Inflammatory responses of immune cells and osteoblasts in orthodontically-induced bone remodelling and root resorption:

in vitro and *in vivo* studies

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Abbreviations

ALP	Alkaline phosphatase
Bax	Bcl-2–associated X protein (a pro-apoptotic regulator)
Bcl-2	B-cell lymphoma 2 (an apoptotic inhibitor)
BMP	Bone morphogenic protein
BMU	Bone multi-cellular units
BSA	Bovine serum albumin
BSP	Bone sialoprotein
Cbfa1	Core-binding factor alpha 1
CD	Cluster of differentiation
CF	Continuous compressive force
c-fms	Colony-stimulating factor 1 receptor
Col 1	Type I collagen
CGRP	Calcitonin gene-related peptide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunoassay
FCS	Fetal calf serum
FGF-2	Fibroblast growth factor-2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCF	Gingival crevicular fluid
HOB	Primary human osteoblast-like cell

IFN	Interferon
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IR	Immunoreactive
MEM	Minimum essential medium
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MTT	Methylthiazole tetrazolium
M-CSF	Macrophage colony-stimulating factor
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
PBS	Phosphate buffered saline
PDL	Periodontal ligament
PGE2	Prostaglandin E2
PGP 9.5	Protein gene product 9.5
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
Runx-2	Runt-related transcription factor 2
SP	Substance P
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase

TNF	Tumour necrosis factor
TRAP	Tartrate resistant acid phosphatase
VEGF	Vascular endothelial growth factor
$2^{-\Delta\Delta Ct}$ method	Comparative C_T method for relative quantification

Abstract

Orthodontic tooth movement is achieved by remodelling of the surrounding bone in response to the application of appropriate compressive force (CF) to the tooth. Considerable evidence supports the roles of immune cells of the periodontal ligament (PDL) and dental pulp during the remodelling process. One essential mechanism for initiation of this process is the inflammatory response to the application of force by immune cells, which migrate from the capillaries of the PDL. Osteoblasts are also involved in inflammation and osteoclastogenesis in the PDL during alveolar bone remodelling. If excessive CF is applied, root resorption may be an unwanted complication of the bone remodelling process. The role of the immune cells in orthodontically-induced root resorption is poorly understood and little is known about the response of osteoblasts to compressive force (CF). Improved understanding of the molecular and cellular events which regulate bone formation and bone resorption in orthodontic tooth movement is fundamental to the development of clinical guidelines offering greater precision in determining optimal CF to achieve tooth movement while avoiding the untoward complication of root resorption induced by the application of excessive force.

The aim of the *in vivo* and *in vitro* studies on which this thesis is based was to investigate the responses to CF of immune cells and osteoblasts derived from human alveolar bone (HOBs).

In Paper I, an *in vivo* study in rats, extensive root resorption was created by the application of two-cycle orthodontic forces. Resorption was observed in the compression zone of the teeth being moved. Recruitment of immune cells, including monocytes, macrophages and MHC class II Ia-expressing cells, but not granulocytes or lymphocytes, was confined mainly to the compressed PDL, whereas the dental pulp was not markedly affected. The results suggest that extensive root resorption causes only minimal pulpal changes.

In the *in vitro* studies (Papers II and III), HOBs were used to study the initial effects of varying magnitudes of CF on cell viability, proliferation, apoptosis and the

expression of molecules involved in inflammation and the bone remodelling process. The results show that the main effect of CF is to inhibit cell-proliferative activity, without a corresponding increase in cell apoptosis. CF induces the expression of inflammatory mediators IL-6 and CXCL8 in a force-dependent manner. Furthermore, initial application of CF on HOBs can simultaneously affect the expression of markers related to both osteogenesis (initial stage: ALP and Col 1) and osteoclastogenesis (RANKL and OPG).

The results of the studies provide evidence of the potentially pivotal roles of both the immune cells and the osteoblasts of the PDL in extensive root resorption induced by orthodontic tooth movement.

List of publications

This thesis is based on the following articles:

I. Tripuwabhrut P, Brudvik P, Fristad I, Rethnam S. Experimental orthodontic tooth movement and extensive root resorption: periodontal and pulpal changes. *European Journal of Oral Sciences* 2010; 118: 596-603.

II. Tripuwabhrut P, Mustafa K, Brudvik P, Mustafa M. Initial responses of osteoblasts derived from human alveolar bone to various compressive forces. *European Journal of Oral Sciences* 2012; 120: 311-318.

III. Tripuwabhrut P, Mustafa M, Gjerde CG, Brudvik P, Mustafa K. Effect of compressive force on human osteoblast-like cells and bone remodelling: an *in vitro* study. *Archives of Oral Biology* 2013; 58: 826-836.

Introduction

General background

Orthodontic tooth movement is achieved by sequential tissue remodelling of the PDL and the supporting alveolar bone, in response to the application of orthodontic force to the tooth. The force creates two zones around the tooth, so-called compression and tension zones. It is proposed that in the compression zone, a reduction in normal strain on the PDL leads to local unloading of bone, followed by bone resorption (Henneman *et al.*, 2008). In the tension zone, stretching of PDL fibres is transferred to the bone. It is likely that the increase in bone loading contributes to osteoblast recruitment and bone formation (Smit and Burger, 2000). This process leads to displacement of the tooth towards the compression zone.

Tooth displacement is activated by the transmission of applied forces from the dental roots to the surrounding tissues, causing an alteration in biological responses, stimulating cells to remodel the surrounding matrices. Histological studies have revealed extensive cellular activity in the compressed PDL, with not only fibroblasts but also osteocytes, endothelial cells and alveolar bone cells involved in the remodelling process (Krishnan and Davidovitch, 2006).

While successful orthodontic treatment achieves tooth movement and remodelling of the supporting tissues, the application of excessive force may lead to adverse effects such as root resorption (Owman-Moll and Kurol, 2000; Sano *et al.*, 2002).

Biology of tooth and periodontium

Tooth

The hard tissues of the permanent teeth are comprised of dentine, cementum and enamel. Cementum, the mineralized tissue covering the root surface, is less readily

resorbed than bone (Lindskog and Hammarstrom, 1980) and unlike bone, does not undergo continuous remodelling, but continues to grow in thickness throughout life (Bosshardt and Selvig, 1997). The principal collagen fibres of the PDL, Sharpey's fibres, are anchored to the root surface by insertion into the cementum and are believed to act as a barrier to orthodontic root resorption (Faltin *et al.*, 2001). While collagen fibres are the main organic component of cementum, there are also non-collagenous molecules, two of which, BSP and OPN, are major components of the interfibrillar matrices (Yamamoto *et al.*, 2010). Based on the presence or absence of cells and the origin of the collagen fibres in the matrix, two forms of cementum are described: acellular extrinsic fibre cementum and cellular intrinsic fibre cementum, (Nanci and Bosshardt, 2006).

Acellular extrinsic fibre cementum, also referred to as primary or acellular cementum, covers the cervical half to two-thirds of the root. Inserted into this cementum are Sharpey's fibres, the principal fibres of the PDL. Cellular intrinsic fibre cementum, also referred to as secondary or cellular cementum, covers the apical third to half of the root and furcation areas. As the matrix contains cells, this cementum has the ability to repair tissues by filling resorptive defects and root fractures. The cementoblast produces collagen (intrinsic collagen fibres). A characteristic feature of cellular intrinsic fibre cementum is the presence in the matrix of cementocytes, i.e. cementoblasts which are embedded in lacunae.

Because of the differences in composition of root cementum, it is possible to discern variations in severity of root resorption in the different root thirds when heavy orthodontic force is applied. Resorption is more likely in the apical third than in the cervical third of the root (Faltin *et al.*, 2001). This may be attributable to the fact that there are fewer Sharpey's fibres inserted into the cementum (hence a weaker protective barrier), greater vascularity, facilitating recruitment of clast cells, and higher metabolic activity in the adjacent PDL (Rygh, 1977; Lindskog and Hammarstrom, 1980).

Dental pulp

The dental pulp is located within the tooth, with a rich vascular supply and abundant innervation from the trigeminal nerve (Fristad *et al.*, 2010). The tissue contains a variety of cell types, e.g., fibroblasts, odontoblasts and various immune cells: macrophages, dendritic cells, MHC class II Ia-expressing cells, and T-lymphocytes (Okiji *et al.*, 1992). The pulpal nerve fibres contain a variety of neuropeptides. The neuropeptides, in particular, CGRP and SP, are thought to be mediators of neurogenic inflammation and involved in tissue homeostasis of the dental pulp (Norevall *et al.*, 1995; Sacerdote and Levrini, 2012). Adaptive changes following any pulpal insults, e.g., trauma or dental caries, are critical to maintaining tissue homeostasis (Fristad *et al.*, 2010). Haug *et al.* (2003) observed mild pulpal inflammation 10 days after insertion of orthodontic appliances in rats, evidenced by the presence of CD43-IR granulocytes in the root pulp. Moreover, other studies have reported increases in apoptotic activity, odontoblastic degeneration, fibrotic changes, and oedema (Unsterseher *et al.*, 1987; Mostafa *et al.*, 1991; Rana *et al.*, 2001; Santamaria *et al.*, 2007).

Periodontium

The periodontium comprises the specialized tissues that surround and support the tooth: alveolar bone, PDL, gingiva and root cementum. The function of the PDL is to provide attachment, stability and nutrition to the teeth as well as mobility and adaption to mechanical force. During orthodontic tooth movement, the teeth and these supporting structures are exposed to repeated mechanical stress.

PDL

The PDL is the specialized connective tissue between the tooth root and the alveolar bone. It consists of various cell types and extracellular matrix. The cells are predominantly fibroblasts. PDL fibroblasts are thought to be mechanoresponsive to compressive, tensile, shearing and masticatory stress and orthodontic forces (Krishnan and Davidovitch, 2009). The PDL also contains some fibroblast-like cells, which act similarly to osteoprogenitor cells, giving rise to bone cells and cementum (Garant,

2003b; Krishnan and Davidovitch, 2009). Collagen is the most abundant protein in the extracellular matrix of PDL: approximately 80 per cent is Col 1, the major component of the principal fibre bundles which exit the cementum and alveolar bone to form the PDL (Garant, 2003b). PDL tissue contains sensory nerves, an intricate network of neural filaments arising from the trigeminal nerve, and blood vessels. Myelinated and unmyelinated fibres are also present. Unmyelinated fibres commonly follow PDL blood vessels and may have a vasomotor function. Other cells include endothelial cells, epithelial rests of Malassez and immune cells.

Experiments of orthodontic tooth movement have demonstrated that application of force induces dynamic changes in the density and distribution of CGRP and PGP 9.5 IR-nerve fibres in the PDL and dental pulp (Kvinnsland and Kvinnsland, 1990; Norevall *et al.*, 1995; Kato *et al.*, 1996; Vandevska-Radunovic *et al.*, 1997b). Bordering the PDL tissues are cementoblasts (root surface cells) and alveolar bone cells (osteoblasts, osteocytes and osteoclasts).

Alveolar bone

The alveolar bone is a part of the jaws, forming and protecting the tooth sockets. The cortical plates are thinnest in the maxilla and thickest in the mandibular molar region. Continual, rapid remodelling of the alveolar bone is associated with tooth eruption and the adaptive demands of mastication (Sodek and McKee, 2000). Fundamentally, the structure of the alveolar bone is comparable to other osseous tissues in the body (Sodek and McKee, 2000).

Bone tissues

Bone is a vital tissue which undergoes constant changes throughout life. It consists of dense outer cortical bone plates that have a supportive function and metabolically more active cancellous/trabecular bone. The composition is 70 per cent inorganic, 20 per cent organic matter and 10 per cent water. The inorganic component is comprised mainly of calcium and phosphate in the form of carbonated hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). The organic component comprises collagenous and non-

collagenous proteins and proteoglycans. Col 1 is the main collagen in mineralized bone (> 95 per cent) (Sodek and McKee, 2000) and constitutes about 90 per cent of the protein in bone (Garant, 2003a). Its function is to provide the structural framework for mineral salts. Non-collagenous proteins e.g. OCN, OPN and BSP are found in the organic bone matrix and are involved in bone matrix mineralization, cellular adhesion and attachment of bone cells to the matrix during bone remodelling (Garant, 2003a). Proteoglycans are found in extracellular matrices in almost all tissues in adults. Bone proteoglycans e.g. biglycan and decorin, have a range of roles due to their compositional diversity; for example, acting as important structural elements by control of collagen fibrillogenesis and functioning as co-receptors for some cytokines.

Bone cells

The osteogenic cell lineage in bone tissues consists of pre-osteoblasts, osteoblasts, osteocytes, bone-lining cells and osteoclasts.

Osteoblasts

Osteoblasts, the most active secretory cells in bone, are cuboidal or slightly elongated in shape. Pre-osteoblasts, or osteoprogenitor cells, are differentiated from the mesenchymal cells of the bone marrow stroma (Garant, 2003a). They cover most bone surfaces: their main function is to secrete the organic matrix of the bone. Newly formed bone matrix, or so-called osteoid, is not calcified immediately: a layer of osteoid separates the actively bone-forming osteoblasts from the bone surface. The osteoid layer subsequently undergoes mineralization.

Differentiation of osteoblasts from multi-potent mesenchymal cells to functional osteoblasts is mediated by transcription factors, including Runx-2, also known as Cbfa1 and osterix. Runx-2 is an important regulator of multiple stages of osteoblast differentiation (Karsenty, 2000), whereas osterix is required to differentiate pre-osteoblasts to functional osteoblasts (Nakashima et al., 2002). It has been demonstrated that heterozygous mutations of Runx-2 in mice cause cleidocranial dysplasia, characterized by clavicular hypoplasia, large fontanelles, and delayed skeletal development (Otto et al., 1997). In osterix null mice, no bone formation occurs (Nakashima et al., 2002). Osteoblasts produce Col 1, the major structural protein of the bone organic matrix and express ALP, a hydrolase enzyme responsible for the breakdown of pyrophosphate, an inhibitor of calcium phosphate deposition (Christenson, 1997). During matrix mineralization, non-collagenous proteins, e.g., OPN and OCN, are expressed by osteoblasts at peak levels. Osteoblasts are also responsible for the degradation of osteoid and the production of many known cytokines e.g. insulin-like growth factor I, transforming growth factor β, and BMP-2, essential for osteoblast differentiation (Huang et al., 2007), RANKL and OPG, involved in osteoclast formation.

Bone-lining cells

Bone-lining cells are the flattened cells which cover the majority of bone surfaces that are not being remodelled (Kular *et al.*, 2012). They are transformed from active osteoblasts in which the morphology has undergone gradual alterations. Compared to osteoblasts, bone-lining cells contain fewer cell organelles and are not as active (Nakamura, 2007). However, these cells modulate osteoclastic activity by digesting non-mineralized collagen protruding from the bone surface prior to bone resorption by osteoclasts, and by depositing a thin layer of fibrillar collagen at the base of resorption pits, which appear to be important sites of various remodelling activities (Everts *et al.*, 2002).

Osteocytes

Osteocytes are the most abundant cells in bone, comprising 90 per cent of cells. They are osteoblasts which have terminally differentiated and been incorporated into the newly formed bone matrix. Osteocytes are stellate or dendritic cells found within lacunae in the bone matrix and exist for a long periods of time before undergoing apoptosis. These cells can communicate directly with osteoblasts, bone-lining cells and osteoclasts through their long processes, which occupy tiny canals called canaliculi (Seeman and Delmas, 2006). There is growing evidence that osteocytes play a crucial role in orchestrating bone homeostasis by regulating osteoblasts and osteoclasts (Tatsumi *et al.*, 2007; Nakashima *et al.*, 2011). Experimental studies using both *in vivo* and *in vitro* models demonstrate that osteocytes are sensitive to mechanical stress applied to bone and have the potential to transduce musculoskeletally-derived mechanical input into biological output (Bonewald and Johnson, 2008). A recent study found that osteocytes themselves can express RANKL; mice lacking RANKL specifically in osteocytes exhibit osteopetrotic phenotypes, indicating that osteocytes may be the major source of RANKL during bone remodelling (Nakashima *et al.*, 2011).

Osteoclasts

Another type of bone cell, the osteoclast, is a multinucleated cell which functions to resorb bone. These cells are formed by fusion of mononucleated cells of hematopoietic origin and appear only in the vicinity of mineralized bone (Lerner, 2000). The osteoclast precursor cells are closely related to, but distinct from, cells in the monocyte-macrophage lineage (Lerner, 2004). Osteoclasts as well as their precursors contain tartrate-resistant acid phosphatase (TRAP), a lysosomal enzyme (Hayman, 2008). Osteoclasts are essential for physiological bone resorption during growth and remodelling of the skeleton, which can be initiated by various potential stimuli, e.g., mechanical loads and hormones (Crockett *et al.*, 2011).

In active bone resorption, an osteoclast is transformed from the resting to the active state by changes in cell polarization, through reorganization of cytoskeletal

components such as actin filaments and microtubules. Osteoclasts exist within small cavities called Howship's lacunae, which are eroded by their own resorptive activity. This transition of the polarized cell generates new membrane domains: the sealing zone, the ruffled border and the basolateral domain. The sealing zone is engaged in attachment of the osteoclast plasma membrane to the bone matrix: attachment is facilitated by adhesion receptors on the cell membrane, or so-called integrins e.g. vitronectin receptor, $\alpha_V\beta_3$ integrin. These adhesion receptors can also bind to several extracellular matrix proteins in bone which serve as adhesion proteins, e.g. OPN and BSP, offering attachment to the bone. Bone resorption occurs with the support of the specific cytoskeletal structure called the ruffled border. This unique organelle appears in contact with the mineralized matrix and in a resorptive state. In the process of bone resorption, vesicles provide an electrogenic H^+ ATPase or proton pump and chloride is channelled into the ruffled border, resulting in acidification of the resorptive site and subsequently focal decalcification of hydroxyapatite in the bone matrix (Teitelbaum, 2011). The decalcification of hydroxyapatite is followed by digestion of the organic matrix by lysozomal enzymes such as cathepsin K and MMP-9 (Wucherpfennig *et al.*, 1994; Inaoka *et al.*, 1995).The basolateral plasma membrane is believed to be a site which can be stimulated by calcitonin, a hormone which inactivates osteoclasts, and by cytokines (Nakamura, 2007).

Odontoclasts

Odontoclasts are multinucleated cells responsible for resorption of cementum and dentine. Odontoclasts are considered to be of the same cell type as the osteoclasts: they have similar ultrastructural and functional characteristics (Sahara *et al.*, 1994; Sahara *et al.*, 1996). Nevertheless, odontoclasts have fewer nuclei and fewer clear zones than osteoclasts (Tanaka *et al.*, 1990). The cellular mechanisms of root resorption appear to resemble osteoclastic bone resorption, evidenced by the presence of H^+ ATPase at the ruffled border in the root resorption site (Oshiro *et al.*, 2001). During physiological root resorption, odontoclasts express cathepsin K and MMP-9, which are specific proteolytic enzymes in osteoclasts (Tsuchiya *et al.*, 2008).

Intercellular communication in bone and regulation of osteoclastogenesis

The significance of cell-cell communication in the bone for the modulation of bone cell function became recognized after it was shown that osteoclast formation and activity are mediated by cells of osteoblastic lineage, as demonstrated in Fig. 1. M-CSF, secreted by osteoblasts, is one of the important regulators of osteoclast formation and differentiation, (Teitelbaum, 2000). This protein binds to its receptor c-fms on osteoclast progenitor cells, activating proliferation and survival of these cells and impacting on their differentiation (Proff and Romer, 2009).

The identification of RANKL and OPG, two well-known cytokines mediating osteoclastogenesis, has led to improved understanding of bone biology (Khosla, 2001). Osteoblastic lineage cells can express RANKL, which triggers osteoclast formation and activity by binding to its specific receptor, RANK, on the surface of osteoclast precursors (Takahashi *et al.*, 1999). In bone, osteoblasts/ stromal cells play a vital role in osteoclastogenesis through the expression of RANKL, which is mainly membrane-bound, whereas soluble RANKL has been shown to be produced by activated T-cells and human PDL cells (Lum *et al.*, 1999; Schoppet *et al.*, 2002; Nishijima *et al.*, 2006). The expression of RANKL by osteoblasts can be induced by IL-11, PGE2, $1,25(OH)_2D_3$, or parathyroid hormone (Yasuda *et al.*, 1998; Mayahara *et al.*, 2012; Shionome *et al.*, 2012). Conversely, osteoblasts also produce a RANKL decoy receptor, OPG. OPG is a secreted member of the tumour necrosis factor receptor family which binds to RANKL, disrupting its interaction with RANK on osteoclast precursors, thus decreasing osteoclastic activity (Suda *et al.*, 1999). Accordingly, it is generally accepted that the ratio of the RANKL/RANK/OPG triad controls the balance of bone formation and resorption.

It has also been shown that the RANKL/OPG axis plays a crucial role in orthodontic tooth movement (Nishijima *et al.*, 2006; Yamaguchi, 2009). Using immunohistochemical techniques, Shiotani *et al.* (2001) demonstrated the presence of RANKL protein in the cytoplasm of osteoblasts, osteocytes, fibroblasts and odontoclasts during tooth movement. RANKL was also detected in odontoclasts in the

vicinity of root resorption induced by heavy orthodontic force (Nakano *et al.*, 2011) and Yamaguchi (2009) found an increase of RANKL/OPG ratio in the GCF of orthodontically-moved teeth.

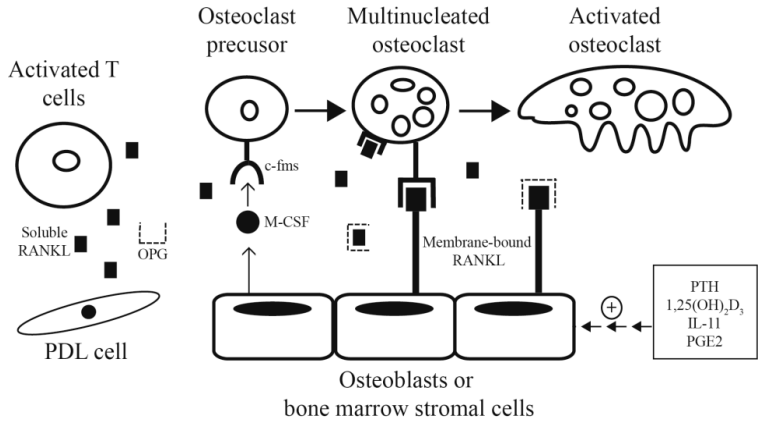

Fig. 1 *Schematic illustration of cell-cell interaction between osteoblast and osteoclast.*

Bone remodelling cycle

Bone remodelling is a complex process of bone resorption by osteoclasts, followed by bone formation by osteoblasts. The remodelling process occurs at so-called bone multi-cellular units (BMU) (Lerner, 2006). The remodelling cycle begins with activation of quiescent osteoblasts (bone-lining cells), by hormonal regulation of calcium homeostasis. Osteoblasts produce and release proteolytic enzymes which degrade the osteoid separating the osteoblastic cell layer and the mineralized bone. This allows the bone-resorbing osteoclasts to adhere to the mineralized bone surface. The recruitment of osteoclasts into BMU is initiated by the activation of receptors on osteoclasts e.g. RANK and c-fms, by the expression of RANKL and M-CSF, respectively, from the osteoblasts. Once the bone resorption lacunae have been moulded, the osteoclasts leave the resorption site and mononucleated cells appear to clean up any remnants of the organic matrix after digestion by the osteoclasts. Simultaneously, growth factors such as IGF-1 and TGF-β are released from the bone extracellular matrix and participate in the recruitment and activation of osteoblasts to the resorption lacunae. Once the osteoblasts fill the resorption lacunae with new bone

in an amount equal to that resorbed, the remodelling process is terminated, and the mineralized bone matrix will be protected by osteoid and a single layer of osteoblasts (Lerner, 2006).

Tissue reactions to orthodontic forces

It has been over a century since Carl Sandstedt first reported the results of histological examination of tissues around orthodontically-treated teeth in dogs (Sandstedt, 1904). In early studies of tooth movement, the investigations sought an understanding of sequential alterations at the tissue and cellular levels. Thus, numerous studies were conducted in a variety of animal species, such as dogs, cats, rats, monkeys and also in humans (Ren *et al.*, 2004). Although there are a few histological studies of experimental tooth movement in humans (Reitan, 1951; Buck and Church, 1972; Kvam, 1972; Kurol and Owman-Moll, 1998), ethical considerations limit human experimental models.

A unique experimental study of tooth movement in humans by Reitan, using light microscopy, demonstrated that the initial phase of orthodontic tooth movement induced local necrosis or hyalinization of the PDL tissue on the compression side, or so called “cell-free zone,” which seems to be almost unavoidable (Reitan, 1951). The details of damaged PDL tissue including degradation of cells and vascular structures were later confirmed by transmission electron microscopy (Rygh, 1972, 1973). In the hyalinized zone, the cells cannot differentiate into osteoclasts and no bone resorption can take place. Tooth movement stops until the adjacent alveolar bone has been resorbed, the hyalinized tissue has been removed and the area repopulated by cells. Elimination of hyalinized tissue is attributed primarily to the phagocytic activity of macrophages and giant cells (Rygh, 1974; Brudvik and Rygh, 1993a, b). It has been proposed that these cells not only participate actively in phagocytosing hyalinized tissues in the compressed PDL, but also, importantly, produce various signalling molecules, including cytokines, which are involved in the remodelling process (Davidovitch, 1995). The adjacent alveolar bone is removed by cells which have

differentiated into osteoclasts. Such resorbing cells are involved not only in elimination of the hyalinized tissue and resorption of alveolar bone, but also in resorption of the root surfaces (Reitan, 1974; Brudvik and Rygh, 1993b, a, 1994a, b).

Mechanobiology of tooth movement

In orthodontic tooth movement, the teeth are displaced within the alveolar bone as a result of remodelling of the PDL and the alveolar bone. This is due to the fact that there is transfer of orthodontic forces to the periodontal tissues, causing alteration of several biological responses in the local tissues. In this section, the mechanical and biological signalling pathways that occur during orthodontic treatment are described according to the theoretical model proposed by Henneman *et al.* (2008).

Matrix strain and fluid flow

After application of orthodontic appliances, the tooth is immediately moved in its socket. The mechanical loading creates a positive strain (tensional deformation) in the PDL of the tension side and a negative strain (compressive deformation) in the PDL of the compression side. On both sides, the induced strain leads to immediate fluid flow in the PDL (Henneman *et al.*, 2008) and the strain induced in the bone matrix causes a fluid flow in the canaliculi. This fluid flow is sensed by osteocytes, which results in maintenance or even an increase of bone mass (Weinbaum *et al.*, 1994). On the other hand, prolonged unloading of bone results in a reduction of canalicular fluid flow, inducing apoptosis of osteocytes and subsequent recruitment of osteoclasts to the site (Burger *et al.*, 2003).

Cell strain

As cells are linked to the ECM by cell-matrix attachments, namely integrins, ECM strain and fluid flow result in deformation of PDL and bone cells. This leads to production of several mediators which stimulate various cell types in the paradental tissues in an autocrine and/or paracrine manner.

Fibroblasts, the main cellular components of the PDL, are thought to be mechanoresponsive to different types of force. When mechanical loads are applied to fibroblasts, signal transduction is induced by transmitting the forces from extracellular matrix to integrin, cytoskeleton, and nucleus, eventually leading to gene transcription and protein translation (Wang *et al.*, 2007).

Cultured human PDL cells have been used extensively in investigations of the effect of mechanical stress on the molecules involved in the bone remodelling process at both mRNA and protein levels. CF stimulates the PDL cells to express various inflammatory cytokines: IL-1β, IL-6, CXCL8, IL-11, and TNF-α, FGF-2, and inflammatory mediators PGE2 (Yamamoto *et al.*, 2006; Nakajima *et al.*, 2008; Li *et al.*, 2011). It has also been shown that the level of RANKL is up-regulated by CF (Kanzaki *et al.*, 2002; Yamamoto *et al.*, 2006; Nakajima *et al.*, 2008). However, findings with respect to the expression of OPG are inconsistent: Kanzaki *et al.* (2002) found a steady level of OPG from PDL cells subjected to different magnitudes of CF, whereas Yamamoto *et al.* (2006) reported that compressed PDL increased expression of OPG. It has also been shown that an increased level of RANKL is mediated by IL-1β, PGE2, and FGF-2 (Yasuda *et al.*, 1998; Chikazu *et al.*, 2001; Shionome *et al.*, 2012). In both *in vivo* and *in vitro* studies, a mediator of angiogenesis, VEGF, was up-regulated in compressed PDL under CF (Miyagawa *et al.*, 2009). The responses of human PDL cells to tensile mechanical strain have also been studied. Bolcato-Bellemin *et al.* (2000) showed that a continuous tensile strain induces the expression of mRNA encoding for MMP-1, MMP-2, TIMP-1 and TIMP-2.

In summary, mechanical loading causes biological changes in the PDL cells, including alterations in the expression of the genes and proteins related to the bone remodelling process.

Moreover, it is suggested that shear stress during orthodontic loading induces canalicular fluid flow, which results in inhibition of osteocyte apoptosis (Tan *et al.*, 2006). This inhibitory effect is believed to be mediated by production of nitric oxide (NO) in response to the shear stress (Tan *et al.*, 2008). In addition, osteocytes respond

to strain by the production of other cytokines, e.g. prostaglandin and TNF-α (Westbroek *et al.*, 2000).

Cellular responses associated with orthodontic tooth movement

When an orthodontic force is applied to the teeth, immediate changes are observed in periodontal tissues (Andrade *et al.*, 2012). A prerequisite for the achievement of tooth displacement during orthodontic therapy is an aseptic inflammatory response i.e. an increase in vascular permeability and leukocyte diapedesis (Krishnan and Davidovitch, 2006; Meikle, 2006). These migratory immune cells, together with local paradental cells, e.g. fibroblasts and osteoblasts, produce various inflammatory molecules such as cytokines and chemokines, to communicate with neighbouring cells in the paradental tissues in an autocrine or paracrine manner, as described above (Krishnan and Davidovitch, 2006; Ren and Vissink, 2008). A previous study has demonstrated that anti-inflammatory medication, such as aspirin and ibuprofen, can diminish the rate of tooth movement (Arias and Marquez-Orozco, 2006), whereas stimulating production of inflammatory mediators through small perforations of cortical bone can increase the rate of tooth movement (Teixeira *et al.*, 2010).

As stated above, mechanical loading stimulates cellular production of mediators, which allow communication between the cells in the remodelling area, resulting in cell differentiation and function and subsequently remodelling of PDL and bone matrix (Fig. 2). Anatomically, osteocytes are in contact with osteoclast precursor cells through their long processes. Recent studies demonstrated that osteocytes are a primary source of RANKL involved in osteoclast differentiation and function (Fig. 2, arrow 1) (Nakashima *et al.*, 2011; O'Brien *et al.*, 2013). In addition, activated osteocytes can affect precursor cells in the PDL to differentiate into osteoblasts through mediators e.g. BMP-2, BMP-6, BMP-9 and platelet-derived growth factor produced by the osteocytes (Fig. 2, arrow 2) (Henneman *et al.*, 2008).

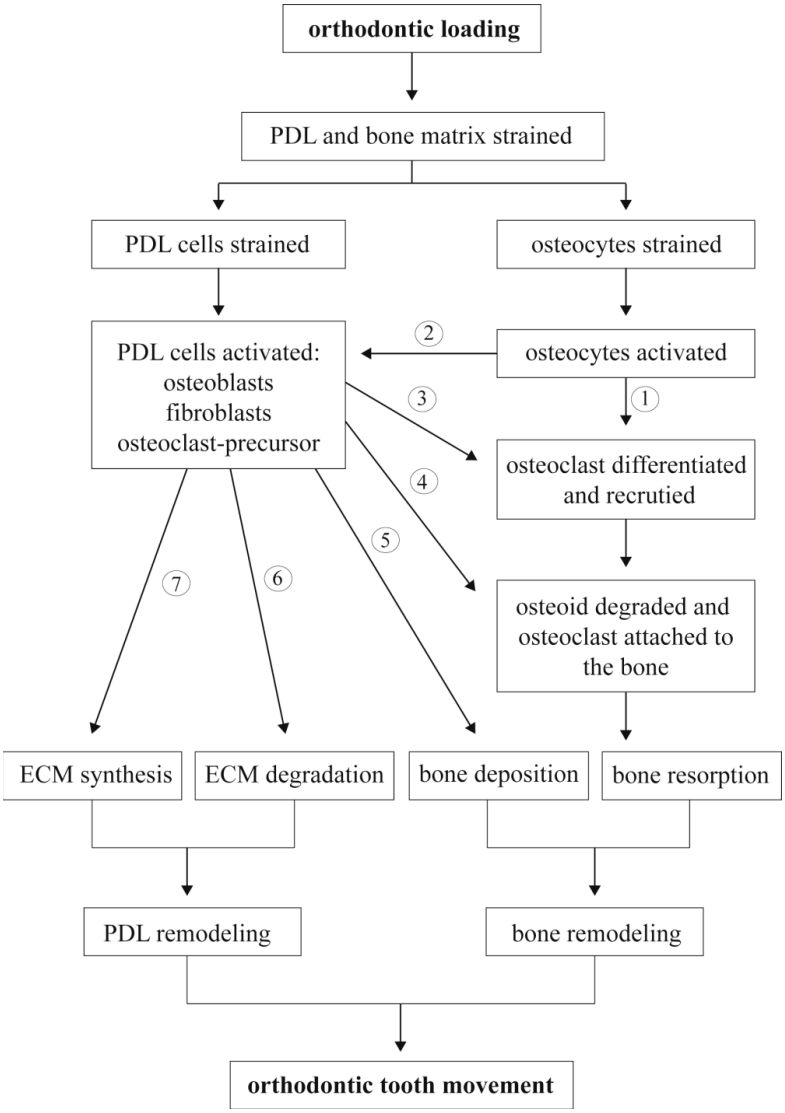

Fig. 2 *Schematic illustration of a theoretical model of tooth movement, adapted from Henneman et al. (2008)*

Concomitantly, the cells of the periodontium e.g. PDL fibroblasts and osteoblasts play an important role in osteoclast formation, through the expression of essential mediators e.g. RANKL, M-CSF and OPG (Fig. 2, arrow 3) (Kawasaki *et al.*,

2006; Dunn *et al.*, 2007; Yamaguchi, 2009; Nakano *et al.*, 2011). These mediators are generated by activated osteocytes and by osteoblasts and fibroblasts localized in the compressed PDL of orthodontically-moved teeth (Shiotani *et al.*, 2001; Oshiro *et al.*, 2002).

Another important function of osteoblasts in bone resorption is to release MMPs, which degrade the non-mineralized osteoid layer to allow attachment of differentiated osteoclasts to the bone matrix (Fig. 2, arrow 4) (Chambers *et al.*, 1985).

Moreover, *in vitro* studies show that PDL cells subjected to mechanical stretching induce mRNA expression of ALP, BMP-2, BMP-4, and Runx-2, important regulators of osteoblast differentiation and bone formation (Fig. 2, arrow 5) (Ozaki *et al.*, 2005; Enokiya *et al.*, 2010; Yang *et al.*, 2010). Previous studies have demonstrated that NO is produced in response to mechanical force in osteoblasts and osteocytes (Bacabac *et al.*, 2004; Tan *et al.*, 2009). Constitutive endothelial nitric oxide synthase (eNOS) has been observed in the tension zone of orthodontically-moved teeth 24 hours after insertion of orthodontic appliances in rats, suggesting that eNOS mediates bone formation in this area (Tan *et al.*, 2009). Moreover, it is shown that osteocytes produce NO to inhibit the activity of osteoclasts at the bone apposition site of orthodontically-moved teeth (Yoo *et al.*, 2004; Tan *et al.*, 2009).

The degradation of ECM during periodontal tissue remodelling is achieved by enzymes e.g. MMPs and cathepsins, produced from PDL fibroblasts and osteoblasts in response to mechanical stress (Fig. 2, arrow 6) (Bolcato-Bellemin *et al.*, 2000; Yamaguchi *et al.*, 2004).

Mechanical stress also contributes to ECM synthesis by collagen production from PDL cells and osteoblasts in response to mechanical loading in both the compression and tension zones of orthodontically-moved teeth (Fig. 2, arrow 7) (Bumann *et al.*, 1997).

It has been proposed that the immune system plays a role in the regulation of tissue remodelling in orthodontic tooth movement (Vandevska-Radunovic *et al.*, 1997a; Nakamura *et al.*, 2001). Previous studies have shown that orthodontic forces

induce dynamic changes in monocytes, macrophages, dendritic cells, and MHC Class II Ia-expressing cells in the PDL of the teeth being moved (Vandevska-Radunovic *et al.*, 1997a; Nakamura *et al.*, 2001; Baba *et al.*, 2011). Vandevska-Radunovic *et al.* (1997b) found that the high influx of macrophages was co-incident with the proliferation of blood vessels located adjacent to the hyalinized tissues: the adjacent immune cells participate in bone/root resorption by acting as scavenger cells, antigen-presenting cells and also cells producing bone resorptive cytokines, e.g., IL-1 and TNF-α (Bletsa *et al.*, 2006).

There is ample evidence to support the crucial roles of neurovascular mechanisms in the inflammatory response to the application of orthodontic force. The nerve fibres of the PDL have two main functions during orthodontic tooth movement: transmission of nociceptive impulses centrally and release of neuropeptides peripherally. Each may be crucial in modulating the local inflammatory responses, mainly by interacting with cells of the vascular system (Davidovitch, 1991). The release of neuropeptides is stimulated by the mechanical stress applied to the tooth, inducing gradual fluid movement in the PDL tissue, resulting in nerve fibre distortion, which leads to neuropeptide release from the nerve endings. It has been shown that during orthodontic tooth movement, the PDL and dental pulp release vasoactive neurotransmitters, e.g. CGRP and substance P, indicating their involvement in tissue remodelling (Kvinnsland and Kvinnsland, 1990; Norevall *et al.*, 1995; Caviedes-Bucheli *et al.*, 2011). These neuropeptides increase vascular flow and permeability, leading to plasma extravasation, activate the immune system and recruit and/ or modulate inflammatory cells, e.g. macrophages and lymphocytes (Caviedes-Bucheli *et al.*, 2008). The recruited inflammatory cells interact with paradental cells of orthodontically-moved teeth, increasing the production of bone resorption-associated cytokines and chemokines, leading to the initiation of bone resorption (Andrade *et al.*, 2012).

At present, identification of the roles of cytokines involved in tissue remodelling during orthodontic tooth movement presents a challenge. Numerous

cytokines, particularly IL-1, IL-6, IL-8, TNF-α, RANKL, and OPG are reported to play an important role in the development of acute and chronic inflammatory responses in the paradental tissues of orthodontically-moved teeth (Davidovitch, 1991; Yamaguchi, 2009).

IL-1 is involved in the regulation of immune responses, inflammatory reactions, and also stimulation of osteoclast function through the IL-1 type I receptor, expressed by osteoclasts (Krishnan and Davidovitch, 2006). A recent study demonstrated that administration of IL-1 receptor antagonist diminishes orthodontic tooth movement in mice, indicating the involvement of IL-1 in bone resorption during orthodontic tooth movement (Salla *et al.*, 2012). Up-regulation of IL-1 mRNA and protein has been shown in compressed rat PDL and gingiva, respectively, of orthodontically-treated teeth (Alhashimi *et al.*, 2001). Luppanapornlarp *et al*. also reported an increased level of IL-1 protein in the GCF of patients undergoing orthodontic treatment (Luppanapornlarp *et al.*, 2010).

IL-6 regulates the tissue remodelling process by directly interacting with bone cells. Its functions are strongly related to osteoclast formation, osteoclast activity, and bone resorption (Kwan Tat *et al.*, 2004). It is produced by many cell types, including osteoblasts (Koyama *et al.*, 2008). In a rat model, Alhashimi *et al.* (2001) demonstrated an increased level of IL-6 mRNA in the compressed PDL of orthodontically treated teeth after three days of force application. In patients undergoing orthodontic treatment, application of orthodontic force stimulated the secretion of IL-6 protein in the GCF (Basaran *et al.*, 2006a).

IL-8 has been renamed CXCL8, according to the new nomenclature for chemokines (Zlotnik and Yoshie, 2000). CXCL8 mediates angiogenesis and inflammatory responses. It is known as a cytokine-induced neutrophil chemo-attractant and is produced by a variety of cells, including macrophages, neutrophils, endothelial cells, osteoblast lineage cells and tumour cells from oral squamous cell carcinoma (Bendre *et al.*, 2003; Koyama *et al.*, 2008; Hwang *et al.*, 2012). Asano *et al.* (2011) reported that heavy force during orthodontic tooth movement induced the expression of CXCL8 protein in odontoclasts and PDL fibroblasts in the compression zone.

Basaran *et al.* (2006a) found an increased release of CXCL8 protein in the GCF of orthodontic patients.

TNF-α is a cytokine associated with acute and chronic inflammation, and also with bone resorption. It is one of the potent cytokines mediating osteoclastogenesis. An *in vivo* study has shown that local delivery of TNF-α increases the number of osteoclasts (Gaspersic *et al.*, 2003). An experiment in tumour necrosis factor receptor-deficient mice revealed a slower rate of tooth movement in response to orthodontic loading, indicating the important role of TNF-α during tooth movement (Yoshimatsu *et al.*, 2006). In a rat model, Bletsa *et al.* (2006) demonstrated expression of TNF-α protein in the gingiva and in the compressed PDL at the early stage of tooth movement. Elevated levels of TNF-α have also been detected in the GCF of orthodontically-moved teeth (Basaran *et al.*, 2006b).

Prostaglandins are ubiquitous mediators of local homeostasis and bone resorption in a variety of pathological conditions, including periodontitis, trauma and cancer (Saito *et al.*, 1991). They are local hormone-mimicking chemicals, produced by mammalian cells, including osteoblasts, within seconds of cell injury (Yamaguchi and Kasai, 2005). Among several subclasses of prostaglandins, PGE2 has been shown to act as a potent stimulator of both bone resorption and formation (Kaneki *et al.*, 1999; Mitsui *et al.*, 2005) and has been detected in the GCF of patients with periodontitis (Biyikoglu *et al.*, 2006) and those undergoing orthodontic treatment (Dudic *et al.*, 2006; Chibebe *et al.*, 2010).

In addition to the cytokines, chemokines provide important signals for trafficking, differentiation and activity of immune cells and bone cells (Yano *et al.*, 2005; Schall and Proudfoot, 2011). The chemokines belong to a family of small cytokines or signalling proteins with the ability to induce cell migration. It is this characteristic which distinguishes chemokines from cytokines. There is growing evidence that some chemokines, e.g. CCL2, CCL3, CCL5, CXCL8 and CXCL12 are involved in bone remodelling during orthodontic tooth movement (Garlet *et al.*, 2008; Andrade *et al.*, 2009; Asano *et al.*, 2011).

It is now known that osteoclast formation and function are increased in inflammatory conditions with associated bone loss, such as rheumatoid arthritis, through the action of inflammatory cytokines e.g. TNF-α and RANKL (Crockett *et al.*, 2011). In the same way, orthodontic loading stimulates fibroblasts, osteoblasts and other paradental cells to release inflammatory mediators, e.g. IL-1 and TNF-α (Bletsa *et al.*, 2006). These inflammatory cytokines are capable of inducing osteoblasts to release chemokines, such as CCL2, CCL3 and CCL5 (Silva *et al.*, 2007). Together with CXCL 12, RANKL and TNF-α, they can induce chemotactic recruitment of osteoclast precursors to bone resorption sites, where these cells differentiate into mature osteoclasts (Yu *et al.*, 2004; Wright *et al.*, 2005). Production of RANKL and M-CSF from osteoblasts is enhanced by PGE2 and some cytokines e.g. IL-1, IL-6, CXCL8, and TNF-α. The RANKL and M-CSF bind to their respective receptors RANK and c-Fms, expressed on osteoclast precursor cells. The increased levels of RANKL and M-CSF result in up-regulation of osteoclastogenesis. Osteoclastogenesis can be down-regulated when OPG, produced by osteoblasts and PDL cells, binds to RANKL, blocking the RANK/RANKL interaction. Compared to the level of RANKL on the compression side of orthodontically-moving teeth, the OPG level is lower, enhancing osteoclastogenesis in this area (Nishijima *et al.*, 2006). Moreover, orthodontic loading at the compression sites causes microdamage to alveolar bone, compromising osteocyte integrity and disrupting canalicular fluid flow. These affected tissues induce osteocyte apoptosis, which enhances bone resorption adjacent to the damaged area by up-regulation of RANKL.

Aims of the investigation

In the lag phase of orthodontic tooth movement, hyalinized tissue appears in the PDL. This necrotic tissue is digested by immune cells and replaced by loose connective tissue. Osteoclasts are then recruited to the area and bone resorption begins. The role of immune cells in digesting the hyalinized tissue is crucial. Root resorption may be regarded as a side effect of this scavenging activity by macrophages and multinucleated giant cells to remove the hyalinized tissue: once these cells reach the root surface they start to remove the cementum. To date, little is known about the specific immune cells involved, or their distribution during orthodontically-induced root resorption. The role of the osteoblast in the inflammatory process is recognized, as is osteoclastogenesis in the PDL during remodelling of the PDL and alveolar bone. However, there is only limited information about the role of the osteoblast in the inflammatory response to the application of excessive orthodontic forces leading to root resorption.

The overall aims of the studies on which this thesis is based were to investigate responses to CF of immune cells (*in vivo*) and HOBs (*in vitro*). The *in vivo* study was undertaken in order to investigate the distribution of immune cells and changes in the blood vessels and nerve fibres of the PDL and dental pulp after extensive root resorption. The aim of the *in vitro* studies was to investigate the response of HOBs to CF of varying magnitude and duration.

The specific aims of the individual studies were:

- To investigate changes in immune cells (monocytes, macrophages, MHC-class II Ia expressing cells, granulocytes, lymphocytes), blood vessels and nerve fibres in the PDL and dental pulp of orthodontically-induced extensive root resorption in rats.
- To investigate initial responses of HOBs to CF of varying magnitude and duration *in vitro,* by determining the expression of molecules involved in bone

formation (ALP, Col 1, OPN, OCN, transcription factor Runx-2), bone resorption (RANKL and OPG), and the inflammatory mediators PGE2, IL-6 and CXCL8.

Materials and Methods

The work described in this thesis is based on both *in vivo* (Paper I) and *in vitro* (Papers II and III) studies. Full details of the materials and methods are described in the attached original articles. An outline of the thesis together with methods used is summarized in Table 1.

Table 1 *Summary of the methods used in the present thesis*

Paper	Experimental model	Evaluation	Method
I	*In vivo*	-Immune cells - Blood vessels - Nerve fibres	- Immunohistochemistry
II	*In vitro*	- Cell viability - Cell proliferation - Cell apoptosis - Inflammatory mediators	- Light microscopy - MTT assay - RT-PCR - Multiplex assay
III	*In vitro*	- Markers related to bone remodelling	- RT-PCR - ELISA - ALP activity assay - Immunofluorescent staining

In vivo study (Paper I)

Animal experiments

The material comprised fifteen 7-week-old male Sprague-Dawley rats. The experimental tooth movement was conducted according to the method previously described by Brudvik and Rygh (1993a). Activation of the spring exerted a force of 50 grams (Fig. 3).

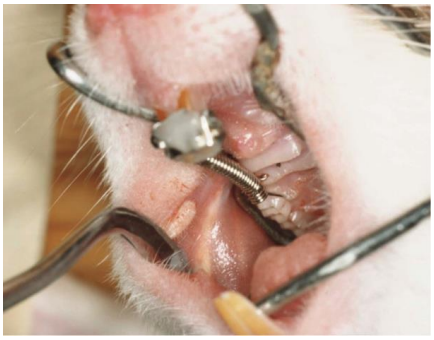

Fig. 3 *Macroscopic illustration of orthodontic appliances used to move the maxillary first molar mesially. A closed coil spring is ligated between an eyelet on the incisor band and the mesial surface of the first molar.*

In order to create extensive root resorption, two cycles of force activation were applied (Fig. 4).

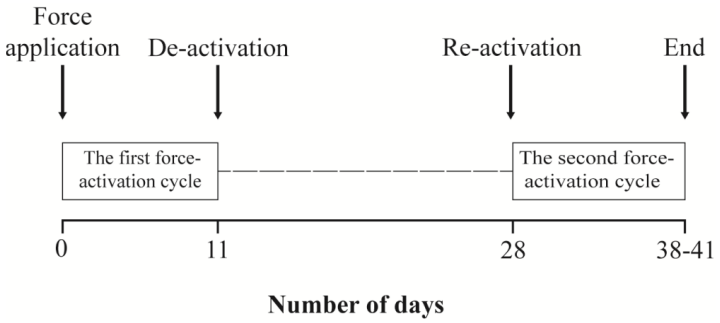

Fig. 4 *Schematic illustration of the experimental orthodontic tooth movement inducing extensive root resorption in rats*

Immunohistochemistry

The specimens were embedded in Tissue-Tek optimum cutting temperative compound (Sakura Finetek, Zoeterwoude, the Netherlands) and 30-µm-thick sagittal sections were made in a –20 ºC cryostat. Alternate serial sections from the right maxilla were placed on pre-coated glass slides (SuperFrost Plus; Menzel-Glaser, Braunschweig, Germany) and processed for immunohistochemistry. Lists of primary antibodies, CD designations, and their specificities used in Paper I are described in Table 2.

The staining protocols for monoclonal and polyclonal antibodies are described in Paper I. All antibodies were visualized by nickel-enhanced 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) + 0.003% hydrogen peroxide. The sections were counterstained with Richardson's stain, and coverslipped with Eukitt (O. Kindler, Freiburg, Germany).

Table 2 *Primary antibodies used for immunohistochemistry*

Antibody name/clone number	**CD Designation**	**Dilution**	**Source**	**Specificity**	**References**
Monoclonal ED1	68	1:2000	Serotec, Oxford, UK	monocytes, macrophages, dendritic cells, osteoclasts, odontoclasts	(Haug *et al.*, 2003)
Monoclonal OX6		1:2000	Abcam, Cambridge, UK	MHC class II Ia-expressing cells, macrophages, dendritic cells, B-lymphocytes	(McMaster and Williams, 1979; Fukumoto *et al.*, 1982)
Monoclonal W3/13	43	1:2000	Serotec, Oxford, UK	granulocytes, T-lymphochytes, plasma cells	(Haug *et al.*, 2003)
Polyclonal PGP 9.5		1:4000	Chemicon, Temecula, CA, USA	general neuroplasmic marker	(Vandevska-Radunovic *et al.*, 1997b)
Polyclonal laminin		1:7000	Sigma-Aldrich, St. Louis, MO, USA	Blood vessels	(Vandevska-Radunovic *et al.*, 1997b)

Quantification of results

The sites selected for quantification were the radicular pulp of the distal root and the PDL tissue at the mesial aspect of the distal root of the orthodontically-moved first molars, where extensive external root resorption was detected (Fig. 5). The second molars, with no evidence of root resorption, served as control teeth. Expression of cells IR to the antibodies against CD68-IR cells, MHC class II Ia-expressing cells, and CD43-IR cells was quantified by computer-assisted image software (NIS-Element software version BR 3.0; Nikon Instruments, Tokyo, Japan). Final data were calculated as the percentage of area occupied by positive cells per total area of the investigated field.

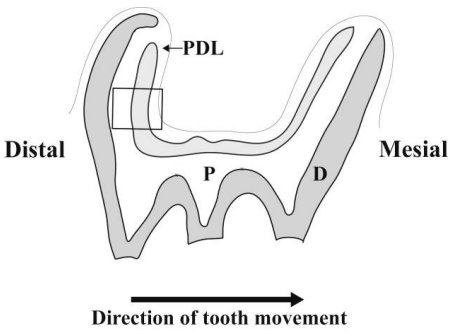

Fig. 5 *Schematic illustration showing area of investigation (box) at the distal root of the first molar. A similar region was investigated in the second molars.*

Statistical analysis

The data were analysed using the Wilcoxon Signed Rank Test for comparison between groups. Results were considered significant at $P < 0.05$. A qualitative evaluation was performed to describe PGP 9.5 and laminin staining.

In vitro studies (Papers II and III)

In Papers II and III, HOBs derived from mandibular alveolar bone were used to study the responses of osteoblasts to CF.

Cell culture (Papers II and III)

Discarded alveolar bone pieces were harvested from the molar region. The bone specimens were isolated and cultured as previously described (Beresford *et al.*, 1984; Mustafa *et al.*, 2000). The steps of cell isolation and culture are described in Papers II and III and summarized in Fig. 6.

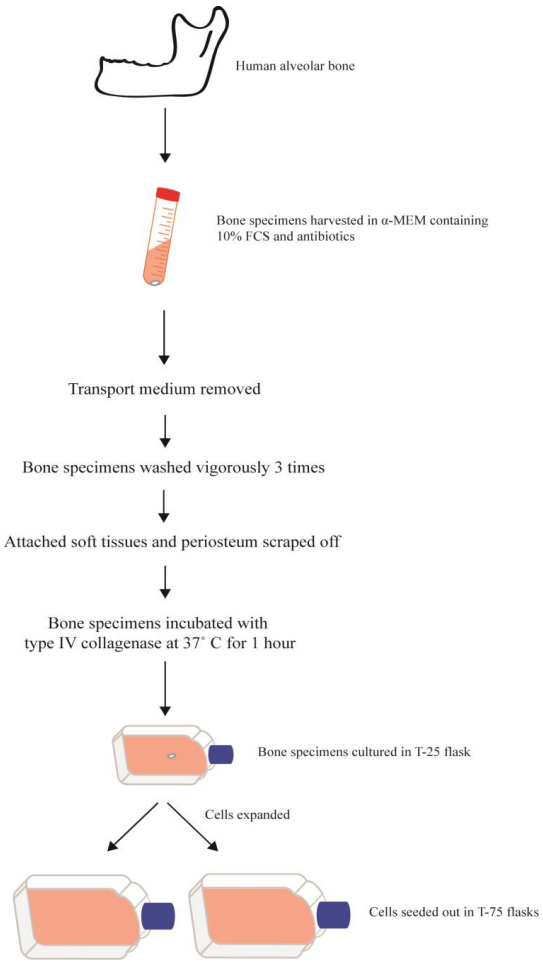

Fig. 6 *Schematic illustration of the isolation of osteoblasts derived from human alveolar bone*

Characterization of bone cells (Papers II and III)

HOBs derived from each donor were kept separately and characterized using ALP activity stain and RT-PCR. Only HOBs from donors showing strongly positive ALP

cells were selected. RT-PCR was used to evaluate the mRNA expression of the bone markers ALP, Col 1, OPN, OCN, and a transcription factor, Runx-2. The cells to be used in the experiments were taken from passages 1-4 and selected on the basis of strongly-positive ALP staining and expression of bone markers.

In order to examine the mineralization potential of the characterized HOBs, cells were cultured with osteogenic stimulatory medium in α-MEM for 11 days. The cells were incubated with 2% Alizarin Red S powder (Sigma-Aldrich) dissolved in distilled water (pH 4.1) (Li *et al.*, 2009).

Application of CF (Papers II and III)

The HOBs were subjected to continuous compression according to a modification of the method previously described by Kanzaki *et al.* (2002), as shown in Fig. 7. Cells were cultured in 6-well plates at a density of 200,000 or 300,000 cells/well. CF varying from 1.0-4.0 g/cm^2 was applied by adding lead weights into glass wells. Control cells were grown on culture plates and covered with thin plastic plates, without lead weights.

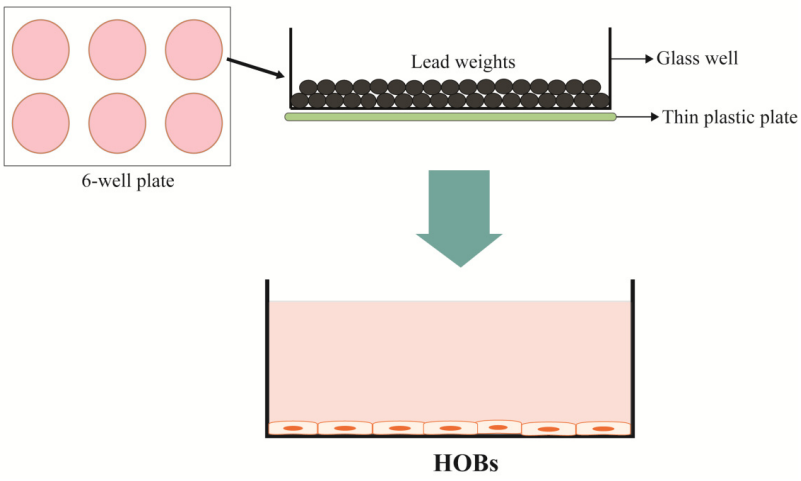

***Fig.** 7 An in vitro compression model, performed in a 6-well plate. HOBs were subjected to varying degrees of CF, determined by the number of lead weights.*

Microscopic evaluation (Paper II)

To study cellular morphology and to determine the percentages of attached HOBs under CF, cells at a density of 200,000 per well were subjected to the weight of the thin plastic plates alone ("control"), and to 2.0 and 4.0 g/cm^2 of CF for 24 and 72 hours. In order to enhance visualization under light microscopy, the compressed cells were stained with crystal violet solution. The final results were calculated as percentages of the area occupied by stained cells per total area of investigated fields.

Cell viability and proliferation in response to CFs (Paper II)

HOBs, seeded in culture plates at a density of 200,000 cells/well, were subjected to "control," 1.0, 2.0, 3.0 and 4.0 g/cm^2 of CF for 1, 3, 24, 48 and 72 hours. At harvest, cell viability and proliferation relative to the controls were determined using MTT assays, as described in Paper II.

Effect of CF on mRNA expression of molecules involved in bone remodelling (RT-PCR, Papers II and III)

The influences of varying magnitudes of CF ("control," 2.0 and 4.0 g/cm^2) for 24 hours on mRNA expression of markers involved in bone remodelling were determined using RT-PCR. All markers and their primers used in this study were summarized in three categories: cell proliferation and apoptosis, bone apposition and bone resorption, as demonstrated in Fig. 8 and Table 3.

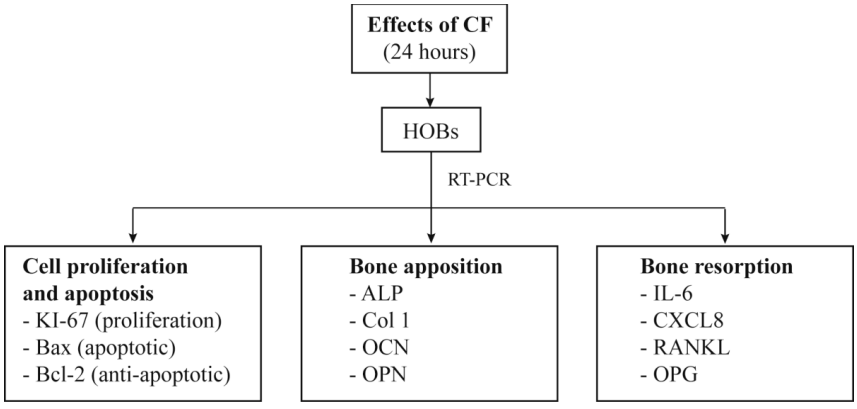

Fig. 8 *Summary of markers used to study effects of CF on HOBs*

Table 3 *Primers used for RT-PCR*

Gene	Accession number
KI-67	Hs01032443_m1
Bax	Hs00180269_m1
Bcl-2	Hs00153350_m1
IL-6	Hs00985639_m1
CXCL8	Hs01567912_g1
ALP	Hs01029144_m1
Col 1	Hs00164099_m1
OPN	Hs00960942-m1
OCN	Hs00609452_g1
Runx-2	Hs00231692_m1
RANKL	Hs00243522_m1
OPG	Hs00900358_m1
GAPDH	Hs99999905_m1

Effect of CF on synthesis of cytokines by HOBs (multiplex and ELISA, Papers II and III)

Protein expression of IL-6 and CXCL8 in the culture medium was assayed using a human cytokine group I 2-plex express assay kit containing coupled magnetic beads for detecting human IL-6 and IL-8 (Bio-Rad, Hercules, CA, USA).

Protein expression of Col 1, RANKL, and OPG and PGE2 release in the culture medium by HOBs subjected to varying magnitudes of CF ("control," 2.0, and 4.0 g/cm^2) and duration was analysed by ELISA (Table 4).

Table 4 *Protein expression of Col 1, RANKL and OPG and PGE2 release, detected at various time-points using ELISA kits*

Protein	Duration (day)	ELISA kit
Col 1	1, 3, 7	Metra CICP Enzyme Immunoassay Kit (Quidel, San Diego, CA, USA)
RANKL	1, 3	Human sRANK-Ligand ELISA developmental kit (Peprotech, London, UK)
OPG	1, 3	Human Osteoprotegerin Instant ELISA (Bender MedSystems, Vienna, Austria)
PGE2	1	Prostaglandin E2 ELISA Kit (Neogen, Lexington, KY, USA)

Immunofluorescent staining (Paper III)

Immunofluorescent staining was used to examine the protein expression of RANKL and OPG by HOBs after the application of CF. HOBs at a density of 300,000 cells/well were cultured on sterile 24 × 24 mm glass coverslips which had been placed in 6-well plates. The cells were subjected to "control" and CF of 2.0 and 4.0 g/cm^2 for 24 and 72 hours. The staining protocol is described in Paper III.

ALP activity assay (Paper III)

The effects of CF on ALP activity were determined both intracellularly and extracellularly. HOBs at a density of 300,000 cells/well were seeded in 6-well plates. The cells were subjected to "control," 2.0 and 4.0 g/cm^2 of CF for 1, 3 and 7 days. ALP activity was analysed using a QuantiChrom Alkaline Phosphatase Assay Kit (DALP-250; BioAssay Systems, Hayward, CA, USA). The protein concentration of each sample was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and ALP activity was normalized based on protein concentration.

Statistical analysis

Significant differences were determined using one-way ANOVA with a multiple comparison test (Bonferroni). Differences between means were considered to be statistically significant when $P < 0.05$. The data presented are from one of two identical experiments performed at least in triplicate.

Results

In vivo study (Paper I)

Orthodontic tooth movement and extensive root resorption

In rats, experimental orthodontic tooth movement by means of two force activation cycles with a passive force period in between resulted in extensive root resorption in all molars subjected to experimental tooth movement (15 rats). Root resorption with pulp exposure was observed in seven rats, and eight rats showed extensive root resorption with thin remaining dentine. In control second molars, there was no evidence of root resorption on the mesial surface of the distal root.

Recruitment of immune cells at sites of extensive root resorption

Significantly greater recruitment of CD68-IR cells and MHC class II Ia-expressing cells was observed in the compressed PDL of the experimental teeth than in the PDL of the control teeth ($P < 0.05$), whereas the density of CD43-IR cells was comparable in the PDL of the control and experimental teeth. Based on morphology, the small CD68-IR cells and MHC class II Ia-expressing cells were dendritic cells and macrophages. The large CD68-IR cells, found in the vicinity of bone-resorbing surfaces and root resorption lacunae, were osteoclasts and odontoclasts, respectively. In the dental pulp, there was no significant difference between the control and experimental teeth with respect to recruitment of CD68-IR cells, MHC class II Ia-expressing cells, and CD43-IR cells.

Expression of PGP 9.5-IR nerve fibres and laminin-IR blood vessels at sites of extensive root resorption

The presence of PGP 9.5-IR nerve fibres could not be detected in the mid PDL of either control or experimental teeth. Sprouting of nerve fibres was not detected at the resorption sites. No morphological differences were observed in the pulpal nerve supply of the control and experimental teeth.

Unlike the PGP 9.5-IR nerve fibres, laminin-IR blood vessels were scattered in the PDL of the control molars and observed densely in the resorption lacunae of the experimental molars.

In vitro study (Papers II and III)

Effects of CF on HOBs: cell viability, proliferation and apoptosis (Paper II)

Based on MTT assays, no magnitude of CF had an effect on the viability or proliferation of the cells one hour after force application. Following the application of 3.0 g/cm^2 and 4.0 g/cm^2 of CF for 3-48 hours, cell viability and proliferation decreased significantly ($P < 0.05$), whereas cells subjected to CF of 1.0 g/cm^2 and 2.0 g/cm^2 had viability and proliferative characteristics comparable to the "control cells." At 24 hours, there was a significant decline in the percentage of attached HOBs and the mRNA expression of KI-67 in HOBs cultured under 2.0 g/cm^2 and 4.0 g/cm^2 of CF, compared to the control ($P < 0.01$). At 72 hours, all magnitudes of CF resulted in significant reductions of cell viability and proliferation ($P < 0.05$) and in the percentages of attached cells ($P < 0.01$). The results of the MTT assay are shown in Paper II. The percentages of attached HOBs and the expression of KI-67 mRNA are summarized in Table 5.

The effects of CF on cell apoptosis were also investigated. Application of CF resulted in a slight increase in the expression of both Bax and Bcl-2 mRNAs. However, the difference was not statistically significant.

Table 5 *Summary of the effects of CF on HOBs, compared to the control, with respect to cell proliferation, measured by MTT assay and mRNA expression of KI-67 (a proliferation marker) and the percentages of attached HOBs*

Time (hour)	Compressive force (g/cm^2)							
	1.0	2.0			3.0	4.0		
	MTT	MTT	KI-67	% cells	MTT	MTT	KI-67	% cells
1	NS	NS	-	-	NS	NS	-	-
3	NS	NS	-	-	*	**	-	-
24	NS	NS	**	**	**	**	**	**
48	NS	NS	-	-	*	**	-	-
72	*	*	-	**	**	**	-	**

Cell proliferation measured by MTT assay (MTT), expression of KI-67 mRNA (KI-67) and percentages of attached cells (% cells). NS: No significant difference *, $P < 0.05$; **, $P < 0.01$.

Effects of CF on the expression of markers related to bone remodelling (Papers II and III)

mRNA level

At 24 hours, application of CF of both 2.0 g/cm^2 and 4.0 g/cm^2 resulted in a significant increase in the expression of ALP, Col 1, IL-6 and CXCL8 mRNAs ($P < 0.01$), compared to the control. Only HOBs subjected to 4.0 g/cm^2 of CF exhibited significantly increased expression of RANKL mRNA compared to the control. CF elicited no change in expression of OPN or OCN mRNAs. Transcription factor Runx-2 and OPG mRNAs were significantly inhibited by CF of both 2.0 g/cm^2 and 4.0 g/cm^2 ($P < 0.05$), compared to the control. The results are summarized in Table 6.

Table 6 *Summary of the effects of CF for 24 hours on the mRNA expression of markers related to bone apposition and resorption, compared to the control*

Bone apposition marker	Compressive force (g/cm^2)		Bone resorption marker	Compressive force (g/cm^2)	
	2.0	4.0		2.0	4.0
ALP	↑**	↑**	IL-6	↑**	↑**
Col 1	↑**	↑**	CXCL8	↑**	↑**
OPN	NS	NS	RANKL	NS	↑*
OCN	NS	NS	OPG	↓*	↓*
Runx-2	↓*	↓**			

↑: Increase; ↓: Decrease; NS: No significant difference *, $P < 0.05$; **, $P < 0.01$.

Protein level

The effect of CF on both extracellular and intracellular ALP activity was investigated. Application of 2.0 g/cm^2 and 4.0 g/cm^2 of CF significantly increased extracellular ALP activity in a force-dependent manner on day 1 ($P < 0.01$), and only 4.0 g/cm^2 of CF significantly enhanced ALP activity on days 3 and 7 ($P < 0.01$). Intracellular ALP activity was significantly reduced on days 1 and 7 ($P < 0.05$) (Paper III).

The production of Col 1 significantly declined on day 1 after force application ($P < 0.05$). After 3 and 7 days, the production of Col 1 by the experimental groups was comparable to the control group (Paper III).

CF of 2.0 g/cm^2 and 4.0 g/cm^2 resulted in a significant decrease in the production of IL-6, CXCL8 ($P < 0.01$) (Paper II) and OPG ($P < 0.05$) in the culture medium, whereas no soluble RANKL was detected (Paper III). Immunofluorescent staining disclosed the presence of RANKL in the cytoplasm of the HOBs of both the experimental and control groups. The RANKL signal was relatively dense in the

cytoplasm of HOBs subjected to 4.0 g/cm^2 of CF. OPG cells with strong positive staining were observed in the control group, but the cells subjected to CF appeared to stain weakly (Paper III).

Compared to the control, the release of PGE2 increased slightly after the application of 2.0 g/cm^2 of CF and increased significantly after the application of 4.0 g/cm^2 of CF ($P < 0.05$) (Paper III).

Discussion

The application of orthodontic force induces dynamic changes in the cells of the periodontium and alveolar bone, on both the compression and tension sides, stimulating remodelling of the PDL and alveolar bone, and ultimately relocation of the tooth. Changes have been observed in various cell types during orthodontic tooth movement, such as cells of the immune, vascular and nervous systems (Davidovitch, 1991), implying that these cells influence the remodelling processes involved in orthodontic tooth movement and root resorption.

The *in vivo* study in the present thesis was undertaken in order to investigate the distribution and number of immune cells, including monocytes, macrophages, dendritic cells, MHC class II Ia-expressing cells and lymphocytes, in the PDL and dental pulp of teeth undergoing extensive orthodontic root resorption. The orthodontic force was achieved by two force activation cycles, with a passive appliance in the intervening period. A previous study in rats, using a similar appliance and the same magnitude of force, demonstrated that the hyalinized tissues had not been completely removed by day 10; some hyalinized tissue persisted for up to 21 days after the initial application of orthodontic force (Brudvik and Rygh, 1994a). It was hypothesized that after the de-activation period, a passive retention appliance would still create some stress in the PDL and the hyalinized tissues would persist. If the orthodontic appliance is re-activated while hyalinized tissue remains, root resorption is likely to continue (Brudvik and Rygh, 1994a). The present finding supports this hypothesis.

Previous studies have demonstrated significant recruitment of monocytes, macrophages, dendritic cells and MHC class II Ia-expressing cells in the compressed PDL of teeth undergoing orthodontic movement (Vandevska-Radunovic *et al.*, 1997a; Nakamura *et al.*, 2001). These macrophages and dendritic cells are assumed not only to participate in phagocytosis of the necrotic hyalinized tissues and antigen presentation, but also to act as a source of bone-resorptive cytokines, e.g., IL-1 and IL-6, which were detected at both mRNA and protein levels in the compressed PDL of the

teeth being moved (Alhashimi *et al.*, 2001; Haug *et al.*, 2003; Bletsa *et al.*, 2006). In Study I, there was a significant presence of MHC class II Ia-expressing cells, monocytes, macrophages and dendritic cells in the compressed PDL of the experimental teeth, but not in the dental pulp. This result suggests that CD68-IR cells and MHC class II Ia-expressing cells play a crucial role in remodelling of the PDL during extensive root resorption. This *in vivo* study clearly showed that inflammation, evidenced by the presence of immune cells in cases of pulp exposure caused by orthodontically-induced root resorption, was confined mainly to the compressed PDL, while the dental pulp was barely affected.

Whereas CD68-IR macrophages and dendritic cells are widely distributed in the normal dental pulp (Okiji *et al.*, 1992), CD43-IR cells, mainly lymphocytes and granulocytes, are uncommon (Haug *et al.*, 2003). The study by Haug *et al.* (2003) disclosed increased recruitment of CD43-IR cells in the dental pulp after 10 days of orthodontic tooth movement, indicating an associated pulpal inflammation. In contrast, in Study I, few CD43-IR cells were observed in the dental pulp of the experimental teeth and there was no significant change in cell recruitment compared to the pulp of the control teeth. This implies that orthodontic tooth movement may create only transient inflammatory responses in the dental pulp.

Orthodontically-induced root resorption is generally an incidental radiographic diagnosis, as the pulp remains asymptomatic, even after extensive resorption (Sasakura *et al.*, 1984; Savage and Kokich, 2002), unless it is secondarily compromised by inflammation or infection. Pulpal insult, including during the early phase of orthodontic force application, causes nerve fibre sprouting and neurogenic inflammation (Vandevska-Radunovic *et al.*, 1997b). In the present study however, no sprouting of nerve fibres was observed in the dental pulp, or in the extensively inflamed root resorption sites.

Earlier clinical studies have shown that pain is often perceived within 24 hours of activation of orthodontic force and subsides during the first week (Ngan *et al.*, 1989; Wilson *et al.*, 1989). This corresponds with the disappearance of nerve sprouting after 38-41 days. In addition, teeth which have undergone extensive root resorption

tend to be more vascularized than normal tissue, evidenced in the present study by dense laminin-IR blood vessels in the extensive root resorption sites.

Lymphocytes and osteoblasts play an important role in inflammatory bone resorption, through RANKL-mediated osteoclastogenesis. The involvement of immune cells in the process of bone resorption following periodontal disease has been demonstrated by the expression of RANKL on activated T- and B-lymphocytes (Kawai *et al.*, 2006). RANKL expressed by T-lymphocytes and osteoblasts triggers differentiation of osteoclast progenitor cells into mature osteoclasts. In Study I, massive bone and root resorption were observed on the compression side of the experimental teeth, but few lymphocytes were observed as a result of aseptic inflammation in this area. It was hypothesized that osteoblasts, but not lymphocytes, are a primary source of RANKL during extensive orthodontic root resorption. In a recent study, PGE2, a strong osteoclast-inducing factor, caused considerably greater enhancement of RANKL expression by primary osteoblasts than by PDL cells. This suggests that primary osteoblasts have a greater potential than PDL cells to induce osteoclastogenesis via RANKL expression (Mayahara *et al.*, 2012). Another recent study has been shown that CF enhances RANKL expression in the osteoblastic cell line MC3T3-E1 in a magnitude-dependent manner (Sanuki *et al.*, 2010).

On the basis of the above findings, osteoblasts derived from alveolar bone were used in the *in vitro* studies, to investigate the cellular responses of osteoblasts and their roles in remodelling of the PDL and alveolar bone under orthodontic force. In order to study the effect of the force on a single cell type, studies II and III were based on a cell culture model.

Recent decades have seen a great expansion of knowledge in the field of cell and molecular biology. Cell culture techniques are increasingly applied in studies of the complex mechanisms underlying orthodontic tooth movement (Kanzaki *et al.*, 2002; Mitsui *et al.*, 2005; Mitsui *et al.*, 2006a; Mitsui *et al.*, 2006b; Yamaguchi *et al.*, 2006; Nakao *et al.*, 2007; Sanuki *et al.*, 2007; Wescott *et al.*, 2007; Koyama *et al.*, 2008; Nakajima *et al.*, 2008; Yanagisawa *et al.*, 2008; Miyagawa *et al.*, 2009; Sanuki

et al., 2010; Li *et al.*, 2011). Kanai *et al.* (1992) established the uniform compression model for vertical application of CF onto human PDL cells. This *in vitro* model is a useful research tool for clarifying cell reactions under CF and has been used to follow the sequence of events on the compression side of teeth undergoing orthodontic movement.

Over the past decade, the application of cell culture models to elucidate the responses of PDL and bone cells to orthodontic forces has increased. Two approaches are currently used: primary cell culture and established cell lines.

A fresh isolate of cells cultured *in vitro* is termed "primary culture". In general, cells in primary culture are heterogeneous, but they consist of many cell types which are representative of the tissues (Pizzoferrato *et al.*, 1994). Examples of primary cultures used in such studies are PDL derived from healthy premolars extracted for orthodontic treatment (Kanzaki *et al.*, 2002; Yamaguchi *et al.*, 2006; Nakajima *et al.*, 2008; Miyagawa *et al.*, 2009) and osteoblasts derived from pieces of alveolar bone collected during surgical removal of third molars (Mustafa *et al.*, 2000). Mustafa *et al.* (2000) demonstrated that osteoblasts derived from alveolar bone can express many known specific bone markers.

Although the primary cells are physiologically similar to *in vivo* cells, there are some disadvantages of using this cell type: availability is unreliable, life span in culture is short and culture is susceptible to contamination. More importantly, primary cells derived from different patients can behave differently in culture conditions, due to donor-to-donor variation (Stewart *et al.*, 2012). The results of the present work exhibited such donor-related variability, hence multiple experiments from different donors were needed to confirm the data. In Papers II and III, after multiple experiments from various donors, similar results were observed from at least two donors in each experiment and the data from one donor were selected as representative. The aim of the present *in vitro* studies was to confirm the effect of CF on primary osteoblasts, rather than to investigate individual biological variation between donors; therefore, the representative data were presented in the results in each experiment. However, biological variation between donors should not be discounted.

Established cell lines are also commonly used in *in vitro* studies. Most such cell lines originate from bone tumours and include osteosarcoma cells. Many studies have been based on established osteoblastic cell lines derived from bone tumours of either rodents or humans, e.g., Saos-2 cells (Mitsui *et al.*, 2005; Mitsui *et al.*, 2006a; Mitsui *et al.*, 2006b; Sanuki *et al.*, 2007; Koyama *et al.*, 2008), MC3T3-E1 (Sanuki *et al.*, 2010; Shionome *et al.*, 2012), ROS 17/2.8 (Yanagisawa *et al.*, 2008), and MG-63 cells (Goga *et al.*, 2006). Established cell lines provide consistency and reproducibility of the results, because the cells are derived from a batch of clonal cells. In addition, they appear to be uniform and make it feasible to have a large number of homogenous cells available for use over long periods of time. Nevertheless, there are reports in the literature of differences between primary osteoblasts and osteosarcoma cell lines.

One such difference was reported by Pautke *et al.* (2004), who revealed the heterogeneity of osteosarcoma cell lines, including Saos-2, MG-63 and U-2, using immunohistochemistry to show different labelling patterns of extracellular matrix proteins of the cells in each cell line. Morphometric analysis shows that compared with cells from osteosarcoma cell lines, normal human osteoblasts are approximately 6 times larger, but about 2-3 times faster in doubling-time. The same authors also found that in contrast to the osteosarcoma cell lines, primary osteoblasts exhibit some extracellular matrix proteins dependent on cell density (Pautke *et al.*, 2004).

In extrapolating the results of experiments using either primary culture or established cell lines to the clinical setting, primary cell culture is generally considered to be more physiologically relevant to actual responses in humans. Although osteosarcoma cell lines retain many bone markers, they are different from those in normal bone cells. From a biological point of view, the use of human oral diploid cells in *in vitro* studies is more reliable than the use of aneuploid cell lines derived from other tissues and species. In this context, it was deemed appropriate to use primary human osteoblasts derived from alveolar bone in the present studies.

There are two well-established methods for isolating cells from tissues: proteolytic digestion and explant-outgrowth. Although the enzyme digestion technique

has been successfully used to obtain osteoblast-like cells from human bone (Evans *et al.*, 1990), the explant-outgrowth technique causes less cell damage from the protease enzymes (Lieberherr *et al.*, 2003). Numerous studies have used the latter technique for cell isolation from human mandibular bone specimens (Mustafa *et al.*, 2000; Mustafa *et al.*, 2001; Mustafa *et al.*, 2002; Mustafa *et al.*, 2003; Idris *et al.*, 2010).

At present, *in vitro* identification of osteoblasts remains contentious: to date, there is no specific marker, able to detect only osteoblasts. ALP has often been used as a biochemical marker to assess osteoblast differentiation and is known to be involved in skeletal mineralization. The presence of ALP and up-regulation of cyclic adenosine monophosphate in response to parathyroid hormone are two characteristic markers that have been used to identify osteoblasts. Nevertheless, it has been reported that PDL fibroblasts also possess these properties, as they have the potential to differentiate into either osteoblasts or cementoblasts (Nojima *et al.*, 1990). A previous study investigated differences in ALP staining of gingival fibroblasts, PDL fibroblasts and HOBs: in normal culture, ALP staining was moderate for HOBs and slight for PDL fibroblasts and gingival fibroblasts (Chou *et al.*, 2002). The PDL cells are heterogeneous, comprising many cell types; it is not clear whether the osteogenic potential is actually attributable to one type of cell or to various cells in the PDL.

Saito *et al.* (2002) reported an interesting study to differentiate PDL cells and osteoblasts both *in vivo* and *in vitro.* The results showed that all PDL cells were positive for ALP, but only 80 and 60 per cent were positive for Col 1 and Runx-2 mRNAs, respectively. No PDL cells were stained by the OCN probe, whereas the MC3T3 osteoblast cell line showed intense staining. Comparable Runx-2 staining was observed in PDL cells and osteoblasts. *In vitro,* RT-PCR disclosed differences between PDL cell lines and MC3T3 cells: the PDL cells were negative for OCN and BSP, whereas MC3T3 cells were positive. (Saito *et al.*, 2002). Although the PDL cells were able to express Runx-2, the expression was less than for MC3T3 cells at both mRNA and protein levels. The PDL cells did not present mineralized nodules, unless they were treated with BMP-2 (Saito *et al.*, 2002).

In an earlier study it had been suggested that osteoblasts should be identified by determining the ability of cells to mineralize and express collagenous and non-collagenous bone-specific proteins, a property which distinguishes osteoblasts and PDL fibroblasts (Robey and Termine, 1985).

In the present study, all cell batches used showed very high ALP staining and the ability to express many bone-related markers, including ALP, Col 1, OPN, OCN and Runx-2. Moreover, mineralized nodules appeared in HOBs cultured in an osteogenic medium for 11 days.

In orthodontic tooth movement, canine retraction and mesialization of molars is nowadays commonly based upon the application of orthodontic appliances with light force, e.g., Nickel-Titanium coil springs, which have been claimed to provide optimum tissue responses and rapid tooth movement (Angolkar *et al.*, 1992). Although force degradation of the springs is less than that of elastomeric chains, Angolkar *et al.* (1992) observed significant degradation of the force from Nickel-Titanium springs 24 hours to three days after the springs were activated.

Accordingly, in the present *in vitro* studies, the osteoblasts were subjected to CF for only short periods of time. With reference to the above-cited studies, the results after 24 hours were of special interest. Under clinical conditions, it is highly unlikely that CF can be sustained over a longer period, hence the maximum duration of application of CF in the present *in vitro* studies was set at 7 days. In a previous study applying a uniform compression model and human PDL cells, a range of force magnitudes, up to 4.0 g/cm^2 of CF, was used. The maximum CF resulted in some damage to the PDL cells (Kanzaki *et al.*, 2002). The same range was applied in the present studies.

Optimal orthodontic CF would achieve rapid tooth movement with minimal undesirable tissue destruction. Proffit (2012) describes the optimal force as the lightest force and resulting pressure that produces a near-maximum response. Greater force would be unnecessarily traumatic and stressful to the anchorage teeth. The present

microscopic findings showed a few atrophic HOBs after application of 4.0 g/cm^2 of CF for 24 and 72 hours. This implies that under the current experimental conditions, a CF of 4.0 g/cm^2 would result in some damage to the cells and could be considered excessive.

Remodelling is a continuous physiological process to maintain bone integrity. During orthodontic tooth movement, the applied forces cause an imbalance in tissue remodelling, not only in the PDL, but also in the alveolar bone. Mabuchi *et al.* (2002) found that the ratios of cell proliferation and apoptosis in the compression and tension sides are closely related to the remodelling of the PDL during orthodontic tooth movement. In Paper II, the effects of CF on cell proliferation and apoptosis were investigated using different parameters. Semi-quantitative image analysis showed that 24 hours after application, CF reduced the percentage of attached HOBs in the culture plate. This was confirmed by a significant decrease in the expression of KI-67 mRNA by HOBs under the CF. In addition, the MTT results revealed that after 3-48 hours, higher CF (3.0 g/cm^2 and 4.0 g/cm^2) resulted in decreased cell viability and proliferation. These findings are in accordance with those of an *in vivo* study by Brooks *et al.* (2009), that after orthodontic tooth movement for 24 hours, half of the compressed PDL cells stained positively to KI-67 protein. An earlier study by Mabuchi *et al.* reported pronounced KI-67 staining on the tension side at 72 hours, indicating proliferative activity of the PDL cells (Mabuchi *et al.*, 2002).

In addition to cell proliferation, the remodelling process also involves cell apoptosis. Apoptosis or programmed cell death is characterized by typical morphological changes: condensation of chromatin, cell fragmentation, and formation of apoptotic bodies. Several methods are used to evaluate cell apoptosis. The caspases, especially caspase-3, are known to act downstream of Bax/Bcl-2 ratios, leading to the induction of apoptosis. A positive correlation has been shown between the Bax/Bcl-2 ratio and caspase-3 activation (Salakou *et al.*, 2007).

In Paper II, the effect of CF on cell apoptosis was investigated by determining expression of Bax and Bcl-2 mRNAs. The results disclosed slightly enhanced expression of both 24 hours after force application, but the difference between the

experimental and the control groups was not significant. The results indicate that application of CF, even at 4.0 g/cm^2, neither disturbs the balance of Bax and Bcl-2 nor enhances apoptosis of the HOBs.

In contrast, an earlier study has reported that CF is able to induce apoptosis in human osteosarcoma cell line MG-63 in a force-dependent manner, through activation of caspase-3 (Goga *et al.*, 2006). This inconsistency in results might be attributable to the use of different cell types in the studies. Such differences between the osteosarcoma cell lines and primary osteoblasts have been reported previously (Perez *et al.*, 2003; Pautke *et al.*, 2004).

During orthodontic tooth movement, it is generally agreed that tension leads to bone formation, whereas compression causes bone resorption. Tooth movement towards the compression side is the result of remodelling of the alveolar bone and the PDL. There is however, ample evidence that during tooth movement, induction of bone formation and resorption, in fact, occur on both sides, but one effect usually predominates (Krishnan and Davidovitch, 2006; Kim *et al.*, 2012).

Three important roles of osteoblasts during bone formation are to induce and to regulate extracellular matrix mineralization, and to mediate bone remodelling. ALP is a hydrolase enzyme responsible for breakdown of pyrophosphate, an inhibitor of calcium phosphate deposition (Christenson, 1997). Col 1 is a major component of the organic extracellular matrix. At the initial stage of bone formation, osteoblasts synthesize and produce ALP and Col 1 in order to provide extracellular matrices suitable for mineral deposition. Later, the non-collagenous proteins OPN and OCN are expressed and participate in mineral deposition.

ALP and Col 1 are commonly used as markers in studying the effects of mechanical stimulation on osteoblasts (Pavlin *et al.*, 2000). Stimulation of osteoblasts by intermittent CF has been reported to promote ALP and Col 1 expression (Roelofsen *et al.*, 1995). Kubota *et al.* (1993) reported that CF also enhanced ALP activity in ROS 17/2.8 cells, but that Col 1 synthesis was unchanged . In a study of CF on MC3T3-E1

cells, both ALP activity and collagen synthesis were suppressed (Ozawa *et al.*, 1990). Moreover, it has been reported that during orthodontic tooth movement in cats, osteoblasts lining the alveolar bone surfaces stained positively for ALP (Krishnan and Davidovitch, 2006). In other studies on ROS 17/2.8 and Saos-2 cells, application of CF for one day upregulated bone-specific gene expression, including bone sialoprotein, OPN, OCN and bone morphogenic proteins (Mitsui *et al.*, 2005; Mitsui *et al.*, 2006b; Yanagisawa *et al.*, 2008).

In Paper III, CF induced upregulation of expression of ALP mRNA and the production of extracellular ALP. However, CF did not alter the expression of OPN or OCN mRNA. The results suggest that CF might affect only the initial stage of bone formation. From the present data, it was evident that the response to CF by primary osteoblasts differs from that of the ROS 17/2.8 and Saos-2 established cell lines.

The present study found reduced production of Col 1 under CF on day one, but the reduction was not associated with the expression of Col 1 mRNA. Cell exposure to stress, e.g., temperature changes and hypoxia, provoke adaptive responses in stress-related gene expression and also reduction in the process of translation to maintain cellular energy (Holcik and Sonenberg, 2005). It is hypothesized that the CF might encourage adaptation of osteoblasts, which would result in decreased expression of Col 1 protein on day 1 after force application.

Runx-2 is a transcription factor involved in osteoblast differentiation and acts as an important regulator of bone formation at multiple stages (Karsenty, 2000). During osteogenesis, two major pathways are recognized: Runx-2-dependent and Runx-2-independent (Ikeda *et al.*, 2005). It has been demonstrated that Runx-2 can induce OCN expression in non-osteoblastic cells *in vitro* (Ducy *et al.*, 1997). Watanabe *et al.* (2007) reported that osteoblasts lining the alveolar bone surfaces in the tension zone of orthodontically-moved teeth stained positively against Runx-2 antibody. In Paper III, CF inhibited Runx-2 mRNA expression, despite marked increases in ALP and Col 1, indicating that the upregulation of ALP and Col 1 might be independent of Runx-2.

Several research groups are currently investigating the interaction between osteoblasts and osteoclasts in orthodontically-induced bone remodelling. In Papers II and III, cytokines/chemokines implicated in osteoclastogenesis, including IL-6, CXCL8, RANKL and OPG, were investigated. After 24 hours of application, CF induced the expression of IL-6 and CXCL8 mRNAs in a force-dependent manner. The increased expression of IL-6 mRNA from HOBs after force application is in agreement with the findings of previous studies on Saos-2 cells (Koyama *et al.*, 2008) and on primary osteoblasts derived from rat mandibles (Yamamoto *et al.*, 2011). However, the increased expression of CXCL8 mRNA in the present study contradicts a recent report that expression of CXCL8 mRNA was unchanged up to 24 hours after force application (Koyama *et al.*, 2008).

Previous reports have indicated that IL-6 and CXCL8 proteins from various cell types, e.g., osteoblasts and peripheral blood mononuclear cells were significantly expressed in the extracellular fluid when exposed to different stimuli, e.g., lipopolysaccharide and inflammatory cytokines IL-1 (Schindler *et al.*, 1990; Silfversward *et al.*, 2004). In addition, increased levels of IL-6 and CXCL8 were found in gingival crevicular fluid of patients undergoing orthodontic treatment (Basaran *et al.*, 2006a; Ren *et al.*, 2007; Leethanakul *et al.*, 2008). In Paper II, a multiplex assay was used to determine protein expression of IL-6 and CXCL8 in the culture medium. Multiplex assay allows simultaneous, independent, quantitative assay of small volumes of samples of multiple analytes and is more economical and less time-consuming than other methods, including ELISA.

The present results reveal a marked decrease in IL-6 and CXCL8 expression under CF compared to the control. The inconsistent results for mRNA and protein levels might be attributable to a reduction in the number of cells available to release the cytokines/chemokines after force application: there were markedly fewer cells under CF than in the control. In addition, up-regulated levels of IL-6 and CXCL8 mRNAs might have exerted negative feedback at the post-transcriptional level, thereby inhibiting the protein expression of IL-6 and CXCL8. Moreover, in the present study,

release of other molecules under CF might also have had a suppressant effect on the expression of IL-6 and CXCL8 proteins.

Possible roles of IL-6 and CXCL8 during the chronic phase of inflammation are induction of leukocyte recruitment at the site of injury and angioproliferation (Bendre *et al.*, 2003; Jones, 2005). It may be speculated that activated alveolar bone-derived osteoblasts are involved in the recruitment of cells in monocyte and macrophage lineage and the increase in blood vessel proliferation in the extensive root resorptive site. The high level of expression of IL-6 and CXCL8 is found in many degenerative diseases of bone, such as rheumatoid arthritis and osteoarthritis (Kaneko *et al.*, 2000). Moreover, an increased level of CXCL8 in the PDL has been observed during orthodontic root resorption in rats (Asano *et al.*, 2011). Recent studies suggest that IL-6 and CXCL8 not only play a role in inflammatory response, but also directly facilitate osteoclastogenesis and osteoclast-mediated bone/root destruction by binding to their specific receptors, IL-6R and CXCR1 respectively, on osteoclasts and their progenitors (Bendre *et al.*, 2003; Axmann *et al.*, 2009; Asano *et al.*, 2011).

Identification of the cytokines RANKL and OPG, which mediate osteoclastogenesis by HOBs, led to improved understanding of bone biology. The effect of CF on RANKL and OPG expression was investigated in Paper III. At 4.0 g/cm^2 of CF, expression of RANKL mRNA was up-regulated, suggesting that CF can stimulate RANKL expression by HOBs. In accordance with a previous *in vivo* study (Shiotani *et al.*, 2001), the present study also demonstrated that CF induces protein expression of RANKL in the cytoplasm of HOBs. Quantitative analysis by ELISA failed to detect any production of soluble RANKL in the culture medium in the present study. Under the *in vitro* conditions it is possible that HOBs were able to express only the membrane-bound form of RANKL. Moreover, the present study showed that CF suppressed OPG expression in HOBs, at both mRNA and protein levels. Thus the results indicate that CF up-regulates RANKL expression and down-regulates OPG expression by HOBs. It may be concluded that in bone and root resorption induced by inflammation, alveolar bone-derived osteoblasts, but not lymphocytes, might be a primary source of RANKL.

The release of the inflammatory mediator PGE2 from HOBs was also investigated. Several studies have demonstrated that an increased level of RANKL is dependent on PGE2 production in pre B-cells and in PDL cells (Kanematsu *et al.*, 2000; Kanzaki *et al.*, 2002). In Study III, the expression of RANKL and PGE2 was significantly up-regulated when HOBs were subjected to 4.0 g/cm^2 of CF. These findings are consistent with those of previous reports. However, further study is warranted to determine whether the up-regulation of PGE2 in the culture medium has a positive correlation with the expression of RANKL in HOBs.

In the present study, HOBs were subjected to 1.0 – 4.0 g/cm^2 of CF for up to 72 hours. Comparison of cell viability and proliferation in the experimental and control groups showed that cells subjected to this range of compressive force were still in good condition even after 72 hours and had not been damaged by CF.

The *in vitro* results show HOBs are mechanoresponsive: they react to CF through mechanotransduction mechanisms, by which the cells convert mechanical signals into a series of biological events, such as the expression of numerous genes, including those responsible for inflammatory responses and bone remodelling. Further study is warranted to elucidate the intracellular signalling pathways of mechanotransduction mechanisms leading to gene transcription.

Conclusions

- The application of orthodontic appliances using two force activation cycles in rats, with a passive intervening period, resulted in extensive root resorption. At the resorption sites, there were substantial influxes of MHC class II Ia-expressing cells, monocyte-macrophage lineage cells and dendritic cells, but not of granulocytes or lymphocytes. The results confirm the role of the inflammatory response of the cells of the PDL in tissue remodelling associated with extensive root resorption.

- Inflammation associated with extensive root resorption appeared to be confined to the compressed PDL. The dental pulp was not inflamed, even in cases of pulpal exposure. The clinical implication of this finding is that in cases of extensive orthodontic root resorption, provided there is no infection, endodontic treatment is not indicated.

- HOBs exhibit several known bone markers, thus expressing osteogenic potential, and behave differently from established cell lines in response to CF. These *in vitro* studies demonstrated that cell culture of primary osteoblasts is appropriate for such investigations.

- The application of varying magnitudes of CF (0-4 g/cm^2) *in vitro* inhibited mainly cell-proliferative activity, without inducing cell apoptosis. However, the use of heavy force (4.0 g/cm^2) should be avoided due to the risk of cell damage.

- Initial application of CF to HOBs stimulates the expression of molecules involved in the tissue remodelling process. This may reflect the role of osteoblasts in tissue remodelling during orthodontic tooth movement.

- In response to CF, HOBs express the inflammatory mediators IL-6 and CXCL8 and RANKL. This suggests that osteoblasts have important roles in the inflammatory response phase of the bone-resorption process which is fundamental to orthodontically–induced tooth movement.

Future perspectives

At present, it seems likely that new insights into this field of study will be acquired from experiments which integrate the findings of *in vivo* and *in vitro* studies to explain the biological responses of cells adjacent to orthodontically-moved teeth at the tissue, cellular and molecular levels. Many questions in this field of research remain unclarified, among them the effects of CF on the interaction of osteoblasts with other cell types, e.g., endothelial cells and cells of epithelial rests of Malassez, in the periodontal tissues.

To date, most studies of gene expression in primary human cells have been based on 2D culture, in which the cells proliferate over the culture plate. It is suggested that 3D culture is preferable, as the shape of the cells more closely resembles those *in vivo.*

Another issue that is not clearly understood is the healing process of the root surface in the tooth undergoing extensive root resorption. Future investigations should focus on molecular determinants initiating the healing process in the affected teeth, leading to improved understanding of the underlying mechanism.

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