly mediated by neutrophils (polymorphonuclear leukocytes), and is modulated by molecules that induce vasodilatation (bradykinin and prostaglandins) increase vascular permeability (bradykinin, histamine and leukotriene) and possess chemotactic properties (complement products) to recruit additional inflammatory cells. Neutrophils only live for a couple of days, so if the inflammation persists for a longer time, they are gradually replaced by longer lived monocytes. The subsequent immune response is initiated when antigen-presenting cells become involved and present the foreign antigens or microorganisms to immunocompetent cells. This leads to the expansion of antibody-secreting plasma cells and the development of a chronic lesion. Chronic inflammation is mainly mediated by mononuclear cells such as monocytes and macrophages. These cells can be further stimulated to maintain inflammation through the action of an adaptive response involving lymphocytes (T-cells, B-cells), and antibodies. The adaptive response is more precise, reacting on specific antigens, but also takes a longer time.

During physiological conditions, the skeleton is continuously remodelled, by bone-forming osteoblasts and bone-resorbing osteoclasts. In pathological processes, the activities of the bone cells are un-coupled, leading to either increased bone resorption or increased bone formation. In some diseases, the amount of resorbed bone exceeds that of formed, leading to bone loss. This is often seen in patients with inflammatory processes in the vicinity of the bone tissue (e.g. periodontal disease, rheumatoid arthritis, osteomyelitis *etc*.). In some situations, however, more bone is produced than resorbed, leading to sclerosis of the bone. This can be seen in patients with apical periodontitis, but not normally in patients with marginal periodontitis. However, there are som evidence showing that increased isotope uptake have been observed also in patients with marginal periodontitis (Jeffcoat *et al*., 1991), indicating that increased bone formation also is a part of this process. The isotope used is incorporated into the skeleton during osteoblastic bone formation, and a high uptake indicating high rates of bone formation. This suggests that, accompanying the inflammation induced bone resorption, there is also increased bone formation. There are some evidence indicating that prostaglandins, as well as PTH (Hodsman *et al*., 2005), can stimulate not only bone resorption, but also bone formation (Hartke and Lundy, 2001; Pilbeam *et al*., 2002; Raisz and Woodiel, 2003; Vrotsos *et al*., 2003).

In several inflammatory diseases such as rheumatoid arthritis, periodontitis and osteomyelitis, increased osteoclast differentiation and activity can be stimulated by several bone resorbing pro-inflammatory cytokines, leading to resorption of bone tissue in areas adjacent to the inflammatory site. The group of bone-resorbing cytokines currently includes IL-1, IL-6, IL-11, IL-17, TNF-α, leukemia inhibitory factor (LIF) and oncostatin M (OSM)

(Martin *et al*., 1998; Horowitz and Lorenzo, 2002; Nakamura and Jimi, 2006) (Fig. 6). In an inflammatory process, there are also a number of factors leaking out from the blood vessels, due to the increased permeability, that are important for the regulation of bone metabolism. These factors are produced in the liver, and as long as they circulate in the bloodstream they are inactive, but as soon as they leak out in the inflammatory site, they are activated locally. Important such factors are the molecules generated from the kallikrein-kinin system and the coagulation cascade (i.e. kinins and thrombin). The pro-inflammatory peptide BK, as well as other kinin analogues has been reported to stimulate bone resorption (Lerner *et al*., 1987a; Ljunggren and Lerner, 1990; Worthy *et al*., 1990; Lerner, 1997; Couture *et al*., 2001; Moreau *et al*., 2005). Importantly, kinins, acting on both B1 and B2 receptors, not only stimulate bone resorption *per se*, but also synergistically potentiate the bone resorptive effect of IL-1 and TNF-α (Lerner, 1991; Lerner and Modéer, 1991; Lerner and Lundberg, 2002), by a prostaglandin dependent mechanism (Lerner *et al*., 1989). The effects of cytokines stimulating osteoclast formation and bone resorption seem to be prevented by other cytokines that inhibit the process of bone resorption (Fig. 6). Such cytokines are IL-4, IL-10, IL-12, IL-13, IL-18, interferon-β (IFN-β) and IFN-γ, and all are able to inhibit osteoclast formation and bone resorption (Horowitz and Lorenzo, 2002; Palmqvist *et al*., 2006). Thus, a balance between stimulatory and inhibitory mediators of inflammation, together with the regulation of their receptors, as well as signal-transducing mechanisms, will determine the amount and the activity of the osteoclasts formed, and thus the degree of bone resorption. The important role of osteoclasts in bone loss has been shown in several inflammatory induced diseases, and here follows an introduction to two of those diseases, namely rheumatoid arthritis and periodontal disease.

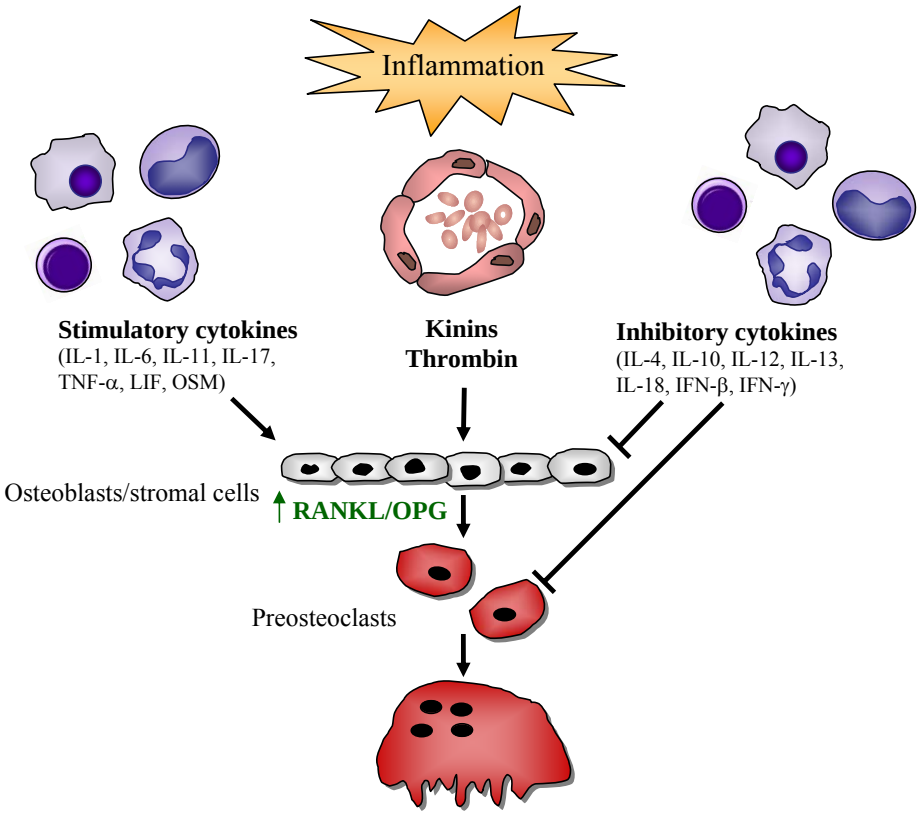

Figure 6. Different cytokines and inflammatory mediators have been shown to regulate osteoclast formation and bone resorption. The stimulatory mediators exert their effects by stimulating the RANKL/OPG-ratio in periosteal osteoblasts. The inhibitory cytokines are able to cause their effects either indirectly, by affecting the osteoblasts, or sometimes, directly, by affecting the osteoclast precursors.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, inflammatory, autoimmune disorder characterized by joint inflammation, synovial lining hyperplasia, pain, cartilage destruction and bone resorption. There are symptoms that distinguish rheumatoid arthritis from other forms of arthritis: 1) Inflammation and soft-tissue swelling of several joints at the same time. 2) Arthritis of hand joints. 3) The joints are usually affected asymmetrically in the beginning, and then in a symmetrical fashion as the disease progresses. 3) The pain of rheumatoid arthritis is usually worse in the morning (morning stiffness), but generally improves with use of the affected joints. 4) As the pathology progresses the inflammatory activity leads to destruction and erosion of the joint surface, which worsens the range of movement and leads to deformity.

During initiation of the disease, the synovial membrane becomes hyperplastic because of the accumulation of fibroblasts and cells of hematopoietic origin. This modification of the

synovial membrane is followed by the destruction of adjacent structures, such as articular cartilage and bone, due to the invasive properties of synovial tissue. This pathologic events leading to arthritis is governed by several proinflammatory cytokines that allow communication between inflammatory cells, leading to concerted actions such as the tissue invasion. IL-1, TNF-α and IL-6 are highly expressed in inflamed synovium and in synovial fluid from patients with RA, and have been implicated in the pathogenetic mechanism leading to bone resorption in this disease (Choy and Panayi, 2001; Udagawa *et al*., 2002; Dinarello, 2002; Firestein, 2003; Gravallese, 2003; Kay and Calabrese, 2004). These proinflammatory cytokines are of particular clinical importance, because their blockade improves the features of RA (Dinarello, 1994b; Feldmann and Maini, 1999; Dayer, 2002; Nishimoto *et al*., 2004; Cohen *et al*., 2004; St Clair, 2002; Zwerina *et al*., 2006a).

Kinins have significant stimulatory effects on TNF-α and IL-1 release from macrophages, and this release may be mediated by the activation of B1 receptors (Tiffany and Burch, 1989). TNFs and IL-1 can stimulate bone resorption, cartilage damage, hypertrophic synovitis, inflammatory cell migration, angiogenesis and pannus formation. TNF receptors have been localized in synovial tissue and cartilage-pannus junction in patients with RA, and this may play a major role in the genesis of the disease (Deleuran *et al*., 1992). In synovial fluid from patients with RA, increased kinin levels, increased kallikrein activity, as well as kininogens, has been demonstrated (Kellermeyer and Graham, 1968; Selwyn *et al*., 1989; Worthy *et al*, 1990; Sharma and Buchanan, 1994). These findings, together with the observations that kinins can stimulate bone resorption in neonatal mouse calvariae, indicate that also kinins could be responsible for osteoclastic activation in inflammatory conditions (Lerner *et al*., 1987a; Lerner and Lundberg, 2002). The kinin induced bone resorption is associated with an induced production of PGE_2 (Ljunggren and Lerner, 1990; Ljunggren *et al*., 1991b). Non-cytokine inflammatory mediators such as PGE_2, PGI_2 and leukotriens are released by kinin stimulation (Sharma & Mohsin, 1990; Sharma, 1991a,b). High levels of extracellular phospholipase A_2 have been detected from human synovial fluid (Hara *et al*., 1989), and this might be released from macrophages, granulocytes and lymphocytes. PLA_2 is activated by kinins to release the arachidonic acid metabolites, prostaglandins and leukotriens, in the process of RA (Sharma, 1991a,b). Kinins are capable of stimulating prostaglandin release in different cell types, including osteoblasts, fibroblasts and endothelial cells (Conklin *et al*., 1988; Lerner *et al*., 1989; Lerner and Modéer, 1991; Ljunggren and Lerner 1990; Ljunggren *et al*., 1990, 1991b).

The proinflammatory and destructive properties of TNF and IL-1 are mediated through activation of several intracellular signal transduction pathways. Among these molecules p38 MAPK is proven to be one of the most important signals for TNF-mediated inflammatory responses (Geng *et al*., 1996; Kotlyarov *et al*., 1999; Schett *et al*., 2000; Revesz *et al*., 2004). Interestingly, inhibition of p38 MAPK leads to inhibition of the arthritis caused by overexpression of TNF, and, in adddition, inflammatory bone loss depends on activation of p38 MAPK, since osteoclastogenesis in the inflamed synovium depends on intact activation of the p38 MAPK signalling pathway (Zwerina *et al*., 2006b).

Periodontal disease

Periodontal diseases are mainly initiated by bacterial infections inducing an inflammatory response, that in some situations are leading to destruction of the periodontal ligament, as well as the underlying alveolar bone tissue (Offenbacher, 1996). It is one of the most common infectious diseases among adults, and it can be influenced by a variety of modifying factors, including the microorganisms present in the subgingival lesion, systemic disorders that might have negative effects on the host response, environmental factors (e.g. plaque and smoking) or one's inherited or genetic susceptibilty to periodontitis.

Results from human and animal research have clearly shown that PGE_2 levels within periodontal tissues and within the gingival crevicular fluid (GCF) correlate with the clinical expression of periodontal disease (Offenbacher *et al*., 1993). This indicates that NSAIDs might be able to suppress inflammation and alveolar bone resorption in periodontitis, and there are several studies showing that different NSAIDs can inhibit the periodontal ligament destruction, as well as the rate of alveolar bone loss, both *in vitro* and *in vivo* (Nyman *et al*., 1979; Williams *et al*., 1984, 1988a,b; Offenbacher *et al*., 1987, 1992; Salvi *et al*., 1997). Also the effect of more selective COX-2 inhibitors, on alveolar bone loss, have been studied in rats (Bezerra *et al*., 2000; Holzhausen *et al*., 2002), showing a significant reduction in bone loss compared to controls (Salvi and Lang, 2005).

In addition, increased kinin levels have been found in inflammatory exudates collected from dogs with periodontal disease (Montgomery *et al*., 1986). These observations, together with findings that kinins can stimulate bone resorption in neonatal mouse calvariae, as well as synergistically potentiate the bone resorptive effect of IL-1 (Lerner, 1991), have raised the possibility that also kinins should be regarded as candidates responsible for osteoclastic activation in inflammatory conditions such as periodontal disease and rheumatoid arthritis (Lerner *et al*., 1987a; Lerner and Lundberg, 2002). The synergistic stimulation of IL-1 induced bone resorption caused by kinins is associated with a synergistic potentiation by kinins on IL-1 induced production of prostaglandin E_2 (PGE_2) and PGI_2 as well (Lerner, 1991; Lerner and Lundberg, 2002). IL-1, TNF-α and IL-6 have been found to be generously expressed in inflamed gingiva, and increased levels have been found in the crevicular fluid from patients with periodontitis (Okada and Murakami, 1998; Mogi *et al*., 1999; Boch *et al*., 2001; Graves and Cochran, 2003). Graves and Cochran have also shown that a lot of the damage that occurs during periodontal tissue destruction can be caused by IL-1 and TNF activity. This destruction might be a hyperreaction of the host response to the periodontal pathogens, caused by an extensive production of IL-1 and TNF (Graves and Cochran, 2003; Nakamura and Jimi, 2006). Thus, the same types of cytokines seem to be responsible for the stimulation of bone resorption in periodontitis, as well as in rheumatoid arthritis (Lerner, 2006). There are also observations indicating that upregulation of RANKL mRNA, in both epithelium and inflammatory cells, might be associated with the activation of osteoclastic bone resorption in periodontitis (Liu *et al*., 2003). In addition, it has recently been shown that transvascular dissemination of Porphyromonas gingivalis from a sequestered site is dependent on activation of the kallikrein-kinin pathway, indicating that activation of the kinin system is involved in the connection between human periodontitis and systemic diseases (Hu *et al*., 2006).

Intracellular signalling

Signal transduction is a process in which a cell converts one type of signal/stimulus into another. These processes often involve a sequence of reactions inside the cell, which are performed by enzymes and linked through second messengers. These are very fast processes (milliseconds-seconds). Signal transduction usually involves the binding of extracellular factors to receptors embedded in the outer cell membrane and trigger events inside the cell. This often happens via a change in the shape of the receptor that occurs when the signal molecule "docks" or binds. Receptors respond only to the specific ligand for which they have affinity, and molecules that are different in shape have no effect or maybe act as inhibitors. Often, the intracellular events triggered by the external stimulus are considered distinct from the event of "transduction" itself, because this term refers only to the step that converts the extracellular stimulus to an intracellular signal. There are a variety of intracellular signalling molecules including heterotrimeric G protein, cyclic AMP (cAMP), Ca^{2+}, phosphatidylinositol-triphosphate (PIP_3), diacylglycerol (DAG), inositol-triphosphate (IP_3), and several protein kinases and phosphatases. Some of these are also referred to as second messengers.

A transcription factor is a protein that acts as a regulator of gene expression, specifically regulating the activation or inhibition of transcription in the nucleus. They mainly exert their effects through binding to specific response elements, either through direct binding to DNA or through binding other DNA-bound proteins, in the promoter sequence of target genes and thereby regulate transcription. Here follows an introduction to the transcription factor pathways studied in this thesis.

Activating protein-1 (AP-1)

One of the first transcription factors identified was AP-1. After phorbol ester stimulation AP-1 was found to interact with specific DNA sequences in the gene promoters (Angel *et al*., 1987; Lee *et al*., 1987). Much of the current knowledge about the characteristics of transcription factors comes from the discovery and study of this transcription factor. AP-1 controls both basal and inducible transcription of several genes containing the AP-1 sites (Angel and Karin, 1991). AP-1 proteins are generally expressed and utilize their function in almost all cell types. It is a dimeric protein predominantly consisiting of members of the Jun (i.e. c-Jun, JunB and JunD) and Fos (i.e. c-Fos, FosB, Fra-1 and Fra-2) families of nuclear phosphoproteins. Jun proteins exist as homo- and heterodimers, whereas the Fos proteins, which cannot homodimerize, form stable heterodimers with Jun proteins and thereby enhance their DNA-binding activity (Halazonetis *et al*., 1988). Jun-Jun and Jun-Fos dimers bind to the AP-1 site. Each of these proteins is differentially expressed and regulated, meaning that every cell type has a complex mixture of AP-1 dimers with different functions (Wagner, 2001).

The activity and abundance of AP-1 is mainly regulated by kinases and extracellular stimuli including cytokines, growth factors, stress signals and infections, as well as oncogenic factors. Phosphorylation of AP-1 is achieved by JNK, as well as other kinases and this event increases the transcriptional activity and the stability of AP-1, because of the prevention of ubiquitination and subsequent proteasomal degradation. JNK is a member of the mitogen-activated protein kinase (MAPK) family (Davis, 2000). Activated by the MAPK cascade, JNK translocates to the nucleus, where Jun is phosphorylated and thereby enhancing the transactivation potential (Karin *et al*., 1997; Wagner, 2001). The extracellular stimuli lead to activation of a MAP kinase via a signaling cascade, and mainly regulate AP-1 through control of *de novo* formation of both Jun and Fos proteins (Hess *et al*., 2004) (Fig.7).

There are studies showing an important role for AP-1 components, primarily members of the Fos family, in osteoblasts (Grigoriadis *et al*., 1995; Wang *et al*., 1991; Eferl and Wagner, 2003; Eferl *et al*., 2004; Sunters *et al*., 2004). There are some evidence that suggest that members of the AP-1 family are involved in the regulation of osteoblast differentiation, including the observations that functional AP-1-binding sites have been found in the promoter region of several genes expressed in osteoblasts, such as alkaline phosphatase, α1(I) collagen and osteocalcin (McCabe *et al*., 1996). Various members of the AP-1 family have also been shown to be expressed in osteoblast cultures and can be detected at sites of active bone formation *in vivo* (Dony and Gruss, 1987). Members of the Fos family are also involved in osteoclastogenesis, since c-Fos-deficient mice lack osteoclasts and therefore develop severe osteopetrosis that is characterized by increased bone mass due to reduced bone resorption (Johnson *et al*., 1992; Wang *et al*., 1992). Fos expression in osteoclasts is induced by binding of RANKL to its receptor RANK (Wagner, 2002), resulting in the recruitment of TRAF-family proteins, which activate MAPK pathways that include JNK. There is also evidence that activation of JNK alters osteoclastogenesis through both Jun-dependent and Jun-independent mechanisms (David *et al*., 2002), showing that Jun proteins contribute partially to this process. JunB has also been shown to be important for efficient osteoclast differentiation, since mice missing JunB have decreased osteoclast formation (Kenner *et al*., 2004).

Nuclear factor κB (NF-κB)

In 1986, Sen and Baltimore detected a nuclear DNA binding protein that bound to a sequence prestent in the intronic enhancer element of the immunoglobulin κ light chain (Igκ) gene, in B-cells. This protein was called nuclear factor κB, NF-κB (Sen and Baltimore, 1986). Despite its initial discovery in B-cells, NF-κB is now known to be present in many other cell types, although in an inactive cytoplasmic form. Upon cellular stimulation, NF-κB can be induced to translocate into the nucleus where it binds to, as well as regulates several enhancers and promoters. NF-κB is a family of dimeric transcription factors that share a highly conserved amino-terminal domain called "Rel homology domain" (RHD), responsible for dimerisation, DNA binding, nuclear import and interactions with the family of inhibitory κB proteins (IκBs). In mammalian cells, NF-κB exists as homo- and heterodimers of the family members p50, p52, p65 (also called RelA), RelB and c-Rel (Ghosh *et al*., 1998).

In contrast to several other transcription factors, NF-κB is always present in the cytoplasm waiting for a signal, which leads to its activation. This makes a rapid activation possible, since *de novo* synthesis is not required. In unstimulated cells, the NF-κB transcription factors are kept inactive by members of another protein family, namely the inhibitor of NF-κB (IκB) family, consisting of IκBα, IκBβ, IκBε, IκBγ and Bcl-3. A common feature of the IκB family members is that they accommodate protein interaction motifs called ankyrin repeats, mediating the interaction with the RHD of NF-κB. The initial step in the NF-κB pathway is the activation of IκB kinase (IKK) that subsequently phosphorylates IκB on two amino-terminal serine residues (Karin and Delhase, 2000). IKK is composed of two catalytic subunits (IKKα and IKKβ), and a regulatory subunit (IKKγ; also called NEMO). IKKα and IKKβ are both capable of phosphorylating IκB *in vitro,* and IKKγ is thought to link the IKK complex to more upstream signalling molecules that regulate its activity. The phosphorylation of IκB enables the binding of ubiquitin, leading to rapid proteasomal degradation (Wei *et al*., 2001). Dissociation of IκB from NF-κB reveals the nuclear localisation sequence that promotes nuclear import of NF-κB. In the nucleus, NF-κB regulates the expression of genes containing the specific κB binding site (Miyamoto and

Verma, 1995) (Fig. 7). Since one of the NF-κB target genes is IκBα, the NF-κB activation results in new formation of its own inhibitor, and IκBα therefore promotes an effective negative feedback regulation of the activity of NF-κB. IκBα also appears to be involved in the nuclear export of NF-κB, since there are studies demonstrating the shuttling of IκBα between the cytoplasm and the nucleus, thereby mediating the export of the inactivated NF-κB dimer from the nucleus (Huang *et al*., 2000).

Activation of NF-κB has been suggested in the pathogenesis of inflammatory diseases, and patients with rheumatoid arthritis, asthma, periodontitis, atherosclerosis and inflammatory bowel disease have enhanced levels of NF-κB in the affected tissues. Proteins encoded by certain NF-κB target genes are probably of importance for these inflammatory responses (Yamamoto and Gaynor, 2001). The NF-κB pathway is very important for osteoclastogenesis, demonstrated by the observations that mice lacking both NF-κB subunits p50 and p52 are osteopetrotic, with bone marrow cavities filled with unremodelled osteocartilaginous matrix (Franzoso *et al*., 1997; Iotsova *et al*., 1997). The p50/p52 knockout mice lack both mature osteoclasts and TRAP-positive mononuclear progenitor cells. The osteopetrotic phenotype of *p50/p52-/-* mice can be prevented by bone marrow transplantation, but not by co-culture of wild-type osteoblasts with spleen cells from p50/p52-deficient mice, indicating that it is the lack of p50/p52 expression in osteoclast progenitor cells that is the cause of the defective osteoclastogenesis (Lerner, 2004).

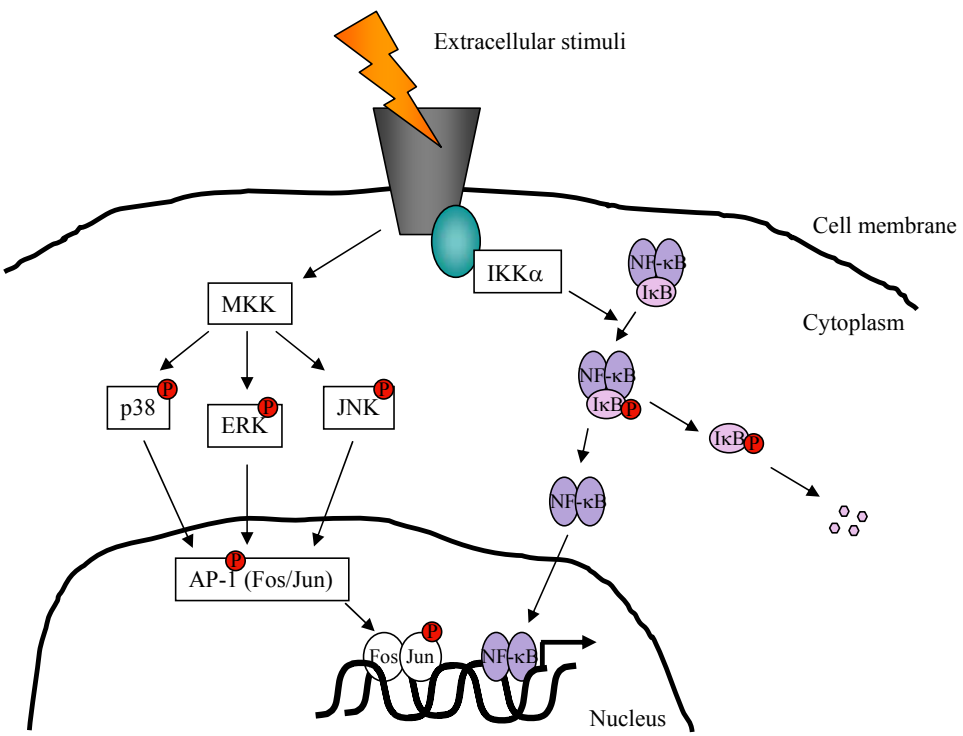

Figure 7. Schematic illustration of the signalling pathways for NF-κB and AP-1.

Signal Transducers and Activator of Transcription (STAT)

The STAT family of proteins regulates many events of cell differentiation, growth and survival (Darnell *et al*., 1994; Horvath, 2000). There are seven mammalian STAT family members identified today (i.e. STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6). The transcription factors of this family are activated by the Janus Kinase (JAK).This kinase was initially called JAK after "just another kinase", but later, it was said to be named after Janus, the Greek gatekeeper of heaven, described to have two faces. The kinase is named after him because of its two phosphate-transferring domains.

Cytokines are secreted proteins regulating several biological functions by binding to cell surface receptors, leading to an activation of signal transduction pathways. A number of cytokines activates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway in this event. Extracellular binding of cytokines leads to activation of the intracellular JAK that phosphorylates the specific tyrosine residue in the STAT protein. This event promotes the dimerization of STAT monomers via their Src homology 2 (SH2) domains, and the phosphorylated dimer is then translocated rapidly into the nucleus. Once inside the nucleus the active STAT dimer binds to the promoter region of genes containing a gamma activated site (GAS) motif and activates transcription of this protein. The STAT protein can be de-phosphorylated by nuclear phosphatases leading to inactivation of STAT and subsequently the transcription factor becomes transported out of the nucleus (Levy and Darnell, 2002; Silva, 2004). To ensure an appropriate and controlled cellular response, intracellular signalling is regulated by inhibitors. There are three families of proteins (i.e. the SH2-containing phosphatases (SHP), the protein inhibitors of activated STATs (PIAS), and the suppressors of cytokine signaling (SOCS)) that inhibit specific aspects of cytokine signal transduction, and these inhibitors control different steps in the intracellular signalling cascade (Sasaki *et al*., 1999; Wormald *et al*., 2004).

STAT6

STAT6 is a member of the STAT protein family. In 1988, Boothby *et al*., reported, for the first time, the presence of a DNA binding factor in B-cells that could be increased by IL-4 (Boothby *et al*., 1988). Classically, STAT6 is tightly connected to IL-4 and IL-13 signalling. There are three different receptors known to bind to and transduce signals of IL-4 and/or IL-13. Signalling is mediated predominatly through IL-4Rα and results in activation of the JAK/STAT and IRS-1/IRS-2 pathways. The activation of JAK1, leads to phosphorylation of tyrosine residues of IL-4Rα, which subsequently binds to the transcription factor STAT6. Due to activation/phosphorylation, STAT6 homodimerizes and translocates into the nucleus, where it binds to sequences found within promoters of the responsive genes (i.e. IL-4 and IL-13 regulated genes) (Kelly-Welch *et al*., 2003; Hebenstreit *et al*., 2006). STAT6 seems to be important for the regulation of gene expression, immunoglobulin E (IgE)-production from B-cells and the development of T-helper Type 2-cells (Nelms *et al*., 1999). STAT6 binding to DNA alone is usually not enough to stimulate the activation of a specific position of a particular gene. Initiation of transcription often requires the interaction of STAT6 with the basal transcription machinery and is depending on other transcriptional co-regulatory proteins. Two important factors proposed are CBP, the binding protein for cAMP response element binding protein (CREB) and p300. Both are generally expressed nuclear proteins that are highly homologous and share several functional properties. Due to this redundancy they are often addressed together as CBP/p300. CBP/p300 serves as a bridging factor to the basal transcription machinery (McDonald and Reich, 1999; Gingras *et al*., 1999).

Mitogen-activated protein kinase (MAPK)

Among the most widespread mechanisms of cellular regulation are the MAP kinase signal transduction pathways. MAP kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (i.e. hormones, mitogens, inflammatory cytokines, vasoactive peptides and envirionmental stress) and regulate several cellular activities, such as gene expression, differentiation, mitosis, cell survival and apoptosis (Pearson *et al*., 2001).

The extracellular stimuli lead to activation of a MAP kinase via a signaling cascade (the MAPK cascade), consisting of MAP kinase kinase kinase (MKKK or MAP3K), MAP kinase kinase (MKK or MAP2K) and MAP kinase. When a MAP3K is activated by extracellular stimuli, it phosphorylates a MAP2K on its serine/threonine residues, and subsequently this MAP2K activates a MAP kinase through phosphorylation on its serine and tyrosine residues (Seger and Krebs, 1995). This MAP kinase signaling cascade has been evolutionarily well-conserved. To date, there are four different subfamilies of MAPKs that have been characterized in mammals, i.e. extracellular signal-regulated protein kinases (ERKs), JNKs, p38 MAP kinases and ERK5.

Extracellular signal-regulated protein kinases (ERKs)

ERKs are also known as the classical MAP kinases. They are extensively expressed and involved in the regulation of mitosis and meiosis in differentiated cells. Many different extracellular stimuli, including cytokines, growth factors, virus infection and ligands for G protein-coupled receptors, activate the ERK signalling pathway, and this regulates cell differentiation and cell proliferation. Two similar (85% amino acid sequence identity) protein kinases were originally called ERK1 and ERK2 (Boulton and Cobb, 1991). Transgenic gene knockout mice that lack ERK2 have major defects in early development (Yao *et al*., 2003). In contrast, mice deficient in ERK1 are viable and it is believed that ERK2 can fulfill most of the ERK1 functions in a variety of cell types (Pages *et al*., 1999). Phosphorylation of ERKs leads to the activation of their kinase activity. The molecular events linking cell surface receptors to activation of ERKs are complex. It has been found that Ras GTP-binding proteins are involved in the activation of ERKs (Leevers and Marshall, 1992). Another protein kinase, Raf-1, has been shown to phosphorylate a "MAPK kinase", and therefore qualifying as a "MAPK kinase kinase" (Kyriakis *et al*., 1992). This MAPK kinase was called MAPK/ERK kinase (MEK) (Crews and Erikson, 1992). Phospho-ERK can translocate into the nucleus and activate the transcrition factor Elk-1, leading to c-Fos transcription (Gille *et al*., 1992; Marais *et al*., 1993).

C-Jun N-terminal kinases (JNKs)

The JNKs are also known as stress-activated protein kinases (SAPKs). They are mitogen-activated protein kinases mainly activated in response to stress stimuli, e.g. inflammatory cytokines, ultraviolet irradiation and heat shock, and are involved in cell differentiation and apoptosis. The JNKs consists of three different isoforms. JNK1 and JNK2 are generally spread and JNK3 is mainly found in neuronal tissue. Activation of JNK1 occurs by phosphorylation at Thr183/Tyr185 by MKK4. Activated JNK1 can translocate into the nucleus where it regulates transcription through its effects on AP-1 and other transcription factors. JNK1 is involved in inflammatory conditions and cytokine production.

The involvement of the MAPK pathway in osteoclast formation is indicated by the fact that JNK is not activated in *traf6-/-* mice (Lomaga *et al*., 1999), as well as the

obsevation that RANK overexpression leads to increased activation of JNK and NF-κB (Hsu *et al*., 1999). RANKL seems to preferentially activate JNK1, and lack of JNK1 leads to decreased (50%) osteoclast formation (David *et al*., 2002).

p38 mitogen-activated protein kinase (p38 MAPK)

p38 MAPK is a well known major signalling molecule of inflammation, and is activated by a range of cellular stress stimuli, including osmotic shock, LPS, pro-inflammatory cytokines, UV light and growth factors, and it is involved in cell differentiation and apoptosis. Importantly, p38 MAPK is also involved in the proinflammatory cytokine production by activating transcription factors that bind to the promoter regions of several proinflammatory cytokines, including TNF and IL-1 (Kumar *et al*., 2001). There are four different isoforms of p38 MAPK, and the α and β isoforms are mainly involved in the signalling of cytokines. MKK3 activate p38α by phosphorylation at Thr180/Tyr182. Activated p38α has been shown to phosporylate different transcription factors, including AP-1 and Max.

RANKL stimulates phosphorylation of JNK, ERK and p38 MAPK in an osteclast progenitor cell line (RAW 264.7 cells) that can differentiate to mature osteoclasts in the presence of RANKL (Matsumoto *et al*., 2000). Several pharmacological inhibitors have been used to show that osteoclast formation is associated with p38 MAPK, and there is strong evidence for the important role of this kinase, since the dominant negative form of p38 MAPK inhibited RANKL-induced osteoclastogenesis. RANKL induced stimulation of p38 MAPK seems to be involved in the differentiation of osteoclasts, but not for their function (Li *et al*., 2002). There are also observations indicating that p38 MAPK is a major signalling pathway involved in IL-1β and TNF-α induced RANKL expression in bone marrow stromal cells (Rossa *et al*., 2006).

ERK5

ERK5 is a MAP kinase that has been discovered rather recently and is activated both by growth factors and by stress stimuli and it is involved in cell proliferation and cell differentiation. ERK5 is suspected to mediate the effects of numerous oncogenes. Recently, the targeted deletions of the *erk5* and the *mek5* genes in mice have provided genetic evidence that the ERK5 cascade is a non-redundant signalling pathway essential for normal cardiovascular development (Wang and Tournier, 2006). ERK5 plays also a key role in neural differentiation. ERK5 nuclear translocation is controlled by its own nuclear localizing and nuclear export activities (Nishimoto and Nishida, 2006).

AIMS

The overall aim with this project was to increase the knowledge about the mechanisms involved in the interactions between inflammatory mediators involved in the pathogenesis of inflammatory bone resorbing diseases.

Paper I

The aim of the first study was to characterize the kinin receptors in a human osteoblastic osteosarcoma cell line (MG-63), using different kinins with specific agonistic and antagonistic properties for different kinin receptor subtypes.

Paper II

The aims of the second paper were to study the interactions between kinins and osteotropic cytokines on i) prostaglandin production, ii) expression of enzymes involved in the arachidonic acid cascade, iii) expression of RANKL, RANK and OPG and iv) finally to evaluate the role of NF-κB and MAPK, using the human osteoblastic cell line MG-63 and neonatal mouse calvarial bones.

Paper III

The aim of paper III was to study if the synergistic interactions between kinins and IL-1β or TNF-α respectively, could involve regulation of receptor expression and we therefore studied the effect of cytokines on kinin receptors and, vice versa, the effect of kinins on cytokine receptor expression. Another aim was to investigate the signal transduction pathways involved in these interactions. We used MG-63 cells, primary human gingival fibroblasts ae well as mouse calvarial bones in these studies.

Paper IV

The aim of this paper was to investigate how the inhibitory cytokines IL-4 and IL-13 may affect inflammation induced bone resorption by regulating the expressions of kinin receptors, as well as the enzymes involved in prostaglandin biosynthesis, in osteoblasts, human gingival fibroblasts and neonatal mouse calvarial bone.

METHODS

Animals (Paper II-IV)

CsA mice from our own inbred colony were used in several experiments. Mice homozygous for the STAT6 ^{tm1Gru} mutation in a Balb/c background (C.129S2-Stat6tm1Gru/J; stock no. 002828) and their corresponding wild type mice Balb/cJ (stock no. 000651) were purchased from JAX®MICE, The Jackson Laboratory and were bred in our animal facility unit. The *stat6*$^{-/-}$ mice were confirmed not to express STAT6 by reverse transcriptase polymerase chain reaction (RT-PCR) analyses. Animal care and experiments were approved and made in accordance with accepted standards of humane animal care and use, as considered appropriate by the Animal Care and Use Committee of Umeå University, Umeå, Sweden.

Bone cell culture (Paper I-IV)

MG-63 cells (obtained at passage 87 from American Type Culture Collection) are a human osteoblastic osteosarcoma cell line which expresses several osteoblastic phenotypes including biosynthesis of type I collagen and osteocalcin (Clover and Gowen, 1994). For experiments, cells were seeded at an initial density of 4-5 x 10^4 cells/cm^2 in either 2 cm^2 multiwell culture plates for prostaglandin analysis, or 9.5 cm^2 or 20 cm^2 culture dishes for gene expression analysis or 60 cm^2 for preparation of total cell lysates or nuclear extracts. They were cultured in α-MEM, supplemented with 10% FCS, L-glutamine and antibiotics (bensylpenicillin, gentamycin sulphate, streptomycin), at 37 °C in humidified air containing 5% CO_2. The cells were cultured for 1-2 days until 80-90% confluent monolayers were obtained. Then, the cells were washed 2 x in PBS and 1 x in serum free α-MEM, and subsequently incubated in α-MEM/1% FCS, with or without test substances for different periods of time.

Culture of calvarial bones (Paper II-IV)

Calvarial bones from 5-7 days old mice (CsA, *stat6*$^{-/-}$ and Balb/cJ) were dissected and devided into two halves along the sagittal suture. The bones were preincubated for 18-24 hours in α-MEM containing 0.1% BSA and 1 μM indomethacin, to prevent initial prostaglandin induced bone resorption, caused by handling trauma. Following preincubation, the bones were extensively washed and subsequently incubated for 24 h, in 24-wells culture plates, containing 1.0 ml indomethacin-free α-MEM, supplemented with 0.1% BSA, 1 μg/ml $Fe(NO_3)_3$, 0.1 mg/ml ascorbic acid, L-glutamine and antibiotics, with or without test-substances, at 37 °C in humidified air containing 5% CO_2 (Lerner, 1987). The bones were homogenized and RNA was extracted from individual bones (5-6/group) and used for gene expression analyses. CsA mice were from our own inbred colony.

Isolation of human gingival fibroblasts (Paper III-IV)

Human gingival fibroblasts were isolated from explants of human papillar gingiva obtained by surgery from clinically healthy gingiva as previously described (Lerner and Hänström, 1987). The explants were dissected into small pieces (0.5 cm^2) and placed in

culture flasks containing α-MEM supplemented with 10% foetal calf serum (FCS), L-glutamine and antibiotics (bensylpenicillin, gentamycin sulphate, streptomycin), followed by incubation at 37 °C in humidified air containing 5% CO_2. Cells from passages 4-7 were used in the experiments. The cells growing out from the explants were subcultured in α-MEM/10% FCS and then seeded at a density of 80% in 6-wells plates (9.5 cm^2), and then cultured with or without different test substances. After 24 h of incubation, RNA was extracted for subsequent analysis of gene expression. The study was approved by the Human Studies Ethical Committee of Umeå University and informed consent was obtained by all patients.

Measurements of prostaglandin release (Paper I-IV)

Prostaglandin biosynthesis in MG-63 cells and calvarial bones (paper II) was assessed by analyzing the amounts of PGE_2 and 6-keto-$PGF_{1\alpha}$ (the stable breakdown product of PGI_2; paper I) in the media at the end of the cultures. MG-63 cells were cultured to 70-90% confluent monolayers in 2 cm^2 multiwell dishes. In short-term experiments (< 30 min), the cells were extensively washed and subsequently incubated in serum-free MEM/HEPES medium with or without different test substances. These incubations were performed in air at 37 °C in a water bath. In long-term experiments (1-48 h), the cells were incubated in α-MEM/1% FCS in the absence or presence of test substances, and the incubations were performed at 37 °C in a humidified incubator gassed with 5% CO_2 in air. At the end of the culture period, the media were withdrawn, acidified, frozen and stored at -20°C. PGE_2 and 6-keto-$PGF_{1\alpha}$ were determined using commercially available radioimmuno-assay kits, by following the instructions of the manufacturer.

Radioligand binding (Paper I and III)

MG-63 cells were cultured to 80% subconfluent monolayers in 2 cm^2 multiwell plates. The cells were washed once in PBS/0.1% BSA and once in MEM/HEPES/0.1% BSA.
In paper I, the cells were then incubated in 250 µl MEM/HEPES/0.1% BSA containing 10 µmol/L phosphoramidone, 4 nM [^{3}H]-BK (~ 120 000 DPM/well) or 7 nM [^{3}H]-DALBK (~ 240 000 DPM/well), with or without different kinin receptor agonists or antagonists at different concentrations. Cells were incubated at 4°C for 5-300 min. In displacement experiments, the cells were incubated for 150 min.
In paper III, the cells were preincubated in MEM/HEPES/0.1% BSA with different cytokines (for different periods of time or different concentrations), kinins or in plain control medium. Subsequently, the cells were incubated in 250 µl MEM/HEPES/0.1% BSA containing 10 µmol/L phosphoramidone, 4 nM [^{3}H]-BK (~ 120 000 DPM/well) or 7 nM [^{3}H]-DALBK (~ 240 000 DPM/well), with or without different kinin receptor agonists. Cells were incubated for 150 min, at 4°C. At the end of the incubation, medium was aspirated and cells were washed 5 times with PBS/0.1% BSA. Finally, 500 µl of Ca^{2+}- and Mg^{2+}- free phosphate buffer (137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4 x H_2O ; pH=7.2) containing EDTA (187 mg/l) and trypsin (100 mg/l) was added for 10 min and then the radioactivity in the suspension was analyzed using a liquid scintillation counter.

Protein analysis by ELISA (Paper II)

The protein synthesis of RANKL was assessed by enzyme-linked immunosorbent assay (ELISA), measuring either protein release from cells to conditioned media or cellular protein content in calvarial bone lysates, using a commercially available ELISA kit (Swanson *et al*., 2006). Calvarial bones were dissected from 5-7 days old mice (CsA) and divided into two halves along the sagittal suture. Following preincubation, a total of 6-8 calvarial bone halves per group were individually incubated in 24 well plates in the absence (control) or presence of test substances for 24 h. The amounts of released RANKL protein were measured in conditioned media collected following incubation. For measurement of RANKL protein content in the calvarial bones, the bones were treated with 0.2% Triton-X 100 for 24 hours in room temperature. The bone lysates and the conditioned media were analyzed by ELISA according to the manufacturer's protocol. The sensitivitie of the immunoassay is 5 pg/ml. Vit D_3 was used as a positive control and resulted in an increased protein level of RANKL in both culture media and bone lysates, as expected.

RNA isolation and first-strand cDNA synthesis (Paper I-IV)

After incubation with or without test substances, the MG-63 cells or gingival fibroblasts were washed 2 x in PBS and total RNA was isolated using Trizol LS Reagent or RNAqueous^{TM}-4PCR kit, by following the manufacturer's protocol. The calvarial bones were homogenized (Ultra-Turrax$^{®}$, Jenke & Kunkel KG, Staufen, Germany) before RNA extraction using RNAqueous^{TM}-4PCR kit, by following the manufacturer's recommendations. Extracted RNA was quantified spectrophotometrically and its integrity was analyzed by agarose gel electrophoresis. The RNA isolated was DNase-treated with a commercially available DNA-free^{TM}-kit. Only RNA preparations showing intact species were used for subsequent analysis. 0.25-1.0 μg of total RNA was reverse transcribed into single-stranded cDNA with a 1st strand cDNA Synthesis Kit, using random p(dN)$_6$ or oligo-p(dT)$_{15}$ primers. After incubation at 25°C for 10 min and at 42°C for 60 min, the AMW reverse transcriptase was was finally denatured at 99°C for 5 min, followed by cooling to 4°C for 5 min. The cDNA was kept at -20°C until used for polymerase chain reaction (PCR).

Semi-quantitative reverse transcription polymerase chain reaction (Paper I-IV)

Total RNA (1 μg) from MG-63 cells was reverse transcribed into single stranded cDNA with a 1st Strand cDNA synthesis kit using random p(dN)$_6$ primers or oligo-p(dT)$_{15}$ primers. The cDNA was amplified in polymerase chain reactions (PCR) utilizing a PCR Core Kit and PC-960 G Gradient Thermal Cycler (Corbett Research, Australia). The PCR reaction conditions were performed in a reaction volume of 100 μl containing 2-4 μl template, 0.4 μM of each primer, 2.5 U HotStar Taq DNA polymerase, 1 x PCR buffer, 0.2 μM dNTPs and 1.5 mM $MgCl_2$. The reaction conditions included a hot start at 95°C for 15 min, denaturing at 94°C for 40 s, annealing at individual temperatures for 40 s, followed by extension at 72°C for 40 s. After the last cycle, the samples were incubated at 72°C for 6 min, followed by cooling to 4°C. The annealing temperature was optimized for the individual primer pairs. For normalization, glycerylaldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. For all genes, no amplification was detected in samples where the RT-reaction had been excluded. The PCR products were fractionated by 1.5% agarose gel electrophoresis and visualized using ethidium bromide staining. The identity of the PCR

products was confirmed using QIAquick purification kit and a Thermo Sequenase™ II DYEnamic ET® terminator cycle sequencing premix kit with sequences analysed on an ABI 377 XL DNA Sequencer.

Quantitative real-time polymerase chain reaction (Paper II-IV)

0.25-1.0 μg of total RNA, following DNase treatment, was reverse transcribed into single-stranded cDNA with a 1st Strand cDNA Synthesis Kit using random $p(dN)_6$ primers. The obtained cDNA were diluted 5-20 times, with nuclease-free water and analysed using a TaqMan Universal PCR Master Mix kit. PCR reactions were carried out in 384-well plates with 5, 10 or 20 μl volume per well and each sample analysed in duplicate or triplicate. The concentrations of primers and probes were individually optimized for each gene. Quantitative real-time PCR (q-RT-PCR) analyses were performed using the TaqMan kinetics with fluorescence labelled probes (reporter fluorescent dye VIC or FAM at the 5' end and quencher fluorescent dye TAMRA at the 3' end). The amplifications were performed on an ABI PRISM 7900 HT Sequence Detection System and software (Applied Biosystems, Foster City, CA, USA). The reaction conditions included an initial step for 2 min at 50°C and 10 min at 95°C , followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. No amplification was detected in samples where the RT-reaction had been excluded. To control for variability in amplification due to differences in starting mRNA concentrations, 60S ribosomal protein L13A (RPL13A; human) and β-actin (mouse) were used as internal standards. The relative expressions of target genes were calculated as differences in threshold cycle (Ct) value for target genes compared to internal standards, using the standard curve method. The Ct-values for RPL13A and β-actin did not vary with time or treatment. Most of the oligonucleotide primers and TaqMan probes were designed using Primer Express™ 2.0 (Applied Biosystems), based on the sequences from the Genbank database (http://www.ncbi.nlm.nih.gov). Pre-made TaqMan® Gene Expression Assays for mouse B1- and B2 receptors were ordered from Applied Biosystems.

Preparation of total cell lysates (Paper II and III)

MG-63 cells were cultured to 80-90% confluent monolayers in 60 cm^2 dishes, washed 2 x in PBS and 1 x in serum-free α-MEM, and then incubated in α-MEM (without serum), with or without test substances for different periods of time. After the incubation, the cells were washed twice in PBS before adding the lysis buffer (1% igepal CA-630, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 0.1 mg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, in PBS). The dishes were kept on ice for 15 min followed by scraping and collection of cell lysates. Before using the cell lysates in Western blot, they were concentrated using microcon centrifugal filter devices according to manufacturer's recommendations. Protein concentrations of the cell lysates were measured using the BCA technique with bovine albumin as standard.

Western blot (Paper II and III)

Concentrated cell lysates were mixed with sample buffer (200 mM Tris-HCl, pH 6.7, 20% glycerol, 10% β-mercaptoethanol, 5% SDS, 0.01% pyronin Y) and boiled for 3 min. Protein samples were then loaded on Tris-HCl polyacrylamide gels and electrophoresis was

performed according to the Laemmli method (SDS-PAGE). Electrophoresed proteins were then blotted onto a PVDF-membrane, which was blocked (1% milk, 1% BSA in Tris buffered saline (TBS); 5% milk, 0.1% Tween-20 in TBS(TTBS) or 3% gelatine in TBS) overnight or 2 hours at room temperature. For detection, the membrane was incubated with primary antibody (diluted in 1% milk, 1% BSA, 0.05% TTBS; 2% milk, 0.05% TTBS or 1% gelatine, 0.05% TTBS) for 60 min at room temperature or overnight at 4°C respectively, depending on the blocking time. After incubation the membrane was washed three times, for 10 min, in TBS with 0.05% TTBS, followed by incubation with HRP-conjugated secondary antibody (diluted in 1% milk, 1% BSA, 0.05%TTBS; 2% milk, 0.05%TTBS or in 1% gelatine, 0.05% TTBS) for 60 min at room temperature. Finally, the membrane was washed extensively with TTBS and TBS followed by development using a chemiluminence detection kit according to the manufacturer's protocol.

Preparation of Nuclear Extracts (Paper III)

MG-63 cells were plated at a density of 3-4 x 10^4 cells/cm^2 in culture dishes (60 cm^2) containing α-MEM with 10% FBS, L-glutamine and antibiotics. After 1-2 days, when 80-90% confluent monolayers were obtained, the cells were incubated in the without (control) or with test substances for 5-30 min. After the incubation, the cells were washed with ice cold PBS and scraped. Cell suspensions were centrifuged briefly and pelleted cells homogenized in lysis buffer A (10 mM HEPES, pH 7.9, 0.1 mM EDTA, 10 mM KCl, 625 μg/ml spermidine, 625 μg/ml spermine, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). After 15 min on ice, Igepal CA-630 was added to a final concentration of 0.5%, and the nuclei were collected by centrifugation at 12000 x g for 2 min. Pelleted nuclei were lysed by incubation for 30 min on ice in lysis buffer B (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.42 M NaCl, 25% glycerol, 625 μg/ml spermidine, 625 μg/ml spermine, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). Supernatants were collected by centrifugation at 16000 x g for 10 min. The protein concentration of the samples was determined by the Bradford method and aliquots were stored at -80°C until use in electrophoretic mobility shift assays (EMSAs).

Electrophoretic Mobility Shift Assay (EMSA; Paper III)

Oligonucleotide probes including a κB site (CACTTTTGCGGCAATCCCCACAAT) and an AP-1 site (TCCCGAAAGACTCACTTTTG) of the human B1 promoter (Ni *et al*., 1998) were end-labelled with [γ-^{32}P]ATP using T4 kinase according to manufacturer's instructions. Mutated forms of the NF-κB (CACTTTTGCCTCAATCCCCACAAT) and AP-1 (TCCCGAAAGAGCTCCTTTTG) oligonucleotides were used in competition studies. Annealing of complementary strands of both labelled and unlabelled oligonucleotides was performed before used in EMSA. Reaction mixtures containing 8 μg of nuclear extract, 0.5-1.0 ng of probe (50 000 cpm), 6 μg poly(dI-dC)•poly(dI-dC), 20 nM DTT, and reaction buffer (50 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 5 mM EDTA, 25% glycerol) were incubated at room temperature for 30 min. In antibody supershifts and competition studies, 2 mg/ml of antibody or 50- or 100-fold excess of unlabelled probe was preincubated with reaction mixture without probe for 30 min before addition of ^{32}P-labelled probe. After incubation for 30 min at room temperature, the samples were loaded onto a non-denaturing polyacrylamide gel and subsequently electrophoresed, followed by drying of the gel and autoradiography, a technique using X- ray film to visualize molecules that have been radioactively labeled.

Phosphorylation of proteins analyzed by Cellular Activation of Signalling ELISA (CASE) kits (Paper III)

The effects of IL-1β on the protein phosphorylations of p38 and JNK MAP kinases were analyzed using Cellular Activation of Signalling ELISA, CASE™, kits by following the manufacturer´s protocol. In short, MG-63 cells were seeded (2 x 10^4 cells/well) in α-MEM/10% FCS in 96-well plates, attached overnight and then serumstarved for 24 h. Subsequently, cells were incubated with or without IL-1β (100 pg/ml) in HEPES-buffered MEM for 5 or 15 min at room temperature. Cells were then fixed and separated wells were incubated with primary antibodies recognizing either JNK phosphorylated at threonine 183 and tyrosine 185, p38 phosphorylated at threonine 180 and tyrosine 182, total JNK or total p38, followed by HRP-conjugated secondary antibodies and then colorometric absorbance (450 nm and 595 nm) was quantified using a plate reader. The antibody readings were normalized to the relative cell number by deviding the OD_{450} readings for each well by its OD_{595} reading. To determine the relative extent of target protein phosphorylation, the phospho-protein specific antibody OD_{450}:OD_{595} ratio was normalized to the pan-protein specific antibody OD_{450}:OD_{595} ratio, for the same experimental conditions.

RESULTS AND DISCUSSION

Characterization of bradykinin B1 and B2 receptors in osteoblasts (Paper I)

It has been shown that osteoclastic bone resorption can be stimulated by bradykinin (BK), a B2 receptor agonist, in mouse calvariae (Lerner *et al*., 1987a), and this effect is clearly reduced by inhibitors of prostaglandin formation. In this paper, we report that the human osteoblastic cell line, MG-63, responds to BK, as well as other BK analogues, with a burst of PGE_2 and 6-keto-$PGF_{1\alpha}$-release within minutes. This effect requires the whole nonapeptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), since neither des-Arg^1-BK nor des-Arg^9-BK or des-Arg^{10}-Lys-BK (DALBK) can cause this burst of prostaglandin release. These observations demonstrate that the cleaving of either the amino- or the carboxyterminal arginine from BK leads to inactivation of its capacity to cause a burst of prostaglandin release.

We tested a wide variety of natural kinins, their breakdown products and different kinin analogues as stimulators of rapid (10 min) prostaglandin release, and found the following rank order of response: Lys-BK > BK ≥ Met-Lys-BK >Ile-Ser-BK>[Tyr^8]-BK ≥ [Hyp^3]-BK >>>>> des-Arg^9-BK = DALBK = des-Arg^1-BK = [$Thi^{5,8}$, D-Phe^7]-BK = Sar-[D-Phe^8]-des-Arg^9-BK = Tyr-Gly-Lys-Aca-Lys-des-Arg^9-BK, indicating that B2 receptors are responible for this effect (Marceau *et al*., 1998; Regoli *et al*., 1998). This is further supported by the observations that Hoe 140 (a B2 receptor antagonist), dose-dependently inhibited the stimulatory effect of BK, whereas des-Arg^9-[Leu^8]-BK, des-Arg^{10}-[Leu^9]-Lys-BK or des-Arg^{10}-Hoe 140 (all B1 receptor antagonists) were without effects. Additionally, radioligand binding showed specific binding sites for [^{3}H]-BK, which were inhibited by B2 receptor agonists/antagonists but not by B1 receptor agonists/antagonists. These observations, together with the findings that MG-63 cells express mRNA for the human B2 receptor indicates that these human osteoblastic cells are equipped with functional B2 receptors.

There is also evidence showing that not only B2 receptor agonists, but also B1 receptor agonists (i.e. des-Arg^9-BK and DALBK) are able to induce a bone resorptive response in mouse calvarial bones, cultured for 72 hours. This effect is dependent on prostaglandin biosynthesis since the effect is abolished by COX-inhibitors. Here, the enhancement of PGE_2 release is much more delayed (Ljunggren and Lerner, 1990). Similar to these findings, we demonstrate, in this paper, that des-Arg^9-BK and DALBK cause a delayed PGE_2 and $PGF_{1\alpha}$ response, in the MG-63 cells, with stimulatory effects seen at and after 4 hours. This late prostaglandin response was also observed with other B1 receptor agonists, and the rank order of response, in long term experiments (24 h), for the B1 receptor agonists was: Tyr-Gly-Lys-Aca-Lys-des-Arg^9-BK >> DALBK > Sar-[D-Phe^8]-des-Arg^9-BK > des-Arg^9-BK. BK also caused a PGE_2 response in the 24-hours incubations, but this effect was unaffected by the three different B1 receptor antagonists, but markedly reduced by Hoe 140, suggesting that this enhancement still was caused by the rapid initial B2 receptor mediated burst of PGE_2. In contrast, the delayed response to the B1 receptor agonist DALBK was inhibited by all three B1 receptor antagonists used. These obseravtions indicate that the delayed prostaglandin response is mediated by B1 receptors.

Using radioligand binding and RT-PCR, we could further demonstrate the presence of B1 receptors on MG-63 cells. When we used the B1 receptor agonist DALBK as a ligand, we could observe specific binding sites on these cells that were inhibited by several B1 receptor agonists/antagonists. Using RT-PCR, the B1 receptor mRNA was also shown to be

constitutively expressed in the MG-63 cells. The common view that a B1 receptor agonist-induced response is due to "culture-induced" B1 receptor expression in different *in vitro* systems, is not the situation in the MG-63 cells, since both specific binding and mRNA expression for the B1 receptors were seen in cells without any preculture. It has been observed that B1 receptors not only can be upregulated by cytokines and endotoxin (Campos *et al*., 1999; Phagoo *et al*., 2000; McLean *et al*., 1999) but also by B1 receptor agonists themselves (Phagoo *et al*., 1999), although this seems not to be the case in MG-63 cells since the B1 receptor agonist DALBK does neither increase the mRNA for the B1 receptor, nor the binding of [^{3}H]-DALBK. In contrast, pretreatment with IL-1β or TNF-α markedly enhanced the binding of [^{3}H]-DALBK (paper III).

Thus, human osteoblasts seem to constitutively express B1 receptors linked to stimulation of prostaglandin release, by a mechanism that are temporarily different from the mechanism involved in B2 receptor-induced prostaglandin release. The difference in time-course of action between B1 and B2 receptors has also been observed in a rat paw model of hyperalgesia (Poole *et al*., 1999), in the endotoxemic heart (McLean *et al*., 1999), in rat renal mesenteric arteries (Bagaté *et al*., 1999), in relaxation of rabbit mesenteric arteries (Churchill and Ward, 1986) and dog renal arteries (Rhaleb *et al*., 1989). The delayed response to B1 receptor agonists in several *in vivo* and *in vitro* models may not be due to upregulation of B1 receptor expression but instead to a delayed release of prostaglandins, in contrast to the rapid release caused by B2 receptor agonists. This view is in line with our more recent observations in MG-63 cells, showing that B1 receptors agonists, also in cells that have been pretreated with IL-1β, to upregulate B1 receptors, cause a delayed PGE_2 release (paper III).

In conclusion, our data indicates that human osteoblastic cells are equipped with functional B1 and B2 receptors coupled to prostaglandin release and that the stimulation of prostaglandin release is probably mediated by different molecular mechanisms. Due to our previous findings that both B1 and B2 receptors can stimulate bone resorption in neonatal mouse calvariae and here demonstrate that both receptors are expressed on human osteoblasts, there are a possibility that inflammation-induced bone resorption in diseases such as periodontitis and rheumatoid arthritits may be induced not only by BK and Lys-BK but also by their breakdown pruducts des-Arg^9-BK and DALBK.

Interactions between kinins and pro-inflammatory cytokines (Paper II)

Our observations that different kinins, both B1 and B2 receptor agonists, synergistically stimulated PGE_2 biosynthesis induced by either IL-1β or TNF-α, demonstrate that signalling through both B1 and B2 receptors interact with the receptor signalling of IL-1 and TNF, to cause synergistic stimulation. This synergistic interaction, as assessed by PGE_2 release, was delayed, indicating that this mechanism involves induction of gene expression. Therefore we studied the expression of two important enzymes involved in the conversion of arachidonic acid to PGE_2, namely COX-2 and m-PGES-1. Co-stimulation of IL-1β or TNF-α with either BK or DALBK resulted in a potentiation of COX-2 expression, by the kinins, both in human osteoblastic MG-63 cells, as well as in mouse calvarial bones. This response could be seen at both mRNA and protein levels. The increased expression of COX-2, caused by co-treatment with IL-1β and kinins, was unaffected by indomethacin in MG-63 cells, but clearly inhibited by indomethacin in mouse calvarial bones, indicating that the interactions between B1, B2, IL-1β and TNF-α receptors lead to either PGE_2-independent or dependent induction of COX-

2 expression, in different cells. The kinins also potentiated the IL-1β and TNF-α induced formation of mPGES-1 mRNA, in MG-63 cells. These effects were also independent on PGE_2 formation. No effects were seen on cPGES or mPGES-2 mRNA expression, by the kinins. The enhanced mPGES-1 mRNA levels caused by IL-1β resulted in increased protein levels of mPGES-1, but we could not see any further potentiation, by B1 or B2 receptor agonists, at the protein level.

These data indicate that enhanced expression of COX-2 is involved in the mechanism by which kinins synergistically potentiate IL-1β and TNF-α induced PGE_2 biosynthesis. This observation is further supported by the fact that potentiation of COX-2 expression preceded the synergistic effect on PGE_2 formation. In contrast, the kinin-induced enhancement of mPGES-1 does not seem to be involved in the interactions between kinins and IL-1β.

In an attempt to study intracellular signalling mechanisms we evaluated the importance of the transcription factor NF-κB and three MAP kinases related to activation of the transcription factor AP-1. The NF-κB inhibitor, as well as the specific inhibitors of p38, JNK and ERK MAPK substantially inhibited the PGE_2 release caused by co-treatment with either BK or DALBK and IL-1β, indicating that both NF-κB and MAPKs are involved. NF-κB has been regarded as a key transcription factor regulating COX-2 gene expression in a variety of cell systems (Newton *et al*., 1997; Jobin *et al*., 1998; Yan *et al*., 2002; Nakao *et al*., 2000; Chen *et al*., 2004; Lee *et al*., 2004). In the present study, however, the effect of the NF-κB inhibitor PDTC was associated with only a marginal decrease of COX-2 mRNA and a partial decrease of mPGES-1 mRNA, which indicates that other mechanisms also are involved in the large inhibition of PGE_2 release.

In contrast, p38 and JNK MAPK inhibitors caused a substantial decrease in both COX-2 and mPGES-1 mRNA expressions, whereas the ERK inhibitor was without effect. Similarly, Nie *et al*., found that NF-κB did not play a key role in IL-1β-induced COX-2 transcription in human ASM cells, but may be required for optimized effect (Nie *et al*., 2003). In addition, it has been reported that IL-1β induces the COX-2 expression and PGE_2 formation through JNK and p38 MAPK, in renal mesangial cells (Guan *et al*., 1998). Our date indicates that p38 and JNK MAPK are important signalling pathways in the interactions between kinins and IL-1β on PGE_2 release and COX-2 expression.

In agreement with observations in other cell types (Bradbury *et al*., 2003; Kondo and Togari, 2004), stimulation of B2 receptors caused an increase in COX-2 mRNA in the human osteoblastic cell line MG-63 and mouse calvarial bones. We also observed that a B1 receptor agonist significantly increased COX-2 mRNA in MG-63 cells, as well as in mouse calvarial bones. This indicates that the delayed PGE_2 response after stimulation with B1 receptor agonists might be due to the induction of COX-2.

We next wanted to evaluate if the interactions between the kinins and IL-1β might involve effects on the expression of molecules involved in osteoclast differentiation and activity. Since the RANK-RANKL-OPG system is an important event in inflammation induced bone resorption, including in inflammatory diseases such as periodontitis (Crotti *et al*., 2003; Lerner, 2006) and rhematoid arthitis (Gravallese *et al*., 2000; Shigeyama *et al*., 2000; Romas *et al*., 2002; Gravallese, 2002), we concentrated on these molecules. IL-1β caused a significant increase in the expression of RANKL mRNA and protein in mouse calvarial bones, but had no effect on the mRNA expression of OPG. In contrast, other

observations show that IL-1 increases the OPG expression, in MG-63 cells, primary human osteoblastic cells and human bone marrow stromal cells (Vidal *et al*., 1998; Hofbauer *et al*., 1999; Brändström *et al*., 2001; Pantouli *et al*., 2005). BK itself did not affect the expression of RANKL mRNA or protein in the mouse calvariae, but co-treatment with IL-1β and BK caused a synergistic potentiation of IL-1β-induced expression of RANKL mRNA, as well as protein. Co-treatment did not influence the expressions of OPG or RANK mRNA. These data suggest that enhanced RANKL/OPG ratio expression in osteoblasts is a possible explanation for the synergistic potentiation of bone resorption caused by kinins.

In summary, our observations show that kinins, acting via both B1 and B2 receptors, interact with the signalling of receptors for IL-1β and TNF-α, causing a synergistic potentiation of cytokine-induced PGE_2 synthesis, by a mechanism depending on increased expression of COX-2. Interestingly, these interactions also enhanced the expression of RANKL, one of the most crucial activators of osteoclast differentiation and activity in inflammatory diseases such as periodontitis and rheumatoid arthritis.

Up-regulation of kinin receptors by pro-inflammatory cytokines (Paper III)

Previous findings show that co-treatment of cells with B1 or B2 receptor agonists and either IL-1β or TNF-α, leads to a remarkably large stimulation of prostaglandin formation (paper II; Lerner, 1991; Lerner and Modéer, 1991). This stimulation can partially be explained by potentiation at the level of COX-2 mRNA and protein (paper II). In this paper, we report that the interactions also can be explained by more up-stream effects, at the level of receptor expression. The osteosarcoma cell line MG-63, express B1 and B2 receptor mRNA and specific binding sites for ligands that specifically recognize B1 and B2 receptors (Paper I, III). B1 receptor expression does not seem to be regulated by stimulation of either B1 or B2 receptors, in MG-63 cells. However, B2 receptor expression was down-regulated by activation of B2 receptors, but unaffected by B1 receptor stimulation. This homologous down-regulation of B2 receptors was also observed in intact bone, but not in gingival fibroblasts. Similar to the data obtained in MG-63 cells, B1 receptor mRNA was unaffected by stimulation of B1 and B2 receptors in gingival fibroblasts, as well as in intact mouse calvarial bones. Although, there are observations in the human lung fibroblastic cell line, IMR-90, showing that B1 receptor expression is upregulated by B1, as well as by B2 receptor activation (Phagoo *et al*., 1999, 2001; Schanstra *et al*., 1998), this up-regulation do not appear to be a general phenomenon, since we could not see any regulation of the B1 receptor expression by stimulation of either B1 or B2 receptors, in the three different human and mouse cells used. Moreover, Sabourin *et al*., found no regulation of B1 receptor mRNA or binding, by a B1 receptor agonist, in rabbit aortic smooth muscle cells (Sabourin *et al*., 2001).

The MG-63 cells were also equipped with IL-1R1, but not IL-1R2, as well as both TNFRI and TNFRII. IL-1β and TNF-α up-regulated the expressions for IL-1R1 and TNFRII, but not the expression for TNFRI. None of these receptors were regulated, at the mRNA level, by kinins, neither in the absence or presence of IL-1β or TNF-α. However, the activation of IL-1 or TNF-receptors led to increased expression of both B1 and B2 receptor expression, as assessed by semi-quantitative RT-PCR, q-RT-PCR, as well as by radioligand binding assays, with a more profound potentiation of the B1 receptors. Additionally, no effects on kinin receptor mRNA, or radioligand binding, were observed when the MG-63 cells were stimulated with other pro-inflammatory cytokines known to stimulate bone resorption,

showing a specificity in this event for IL-1 and TNF-α. The increase in B1 and B2 receptor expression by IL-1β or TNF-α was also observed in primary human gingival fibroblasts and in mouse calvarial bones. Increased B1 receptor expression due to IL-1β or TNF-α, assessed by either mRNA analysis or binding assays, have previously been demonstrated in a variety of cell types (Ni *et al*., 1998; Schanstra *et al*., 1998; Zhou *et al*., 1998, 2000; Tsukagoshi *et al*., 1999; Phagoo *et al*., 1999, 2000, 2001; Haddad *et al*., 2000; Newton *et al*., 2002; Sabourin *et al*., 2002). IL-1β or TNF-α-induced mRNA expression and/or binding of B2 receptors have previously been observed to a much lesser extent (Schmidlin *et al*., 1998; Phagoo *et al*., 2000; Haddad *et al*., 2000; Newton *et al*., 2002; Imai *et al*., 2005).

Thus, our observations demonstrate that B1 and B2 receptor expressions, at the level of both mRNA and binding, are increased by IL-1β and TNF-α, in connective tissue cells including the human osteoblastic cell line MG-63, primary human gingival fibroblasts and in intact mouse calvarial bones. The IL-1β- and TNF-α-induced kinin receptor expression might be one important mechanism involved in the synergistic potentiation of prostaglandin synthesis caused by co-treatment with kinins and either IL-1β or TNF-α. In addition, these interactions and down-stream effects on the expression of RANKL mRNA and protein (paper II), might be important in the pathogenic mechanisms involved in inflammation induced bone resorption in disorders like rheumatoid arthritis and periodontal disease.

Next, we wanted to study the molecular mechanism in the regulation of kinin receptors by the cytokines IL-1β and TNF-α. Since the B1 receptor promoter contains binding sites for NF-κB and AP-1 (Ni *et al*., 1998), we made oligonucleotide probes based on the sequences in the NF-κB and AP-1 sites in the B1 promoter. In EMSA analyses we could show that IL-1β, as well as TNF-α enhanced the specific DNA binding of both NF-κB and AP-1, in the MG-63 cells. When we made supershift analyses, we found that the bound NF-κB dimer contained the p52 and p65 subunits, in IL-1β stimulated cells, and p50 and p65 subunits, in TNF-α stimulated cells, suggesting that the activation of B1 receptor expression caused by TNF-α is mediated by the canonical NF-κB pathway, whereas the IL-1β stimulation of B1 receptor expression, is caused by a non-canonical pathway. Regarding AP-1, the bound AP-1 dimer was partially composed by c-Jun, in both IL-1β and TNF-α-stimulated MG-63 cells, a finding in line with the observation that c-Jun is important in the activation of B1 receptor expression (Yang *et al*., 2001). The activation of AP-1, by IL-1β and TNF-α, seems to involve different subunits in their complex, since c-Fos was supershifted in TNF-α- but not in IL-1β-stimulated cells.

The activation of the NF-κB pathway starts with the phosphorylation of the NF-κB-inhibitor IκBα, by IκB kinases, and then IκBα dissociates from the NF-κB/IκBα complex, followed by the ubiquitination of IκBα, and subsequently its proteasomal degradation. Released NF-κB dimers are then translocated into the nucleus and there it binds to its responsible elements in different promoters. The activation of NF-κB, by IL-1β and TNF-α, observed in EMSA analyses was also confirmed in Western blot analyses, where we found a rapid reduction of IκBα protein expression in both IL-1β and TNF-α-stimulated MG-63 cells. In previous studies, the role of NF-κB in the regulation of B1 receptor expression have been evaluated (Campos *et al*., 1999; Phagoo *et al*., 2001; Sardi *et al*., 2002; Sabourin *et al*., 2002; Medeiros *et al*., 2004), but its role in IL-1β-induced regulation of B2 receptors, as well as in TNF-α-induced activation of B1 and B2 receptor expression is not known. We, therefore, investigated the importance of the increased NF-κB activation found in IL-1β and TNF-α-

induced B1 and B2 receptor expression using a pharmacological inhibitor for NF-κB, namely PDTC. Interestingly, PDTC inhibited the IL-1β-induced enhanced expression of B1 receptor mRNA (49%), as well as the expression of B2 receptor mRNA (46%), in the MG-63 cells, indicating that regulation of B1 and B2 receptor expression is at least partially dependent on the activation of NF-κB. However, TNF-α-induced enhancement of B1 mRNA expression was unaffected by PDTC, whereas the TNF-α-stimulated increase in B2 mRNA expression was inhibited by 30%. These data suggest that NF-κB is not the only transcription factor involved in the regulation of B1 and B2 receptors. The data also indicates that there are different mechanisms involved in different cell types and apperantly by different stimuli.

The activation of AP-1, by IL-1β and TNF-α, observed in EMSA analyses led us to investigate the possible role of MAPK in the stimulation of B1 and B2 receptor expression by IL-1β and TNF-α. It has previously only been shown that pharmacological inhibitors for p38 and JNK, but not ERK, can reduce the spontaneous enhancement of B1 mRNA in rat portal vein explants (Medeiros *et al*., 2004), and that the IL-1β-induced hyperalgesia response to B1 receptor agonists *in vivo* can be reduced by a p38-inhibitor (Ganju *et al*., 2001). When we used SB203580 as an inhibitor for p38 MAPK, we found that activation of p38 is important for the stimulatory effects of IL-1β, TNF-α on both B1 and B2 receptor mRNA expression. Similarly, when we used SP600125 as an inhibitor for JNK, we found that activation of JNK also appear to be important for the effects of both IL-1β and TNF-α on the expression of B1 receptor mRNA, as well as TNF-α-induced enhancement of B2 mRNA expression. However, JNK does not seem to be involved in the IL-1β-induced expression of B2 receptor mRNA, in MG-63 cells, and the ERK inhibitor, PD98059, did not affect any of the stimulatory effects of IL-1β or TNF-α, on either B1 or B2 receptor mRNA expression. Thus, our results suggest that IL-1β and TNF-α share some pathways in their regulations of B1 and B2 receptor transcription, but the data also demonstrate that separate pathways seem to be involved, further indicating that B1 and B2 receptor expression is regulated by different pathways in different situations.

We have previously demonstrated that stimulation of B2 receptors in osteoblasts, gingival fibroblasts, periodontal ligament cells and intact bone, causes a rapid burst of prostaglandin release, whereas stimulation of B1 receptors leads to a more delayed prostaglandin response (Lerner, 1997; Lerner and Lundberg, 2002). It is speculated that this might depend on delayed induction of B1 receptor expression during culture or by homologous induction. However, we demonstrate in this paper that stimulation of B1 receptors does not lead to enhanced expression of B1 receptors in the MG-63 cells. We also found that the delayed concentration-dependent PGE_2 release in response to DALBK, sensitive to inhibition by a B1 receptor antagonist, also could be obtained in MG-63 cells pre-stimulated with IL-1β, to increase the number of B1 receptors. These results strongly indicate that the delayed PGE_2 response, caused by B1 receptor agonists, is not due to delayed B1 receptor expression. The different time-courses seen for PGE_2 formation after stimulation with B1 or B2 receptors agonists, indicate that the down-stream signalling mechanisms linked to prostaglandin release are different for the two kinin receptor subtypes.

In conclusion, IL-1β and TNF-α stimulate the expression of both B1 and B2 receptors in connective tissue cells by pathways that involve the activation of NF-κB and MAPK. The enhancement of the kinin receptors, caused by either IL-1β or TNF-α, may be an important mechanism in the synergistic interactions between the the two pro-inflammatory cytokines

and kinins on prostaglandin biosynthesis and bone resorption in inflammatory diseases such as rheumatoid arthritis and periodontal disease.

The effects of IL-4 and IL-13 on cytokine-induced enhancements of COX-2 and kinin receptors (Paper IV)

IL-4 and IL-13 are multifunctional immune cytokines shown to inhibit bone resorption, both dependent and independent of prostaglandins (Rianhco *et al*., 1993; Miossec *et al*., 1994; Kawaguchi *et al*., 1996; Onoe *et al*., 1996; Palmqvist *et al*., 2006). In this paper, we examined if IL-4 or IL-13 could inhibit the synergistic potentiation of PGE_2 release caused by co-treatment with BK and pro-inflammatory cytokines, in the osteoblastic cell line, MG-63. Both IL-4 and IL-13 markedly decreased the IL-1β, as well as the TNF-α induced PGE_2 release and the synegistic stimulation of PGE_2 caused by co-stimulation with BK and IL-1β. The inhibition of IL-1β-induced PGE_2 formation seems to be dependent on the reduction of the expression of COX-2, since the enhanced expression of COX-2 mRNA induced by IL-1β was inhibited by both IL-4 and IL-13, in MG-63 cells, as well as in human gingival fibroblasts. In contrast, the TNF-α induced stimulation of COX-2 was not inhibited by IL-4 or IL-13, in either MG-63 cells or human gingival fibroblasts, indicating that different mechanisms are involved in mediating the effects caused by IL-1β and TNF-α. However, this do not seem to be a general phenomenon since other studies are showing that IL-4 and IL-13 can reduce the TNF-α induced COX-2 level in neonatal mouse parietal bones (Kawaguchi *et al*., 1996) and human osteoarthritic synovial fibroblasts (Alaaeddine *et al*., 1999).

We then evaluated if the more terminal enzyme in the arachidonic cascade, mPGES-1, could be involved in the inhibition of PGE_2 release. Our data show that IL-4 or IL-13 could neither inhibit the IL-1β nor the TNF-α induced expression of mPGES-1 mRNA, in MG-63 cells, similar to findings in human non-small cell lung cancer cells (Cui *et al*., 2006). Thus, our studies suggest that IL-4 and IL-13 have the capacity to inhibit PGE_2 formation, and that the inhibition of IL-1β stimulated PGE_2 synthesis is mainly dependent on COX-2.

To confirm our data and also investigate the mechanisms involved in the inhibitory effects of IL-4 and IL-13, we used calvarial bones from STAT6-deficient mice. The transcription factor STAT6 has been shown to be essential for gene expression by receptors for IL-4 and IL-13 (Kelly-Welch *et al*., 2003; Hebenstreit *et al*., 2006). Similarly, we found that the inhibitory effect, caused by IL-4 or IL-13, on IL-1β induced COX-2 mRNA expression, in calvarial bones from *wt* mice could not be seen in calvarial bones from *stat6-/-* mice. These data indicate that the transcription factor STAT6 is crucial in this event.

We have previously shown that interactions between the pro-inflammatory cytokines, i.e. IL-1β or TNF-α, and kinins also are dependent on cytokine induced potentiation of B1, as well as B2 receptors (paper III), so we next sought to evaluate the effects of IL-4 and IL-13 on the expression of B1 and B2 receptors. We here demonstrate that IL-4 and IL-13 can inhibit both the basal and the IL-1β or TNF-α induced expressions of B1, as well as B2 receptors, in MG-63 cells and human gingival fibroblasts. Other observations show that IL-4 can inhibit the effects of kinin receptors in human umbelicial veins (Sardi *et al*., 2002), and in a hyperalgesic model in rats (Cunha *et al*., 1999). We also used calvarial bones from *stat6-/-* mice to confirm our data and to further analyse the mechanisms involved in the inhibitory effects of IL-4 and IL-13, and the inhibition seen in *wt* mice could again not be observed in

the calvarial bones from *stat6-/-* mice, indicating the importance for STAT6 in the inhibitory regulation of B1 and B2 receptor expression.

In summary, these studies demonstrate that COX-2, as well as B1 and B2 receptor expressions are down-regulated by IL-4 and IL-13, and that the regulation involves activation of the transcription factor STAT6. The inhibitory effects caused by IL-4 and IL-13 might be of importance in the reduction of inflammation induced bone resorption, in diseases such as rheumatoid arthritis and periodontal disease.

CONCLUDING REMARKS

In several inflammatory diseases including rheumatoid arthritis, periodontitis and osteomyelitis, bone resorption occur in areas adjacent to the inflammatory process, mainly due to recruitment and activation of osteoclasts, by locally produced cytokines and other inflammatory mediators. Some of these inflammatory mediators seem to interact with each other to regulate the response of their actions. This thesis aimed to investigate the mechanisms behind the interactions between different inflammatory mediators involved in the pathogenesis of inflammatory bone resorbing disorders.

In conclusion, the findings presented in thesis are:

- Human osteoblastic cells are equipped with functional B1 and B2 receptors coupled to prostaglandin release, and the stimulated prostaglandin release is mediated by different molecular mechanisms.

- Kinins, acting via both B1 and B2 receptors, interact with the signalling of receptors for IL-1β and TNF-α, causing a synergistic potentiation of cytokine-induced PGE_2 synthesis, by a mechanism mainly depending on increased expression of COX-2. Both NF-κB and MAPKs seem to be involved in the intracellular signalling

- Interestingly, these interactions also enhanced the expression of RANKL, a crucial activator of osteoclastogenesis and bone resorption, in inflammatory diseases such as periodontitis and rheumatoid arthritis.

- IL-1β and TNF-α increase the expression of both B1 and B2 receptors in connective tissue cells by pathways including the activation of JNK and p38 MAPK, as well as the transcription factors NF-κB and AP-1.

- The anti-inflammatory cytokines IL-4 and IL-13 down-regulate the expressions of COX-2, as well as B1 and B2 receptor in human osteoblastic cells, human gingival fibroblasts and mouse cavarial bones. These effects are dependent on the activation of the transcription factor STAT6.

ACKNOWLEDGEMENTS

I would like to express my gratitude and warmest thanks to my family, friends and colleagues, who have supported and inspired me during these years. You made this journey possible!! I especially want to thank the following persons:

Prof. Ulf Lerner, my supervisor. Thank you for introducing me to the world of science and sharing with me your fabulous expertise on bone biology.
Dr. Maria Ransjö, my examinator. Thank you for good advice in both research and orthodontic matters.
Dr. Pernilla Lundberg, my dear friend and collegue. Thank you for all your support and good advice. All interesting discussions about both research and odontology have been invaluable. You have really inspired me!!
Dr. Py Palmqvist, my dear friend, collegue and the best of room-mates. Thank you for all great discussions about everything in life, your tremendous support, and always being there when I needed you. You have been invaluable to me during these years.
Dr. Emma Persson, my co-author and friend. Thank you for sharing your great knowledge about molecular biological techniques and intracellular signalling. It's also good to have someone around that understand all the great things about horses!
Susanne Granholm, my former room-mate and friend. Thank you for your support and all the nice chats, as well as our interesting discussions about research.
Cecilia Koskinen I hope you will enjoy your postgraduate studies here at OCB. It is really nice to have you around.
Inger Lundgren, my co-author and friend. Thank you for your personal support and skilful technical assistance during all these years. Your help has been very much apprechiated!
Ingrid Boström Thank you very much for helping me out with the Western blots, when time was short and bands were missing….
Birgit Andertun Thank you for your help and support.
Chrissie Roth Thank you, for being such a nice and helpful person and for your great support during these years.
Anita Lie Thank you for your help with developing better methods and your great problem-solving skills when there are things not working at the lab.
Britt-Inger Gladsky Thank you for taking care of the animals so well.
Rosie Forsgren and Marie Bergström Without your help no experiments would have been possible. Thank you.
Birgitta Wiklund Thank you for all your help and support during the first half of my postgraduate studies. I still miss you at the department.
Susanne Lindgren It is very nice to have you at the department, helping out with the administrative business.
Stephen Matemba, Annika Sahlin-Platt and Peyman Kelk Thank you for all your support.
Dr. Anders Johansson Thank you for your help with photographing my cells, introducing me to Western blot and all other nice discussions we have had through the years.
Assar and Stina Bäckman Thank you for introducing me to real-time q-RT-PCR and learning me how design my own primers and probes. Thank you also for your postitive attitude!! It was really nice to have you at the department and I still miss you a lot.
Arne Olovsson Thank you, for helping me with the cover of this thesis.
Dr. Herschel Conaway Thank you for your inspiring way and all the nice chats.
Prof. Östen Ljunggren Thank you for increasing the knowledge about kinins, their receptors, and their effects on bone. I'm probably the most enthusiastic reader of your thesis…

People at the department of Orthodontics Thank you for always being so nice and encourageing. I'm really glad to have the opportunity to work with you all.
Ronny Fors, my collegue, friend and former room-mate. Thank you for all the help and support, and off coarse all great discussions about orthodontic, as well as research matters.
Eva Holmgren, Alexandra Ioannidis Olsson, Håkan Olsson, Peter Berglund and Anna Eriksson Lorenzo, my collegues and former ST-pals. Thank you for taking such good care of me when I was a new resident at the department of Orthodontics. I have learned a lot from you all.

Umeå Studentkör/Vuxna Röster Singing in these great choirs have given me so much positive energy. Thank you!
Malin Björkman-Hietala Your tremendous support and positive attitude have really ment a lot. Thank you for being such a great friend. You are my true singing and laughing-partner!
Kristina Löfdahl, my dear friend. Thank you for always being there, helping out, as well as all the good dinners and chats about everything in life. You are really apprechiated!
Sofia Lundgren, my good friend and collegue. Thank you for all good advice and support, as well as all great dinnerparties that you have arranged during these years.
Ewa Lampa, my dear friend and collegue. Thank you for all the nice chats about almost everything. Your great support during all these years has been invaluable.

My mother Berit and father Mats Thank you for bringing me up believing that nothing is impossible. Your encouragement and great support have shaped me as a person.
Peter and Andreas, my dear brothers. Thank you for being there!
Johanna, my little sister. Thank you for brighten up the day!
Catharina and Carl Thank you for being the best mother/father in law a girl could have. I am really thankful for all your support during these years, and Calle I think you are one of the few that will really read the whole thesis….
Louise, Susanne and Viktoria You are like my own sisters, and that means a lot to me.
Agneta Thank you for being such a warm and kind person. Your concern about other people is tremendous.
Family and friends Thank you for all your inspiration and support!

Magnus, my wonderful husband. I cannot ever thank you enough for your never ending love and support. You are the best and I love you!!
Lisa and Erik, our marvellous children. You have made me realize what is important in life. I'm so proud of you!

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