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Cellular and extracellular factors in early root resorption repair in the rat

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SUMMARY The aim of this study was to investigate the role of extracellular matrix components, such as collagen type I, fibronectin, and osteopontin (OPN) during cementum repair following experimentally induced tooth movement, and to characterize the cells taking part in the regenerative process. The upper right first molars were moved mesially in 21 three-month-old male Wistar rats using a coil spring with a force of 0.5 N. After 9 days, the appliance was removed and the animals were killed in groups of three immediately after withdrawal of the force and 5, 7, 10, 12, 14, and 17 days later. Three rats served as non-experimental control animals. The maxillae were prepared and processed for histological analysis.

Together with the disappearance of the multinucleated odontoclasts from the resorption lacunae, signs of repair were visible 5 days after the release of the orthodontic force. The first signs of cementum repair were seen on day 10. The newly produced cementum was of the acellular extrinsic fibre type (AEFC) and reattachment was achieved with the principal periodontal ligament (PDL) fibres orientated almost perpendicular to the root surface. The initial interface formed between the old and new cementum, as well as the new AEFC, was characterized by a strong immunoreaction with OPN and collagen I antibody, but only a weak immunoreaction with the fibronectin antibody. Only a small number of mononuclear cells, which were involved in the repair process, showed a positive immunoreaction with the osteoblastic lineage markers runt-related transcription factor 2 and osteocalcin. These same cells stained sparsely with muscle segment homeobox homologue 2, but not with the E11 antibody. Thus, most of the cells associated with this reparative activity on the surface of the lacunae were differentiated PDL cells of the fibroblastic phenotype. Cells with a defined osteoblastic phenotype seemed to be of minor importance in this repair process.

Introduction

Pathological root resorption is regularly observed as an unwanted side-effect following orthodontic tooth movement (Brezniak and Wasserstein, 2002; Krishnan and Davidovitch, 2006).

Orthodontic force application induces local tissue degradation followed by a process that typically includes inflammatory characteristics (Brezniak and Wasserstein, 2002). The cells that are initially involved in removal of necrotic and hyalinized tissue are negative for tartrate-resistant acid phosphatase (TRAP) and possess a macrophage-like character (Jäger *et al.*, 1993). The multinucleated cells, which appear later actively resorbing cementum as well as dentine, are TRAP positive and are called 'odontoclasts' (Sasaki, 2003).

The destructive process of root resorption is typically followed by reparative activity of the periodontal ligament (PDL) tissues. When there is no more hyaline tissue present and/or the force level diminishes, the resorption process stops and the cementum starts to repair (Brudvik and Rygh, 1995a,b). Initially, the odontoclasts lose their resorption activity and detach from the resorbed surface (Sahara *et al.*, 1996). Detached odontoclasts probably die due to apoptosis, as has been shown for osteoclasts at the alveolar bone (Noxon *et al.*, 2001).

After detachment of odontoclasts, an initial uncalcified thin cementoid repair matrix on resting collagenous structures is deposited by repair cells, namely, fibroblast-like and cementoblastic cells (Brudvik and Rygh, 1995a,b; Bosshardt, 2005). Repair cementum which is initially secreted by these cells is of the cellular intrinsic fibre type in humans and of the acellular extrinsic fibre type (AEFC) in rodents (Bosshardt and Schroeder, 1996; Owman-Moll and Kurol, 1998).

The regulatory factors responsible for the switch from resorption to repair, the recruitment and activation of repair cells from the periodontium, and the secretion of repair cementum are largely unknown. At present, it is unclear whether there is one common progenitor cell type for the different periodontal connective tissue cells or whether distinct progenitor subpopulations, for example, for cementoblasts, exist. A variety of chemotactic factors, adhesion molecules, growth factors, and extracellular matrix (ECM) constituents participate in the recruitment of cementoblast progenitors, their expansion, and differentiation, of which many may be available during

periodontal healing (Grzesik and Narayanan, 2002). Thus, it has been proposed that non-collagenous proteins of the ECM are involved in pre-cementoblast chemoattraction, adhesion to the root surface, and cell differentiation and may be of importance in the course of the regeneration process (Saygin *et al.*, 2000). Among others, fibronectin (FN), collagen type I, osteopontin (OPN), and osteocalcin (OCN) have been identified in the cementum matrix (Sasano *et al.*, 2001). After adhesion to the root surface, expansion as well as further differentiation of the progenitor cells is regulated by cytokines and growth factors which, to some extent, are present in the cementum matrix. Growth factors which have been identified in cementum include IGF-I, FGFs, EGF, BMPs, and TGF-beta (Bosshardt and Schroeder, 1996; Götz *et al.*, 2003, 2006a,b; Bosshardt, 2005).

Thus, it was the aim of this investigation to gain more information about the time course of the healing process especially with respect to the role played by components of the ECM. In addition, the fate of the resorbing odontoclasts was analysed using histochemical detection of TRAP while the origin of the reparative cells was studied by characterizing these cells with the help of immunohistochemical demonstration of muscle segment homeobox homologue 2 (msx2), runt-related transcription factor 2 (runx2), OCN, and the osteoblast differentiation marker E11 (Wetterwald *et al.*, 1996).

Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the local district government and the Animal Care Commissioner of the University of Bonn, Germany.

Animals

Twenty-four 3-month-old male Wistar rats with an average weight of 320 g, obtained from Charles River Laboratories, Sulzfeld, Germany, were used. Three rats served as a non-experimental control group. The animals were kept in plastic cages with a standard 12 hour light–dark cycle and fed on a soft diet and water *ad libitum*. During the experiments, the weights of the animals were recorded daily.

Experimental protocol

An orthodontic appliance was inserted under anaesthesia (Jäger *et al.*, 2005). Briefly, the appliance consisted of a stretched closed coil spring (0.012 inch nickel–titanium wire, GAC International Inc., New York, USA) ligated between the maxillary right first molar and the incisors, moving the molar mesially with a force of 0.5 N. After 9 days, the appliance was removed (day 0). Three animals were killed on days 0, 5, 7, 10, 12, 14, and 17 after appliance deactivation. Following sacrifice, the maxilla of each animal was dissected, divided into two halves, and prepared for light microscopic examination (Jäger *et al.*, 2005; Götz *et al.*, 2006b).

Histology and histochemistry

The right maxilla half of each animal were fixed in 4 per cent paraformaldehyde in 0.1 M phosphate buffer, decalcified in neutral 10 per cent ethylenediaminetetraacetic acid, and processed for paraffin histology. Serial sagittal sections were prepared and, for the analysis, central sections of complete lengths of the mesial roots of the first molars were chosen. For the purpose of evaluating root resorption and repair, analysis was concentrated on the mesial interradicular regions of the first molar.

Selected sections were stained with haematoxylin and eosin. In order to identify osteoclasts, odontoclasts, and their precursors, selected tissue sections were stained to demonstrate TRAP according to Barka and Anderson (1962).

Immunohistochemistry

Tissue sections were placed in a *tris*-hydroxymethyl aminomethane-buffered saline solution (TBS) at pH 7.4 for 10 minutes. Endogenous peroxidase activity was then blocked using methanol/H_2O_2 for 10 minutes in the dark. Subsequently, sections were rinsed and then pre-incubated with TBS containing 4 per cent bovine serum albumin (BSA) for 20 minutes to avoid unspecific background staining. Thereafter, sections were incubated with primary antibodies in a humidity chamber. The sources, characterization, and incubation protocols of the different antibodies used are shown in Table 1. Binding of the polyclonal rabbit antibody was visualized using EnVision™ peroxidase anti-rabbit (DakoCytomation, Hamburg, Germany), mouse monoclonal antibodies using EnVision™ peroxidase anti-mouse (DakoCytomation) for 30 minutes at room temperature, and the goat antibody using HRP-conjugated rabbit anti-goat IgG (DakoCytomation) diluted 1:50 in 1 per cent TBS–BSA 30 minutes at room temperature. Finally, tissue sections were stained with 3,3′-diaminobenzidine (Pierce, Rockford, Illinois, USA). After immunohistochemistry, all slides were rinsed and then counterstained with Mayer's haematoxylin, dehydrated, and cover slips were placed for light microscopic analysis.

Specificity controls were run by (1) omitting the primary antibody and incubating slides with TBS or normal horse serum and (2) omitting primary antibodies or bridge and secondary antibodies. Positive controls were carried out using rat tissues known to carry the antigens of interest (e.g. liver, kidney, and long bones).

Results

All animals remained healthy during the study and food and water ingestion appeared to be unaffected by the orthodontic treatment. All rats showed an increase in weight before sacrifice.

To document tooth movement, the distance between first and second molars was measured using calibrated gauges.

Table 1 Specification of the antibodies used for immunohistochemistry.

Antibody	Isotype	Producer	Incubation protocol	Predigestion
Collagen type I	Polyclonal rat	Chemicon (Temecula, California, USA)	1:200, 1 hour(h), room temperature (RT)	None
E11	Monoclonal mouse	A. Wetterwald, (University of Bern, Switzerland)	1:50, 1 h, RT	None
Fibronectin	Monoclonal mouse	Dunn Labortechnik Gmbh (Asbach, Germany)	1:50, 1 h, RT	None
msx2	Monoclonal mouse	Stressgen Biotechnologies Corporation (Victoria, British Columbia, Canada)	1:50, 1 h, RT	None
Osteocalcin	Monoclonal mouse	TaKaRa Bio Inc., (Otsu, Japan)	1:1000, 1 h, RT	None
Osteopontin	Monoclonal mouse	Abcam Plc (Cambridge, UK)	1:100, 1 h, RT	0.4% pepsin, 20 min, 37°C
runx2 (Cbfa1)	Molyclonal goat	Santa Cruz Biotechnology Inc., (Santa Cruz, California, USA)	1:30, overnight, 4°C	None

msx2, muscle segment homeobox homologue 2; runx2, runt-related transcription factor 2.

The amount of separation was 0.4–0.5 mm for animals sacrificed on day 0. This decreased during the experiment, so that no distance could be measured between the molars from day 17 animals and later.

Control maxillae

Maxillae from untreated animals showed a normal histological appearance of teeth and supporting tissues. A few small resorption lacunae on the distal side of the molars containing TRAP-positive odontoclasts indicated physiological distal drift.

Type I collagen immunostaining was found to be widespread in the periodontium, and the PDL matrix, in particular, was intensely stained. In the cellular cementum, peripheral parts of the matrix, cementoblasts, and cementocytes were also immunoreactive. In the alveolar bone, matrix osteocytes, osteoblasts, and osteoclasts were reactive.

OPN was found in the cementum lines, osteoid and osteocytes of bone, cementum lines and cementocytes of cellular cementum, in the acellular cementum, and along the dentine–cementum border. Only weak staining appeared in cementoblasts and focally in PDL cells.

Immunoreactions for FN antibody were generally rather weak. Only the PDL and some cementoblasts were slightly stained.

OCN appeared in the alveolar bone matrix and in osteoblasts as a weak staining in the PDL matrix and in bone areas near the PDL, in cementoblasts, and, more centralized, in the matrix of the cellular cementum. In physiological resorption lacunae, repair cementum was also immunoreactive.

The runx2 immunoreactions were observed in the gingival epithelium, in vessel walls, and in some PDL cells. Osteoblasts and cementoblasts lying over apically existing cellular cementum also showed a positive reaction. Immunostaining was found within the cytoplasm as well as intranuclear.

Msx2-immunoreactive cells were found in the periodontium mainly in the middle and occlusal thirds, but never in the apical third. In addition, osteoblasts and osteocytes in the alveolar and basal bone as well as cementoblasts and most of the cementocytes in the cellular cementum showed immunostaining. There was a concentration of positive cells especially in perivascular areas of bone and PDL vessels. E11 immunostaining included cementoblasts and cementocytes of the cellular cementum, epithelial rests of Malassez, osteoblasts, and peripheral osteocytes in alveolar bone, and, in addition, basal cells of the gingival epithelium and endothelial cells.

Morphological description of the repair process

Experimental days 0–7. The histological changes due to the experimental orthodontic tooth movement of the first maxillary molar has been described previously (Jäger *et al.*, 2005). Resorption processes were mainly restricted to the acellular cementum while apical cellular cementum was rarely affected. The lacunae were localized especially in regions with compression and hyalinization of the PDL. Hyalinized zones and resorption lacunae were primarily observed interradicularly at the mesial aspect of the distal root. This part of the root was typically covered by acellular cementum. Resorption cavities often reached beyond the cementum into the dentine.

On day 0, there were still multiple TRAP-positive resorptive cells within the resorption lacunae, on the root surface or adjacent to the hyalinized zones (Figure 1A). Within the lacunae, not only odontoclasts but also fibroblasts and inflammatory cells were observed. In addition, scattered cementoblast-like cells showing a smaller, round-cuboidal appearance were found within the lacunae.

During the following experimental days, a decrease in the number of resorbing cells and an increase in the number of reparative cells became obvious, but signs of deposited reparative cementum were not observed in this early phase of the experiment (Figure 1B).

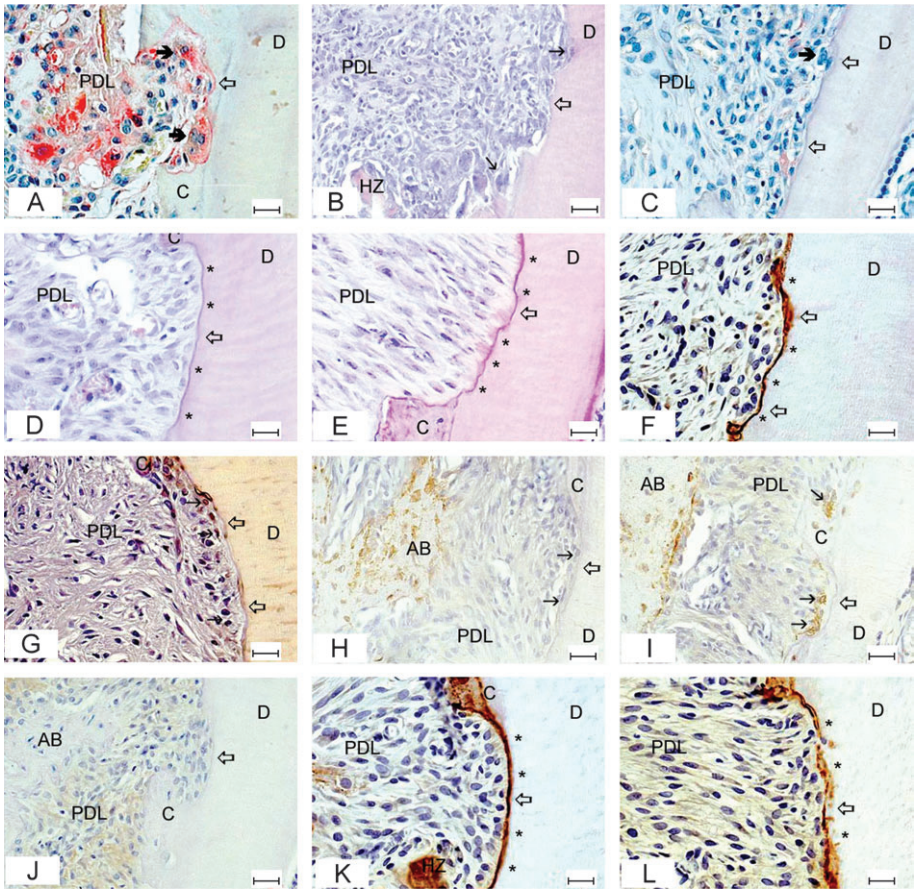

Figure 1 Photomicrographs of the orthodontically induced root resorption lacunae and the healing process on the mesial side of the mesial root of the upper right first molar. (A) Experimental day 0; staining for tartrate-resistant acid phosphatases (TRAP); open arrow: resorption lacunae; black arrows: TRAP-positive odontoclasts (red stain); reduced number of TRAP-positive odontoclasts (red stain) in lacunae. (B) Experimental day 5; no signs of repair cementum in resorption lacunae (open arrow); black arrows: odontoclasts. (C) Experimental day 10; staining for TRAP; open arrow: resorption lacuna; black arrows: TRAP-positive odontoclasts (red stain); reduced number of TRAP-positive odontoclasts (red stain) in lacunae. (D) Experimental day 12; thin seam of repair cementum (asterisks, magenta stain) on the bottom of a lacuna (open arrow). (E) Experimental day 17; broader seam of repair cementum (asterisks, magenta stain) on the bottom of a resorption lacuna (open arrow). (F) Osteopontin (OPN) immunohistochemistry; experimental day 7; distinct staining of original cementum. (G) Osteocalcin immunohistochemistry; experimental day 7; focal immunostaining of cementoblasts (black arrows, brown stain) in a resorption lacuna (open arrow); no immunoreactivity in cells of the periodontal ligament (PDL). (H) E11 immunohistochemistry; experimental day 14; no immunostaining of cementoblasts (black arrows) in acellular cementum resorption lacuna (open arrow; AB, alveolar bone with immunoreactive osteoblasts and osteocytes; brown stain). (I) E11 immunohistochemistry; experimental day 14; immunostaining of cementoblasts (black arrows, brown stain) in cellular cementum resorption lacuna (open arrow); AB, brown stain. (J) Fibronectin immunohistochemistry; experimental day 12; moderate to weak immunostaining in the matrix of the PDL (weak brown stain), open arrow: resorption lacuna. (K) OPN immunohistochemistry; experimental day 12; immunoreactive seam (asterisks, dark brown stain) on the bottom of the resorption lacunae (open arrows), staining of a few cells in the PDL; OPN-immunoreactive layer over the hyaline zone. (L) OPN immunohistochemistry; experimental day 16; weak staining of the PDL matrix (C, cementum; D, dentine; HZ, hyaline zone; magnification bars = 20 μm).

Experimental days 10 and 12. At this stage of the experiment, the number of TRAP-positive cells had further decreased (Figure 1C). However, differences among individual animals and lacunae were obvious.

Signs of deposited reparative cementum were seen for the first time on day 10 (Figure 1D). Using the cementum stain, acellular cementum was coloured violet whereas cellular cementum appeared green. On day 10, there was a thin violet seam layered on few resorption lacunae. During the next few days, this seam increased in width without altering colour.

Experimental days 14 and 17. At these time points, repair cementum deposition had already led to a smoothing of the bottoms of the lacunae (Figure 1E).

Immunohisochemistry

Experimental days 0–7. The immunostaining pattern for type I collagen, in the control specimens, was similar in the experimental animals. On day 0, distinct positive reactions were also found in odontoclasts. The strongest immunoreactions were seen in the periodontal ECM and on PDL fibroblasts. Immunoreactions with the collagen type I antibody were found to be similar on days 0, 5, and 7.

Immunoreactions for the FN antibody were generally fairly weak. Odontoclasts showed no staining. There was slight immunoreactivity for FN in the PDL regions under tension on day 0. In areas of compression, no reaction was obvious. On sections from day 0, some odontoclasts and perivascular areas in the PDL were OPN positive. From experimental day 7 onwards, an OPN-immunoreactive seam with an increasing diameter could be observed on the bottom of root resorption lacunae (Figure 1F). The staining reaction was often more pronounced in the upper and lower marginal corners of the lacunae. In those cases where hyaline zones still existed next to the lacunae, the root was also covered by an OPN-positive seam.

On day 0, directly after active tooth movement, the PDL on the mesial pressure side was devoid of immunoreactivity for OCN except for some PDL cells in the vicinity of hyaline zones. Beginning on day 7, some PDL cells 'streaming' into the lacunae were immunostained. Additionally, a few cells lying inside the lacunae and on the surface of the roots were also stained (Figure 1G). At the same time, many cells within the lacunae and PDL cells neighbouring the lacunae were stained moderately or strongly with the runx2 antibody while PDL cells lying further away reacted only weakly.

Cementoblastic cells within the resorptive lacunae of acellular cementum were negatively stained for the antibody E11 on all experimental days (Figure 1H). Antibody reactions were only found in cementoblasts located in a few cellular cementum lacunae (Figure 1I).

Mechanical stress led to increased msx2 staining of cells within the PDL starting on day 5. In addition, a weak extracellular immunoreactivity was obvious in the PDL. Moderate to strong staining was concentrated in PDL areas near the bone, while areas near the root and the tissue in resorption lacunae were unstained or only weakly reactive.

Experimental days 10 and 12. The initial cementum deposited in the resorption lacunae showed type I collagen immunostaining which became more intense from day 10 to day 12 and remained the same on the following experimental days. At the same time, there was weak immunoreactivity for the FN antibody within the ECM of resorption lacunae on day 10. This positive reaction of the ECM for FN was no longer seen on day 12 or the following experimental days (Figure 1K).

In the further course of the healing process, most of the repair cementum stained positive for OPN (Figure 1L and M) while the early repair cementum did not react with the OCN antibody (Figure 2A). In addition, a few cells inside the lacunae and the nearby PDL were immunostained with the OPN antibody (Figure 1L, M). On experimental day 12, parallel PDL fibres running into the lacunae or into parts of them were observed, indicating the onset of the process of reattachment. These fibres (Sharpey's fibres) were also reactive against the OPN antibody.

As already noted, many cells in the lacunae and neighbouring PDL were stained with the runx2 antibody. Especially in regions of PDL reattachment, a strong immunoreactivity was visible.

On the other hand, only a few cells lying inside the lacunae or on the surface of the root were stained for OCN in sections from animals of these experimental days. These positive cells were mainly located in the apical or occlusal corners of the lacunae.

In addition, only a few reparative mononuclear cells in the repair lacunae and on the root surface demonstrated a reaction against the msx2 antibody (Figure 2B) while there was no positive immunoreaction of these cells with the antibody against E11.

Experimental days 14 and 17. The repair cementum was immunostained for OPN and collagen type I, but not for OCN (Figures 1M and 2A,C). Compared with days 10 and 12, the ECM of the lacunae showed increased type I collagen immunostaining (Figure 2C).

On days 14 and 17, there were no more TRAP-positive odontoclasts on the root surface (Figure 2D). No more OCN-positive cells were found within the lacunae, but the PDL still showed localized weak to moderate immunoreactivity (Figure 2E). The staining pattern for runx2 was similar to that of earlier experimental days and included sporadic cementoblasts and cells lying within the lacunae. On sections from day 17, immunoreactivity was even weaker than on day 14 specimens (Figures 2F, G).

There was still no obvious immunoreaction of the cementoblast-like cells in the lacunae with the antibody against E11 (Figure 1H). On the other hand, msx2-immunoreactive mononuclear cells were visible in the lacunae, but not on the root surface (Figure 2H).

The immunohistochemical findings are summarized in Table 2.

Discussion

Reversal of resorption and repair following the cessation of orthodontic loading

In this study, maxillary first rat molars were moved mesially using a calibrated closed coiled spring, thus inducing typical signs of orthodontic root resorption including resorption lacunae with odontoclasts. The TRAP-positive odontoclasts showed immunoreactivity with OPN and the collagen type I antibody. OPN is a potent regulator of clastic cells via interaction with their integrin receptors influencing cell

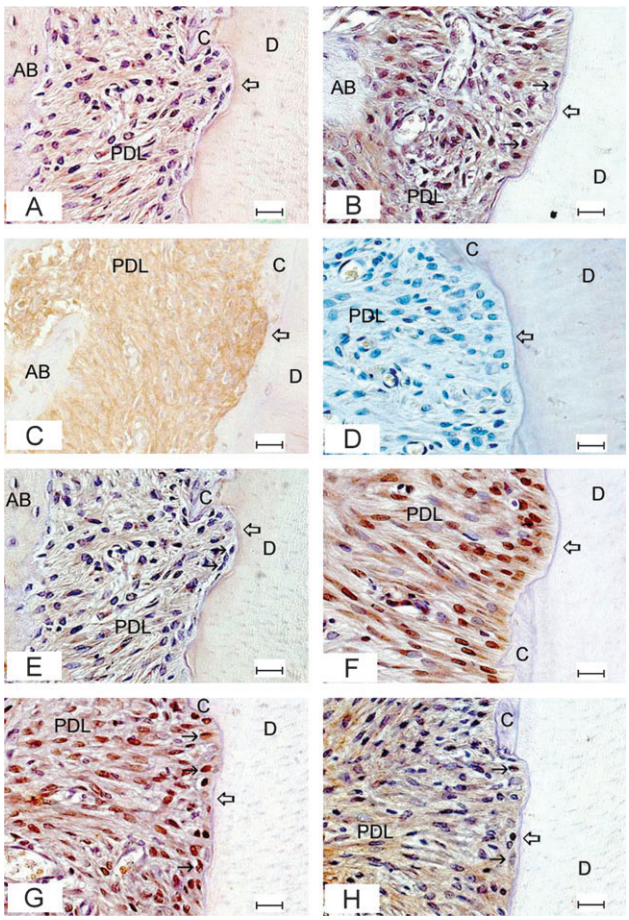

Figure 2 Photomicrographs of the orthodontically induced root resorption lacunae and the healing process on the mesial side of the mesial root of the upper right first molar. (A) Osteocalcin (OCN) immunohistochemistry; experimental day 17; staining of the matrix and focally of cells in the periodontal ligament (PDL, brown stain); open arrow: resorption lacuna. (B) The muscle segment homeobox homologue 2 (msx2) immunohistochemistry; experimental day 12; immunostaining of cells of the PDL (brown stain) and a few cementoblasts (black arrow, brown stain) on the bottom of a resorption lacuna (open arrow). (C) Collagen type I immunohistochemistry; experimental day 14; staining of the PDL, strong staining of the resorption lacuna (open arrow, brown stain) matrix. (D) Tooth roots, mesial sides; experimental day 14; staining for tartrate-resistant acid phosphatases (TRAP); open arrow: resorption lacuna; black arrows: TRAP-positive odontoclasts (red stain); reduced number of TRAP-positive odontoclasts (red stain) in lacunae. (E) OCN immunohistochemistry; experimental day 17; focal immunostaining of PDL cells (weak brown stain); no staining of cementoblasts (black arrows) on the bottom of a resorption lacuna (open arrow). (F) The runt-related transcription factor 2 (runx2) immunohistochemistry; experimental day 12; immunostaining of cells of the PDL(brown stain), also cells on the bottom of a resorption lacuna (open arrow, brown stain). (G) Runx2 immunohistochemistry; experimental day 17; immunostaining of PDL cells and cementoblasts (black arrows, brown stain) on the bottom of a resorption lacuna (open arrow). (H) Msx2 immunohistochemistry; experimental day 17; staining of PDL cells (brown stain); no staining of cementoblasts (black arrows) in resorption lacuna (open arrow; AB, alveolar bone with immunoreactive osteoblasts and osteocytes; C, cementum; D, dentine; HZ, hyaline zone; magnification bars = 20 μm).

adhesion and activation (Giachelli and Steitz, 2000). Whether the odontoclasts themselves are actively involved in the secretion of OPN remains a matter of debate (Shimazu *et al.*, 2002; Bosshardt, 2005). With regard to the positive reaction for type I collagen, it must be borne in mind that this may result from phagocytosis of cells or fibres by the odontoclasts (Sahara *et al.*, 1996).

After release of the orthodontic force, there was a continuous decrease in the number of odontoclasts. Only a very few resorbing cells were observed in the resorption lacunae on day 14 and no TRAP-positive cells were located in the lacunae on day 17. Decompression of the PDL allows for the start of repair mechanisms of the cementum, but the resorption process continues until the removal of necrotic tissue has been completed (Brudvik and Rygh, 1995a,b). Resorption lacunae may present signs of active resorption and regeneration at the same time (Sismanidou and Lindskog, 1995; Kimura *et al.*, 2003). After completion of active resorption, odontoclasts become detached from the surface and show signs of deterioration (Sahara *et al.*, 1996).

Role of ECM proteins in the early repair process

The induction of root resorption lacunae in the rats was almost completely restricted to the upper halves of the roots which are regions originally covered by AEFC. In addition, the repair cementum that was laid down was of the same type. In contrast, Bosshardt and Schroeder (1996) reported that in humans even resorption lacunae on the root originally covered with AEFC were repaired mainly with intrinsic fibre cementum devoid of Sharpey's fibres. Those authors explained this by the fact that only cellular types of cementum are able to fill a resorptive defect in a reasonable period of time. In the rat, AEFC can repair the resorptive defect rapidly because cementoblasts in rats have a higher level of activity (Bosshardt, 2005).

After withdrawal of the odontoclasts from the resorbed root surface, it was observed that the periphery of the resorption lacunae became populated by a particular phenotype of mononuclear cells. These cells closely nestled against the resorbed root and commenced attachment of the initial repair matrix directly to the residual collagen matrix lining the resorption bay. Bosshardt (2005) demonstrated that the first sign of cementum repair involves synthesis of a collagenous–fibrillar material by fibroblast- and cementoblast-like cells. The newly produced collagen fibrils intermingle with the residual collagen fibrils of the resorbed root before the junctional zone becomes obscured by a basophilic, fine granular, and electron-dense material. This zone is named the 'reversal line' and the layer between 'old' cementum or dentine and new synthesized cementum is thought to be free of fibrils and to contain FN and collagen I (Wikesjö and Selvig, 1999). The ability of PDL cells to synthesize these ECM proteins, as well as variability due to alterations in mechanical load, has been demonstrated in cell culture experiments (Howard *et al.*, 1998). In the present investigation, FN was neither detected in cementum nor in dentine. The reason for this absence might be that the

Table 2 Summary of the immunohistochemical findings.

	Periodontal ligament cells	Extracellular matrix in lacunae	Cells streaming into lacunae	Cells in lacunae	Cells on lacunae bottom	Repair cementum
Collagen type I	+	+	+	+	+	+
Fibronectin	Ø	(+)*	Ø	Ø	Ø	Ø
Osteopontin	Ø	Ø	(+)	(+)	+	+/++
Osteocalcin	+	(+)	(+)	(+)	Ø/(+)	Ø
runx2	(+)	Ø	(+)/+	(+)	(+)/+	Ø
E11	Ø	Ø	Ø	Ø	Ø	Ø
msx2	+	Ø	(+)	(+)	Ø	Ø

Ø, no immunostaining; (+), weak immunostaining; +, moderate immunostaining; ++, strong immunostaining.
*Transiently (see text for details).

immunostaining was not sufficiently sensitive to detect the presence of FN in the mineralized matrices. On the other hand, some weak immunoreaction with the FN antibody within the resorption lacunae and within the early repair cementum was observed. Thus, it could be assumed that FN plays a role primarily during initial repair processes. Eckes *et al.* (2000) showed that in the early phases of wound healing, collagen types III and IV, FN, and vitronectin are expressed. Later, these components of the ECM are exchanged for collagen type I. It is possible that in the animals in the present study, FN was exchanged for type I collagen because the immunoreaction with the type I collagen antibody increased within the lacunae from day 14 to day 17.

Kawaguchi *et al.* (2001), as in the present study, determined the appearance of OPN to be among the first events in the course of periodontal tissue repair. The interface between old and new cementum was typically characterized by a planar accumulation of organic matrix demonstrating strong immunoreaction with the OPN antibody. OPN and BSP are the major non-collagenous proteins of bone and cementum (MacNeil *et al.*, 1994, 1995; D'Errico *et al.*, 1997; Bosshardt, 2005). In the cementum, these two phosphorylated glycoproteins are believed to play major roles in the differentiation of cementoblast progenitor cells to cementoblasts and in the regulation of matrix mineralization (Saygin *et al.*, 2000). Ultrastructurally, the two proteins have a similar distribution pattern in bone and cementum, mainly filling in the interfibrillar spaces. Variation in the amount and distribution of OPN in the different varieties of cementum indicates that the architecture of the collagen fibrils ultimately determines the accumulation of the non-collagenous proteins (Bosshardt, 2005). Thus, because its matrix possesses large interfibrillar spaces, strong immunoreactivity was observed in the AEFC (Bosshardt, 2005). In bone, dentine, and cellular cementum, most of the non-collagenous proteins are synthesized and deposited by osteoblasts, odontoblasts, and cementoblasts. However, their source in AEFC is at present not clear. With respect to OPN, for example, although PDL fibroblasts were demonstrated to be able to synthesize OPN in culture (Nohutcu *et al.*, 1996), a relatively high accumulation of the protein was noted in the cementum layer before it was expressed in the adjacent PDL cells (Lekic *et al.*, 1996). These observations suggest that at least some of the OPN in AEFC is derived from a source outside the direct environment, probably from blood circulation (van den Bos *et al.*, 1999).

Cells involved in the early repair process

Among the cells involved in the repair process, cementoblastic cells showed variable immunoreaction with antibodies against the ECM components FN, collagen type I, OPN, and OCN, as well as with the transcription factor, runx2.

OCN is a gamma-carboxy glutamic acid-containing protein which is currently considered to be the most specific and the latest of expressed markers, undetectable in pre-osteoblasts and abundantly expressed only in post-mitotic osteoblasts (Aubin, 1998). In the animals in the present study, immunostaining of the early reparative cells for OCN was found to be variable. (Tenorio *et al.*, 1993) in an immunohistochemical study showed that the cementoblasts responsible for cellular cementum formation were positive with anti-OCN antibodies, but the cells adjacent to acellular cementum were negative. Based on these results, phenotypic differences are suggested between cementoblasts associated with cellular cementum connected to an osteoblastic phenotype and those associated with acellular cementum, which are thought to be PDL cells achieving close contact to the dental root surface. On the other hand, other authors have claimed that both types of cementoblasts express OCN messenger RNA transcripts (Kagayama *et al.*, 1997; Sasano *et al.*, 2001). These authors found OCN staining in cells lining the root surface in both 2- and 3-week-old rats. Almost all the cells lining the cellular cementum were positive for OCN. In contrast, OCN-positive cells lining acellular cementum and root surface devoid of cementum appeared only at specific sites of the root, for example cells at the interradicular area.

The transcription factor, runx2, is a key determinant of osteoblastic lineage (Komori, 2003). It promotes differentiation of undifferentiated mesenchymal cells to osteoblasts by regulating transcription of ECM proteins (e.g. collagen I, OPN, OCN, etc.). Positive immunostaining for the runx2 antibody in some PDL cells and in many reparative cells within the resorption lacunae was found in the present study. Cells lying directly on the root surface reacted to a lesser degree. Recent data have shown that runx2 is also strongly expressed in the early stages of tooth development and is involved in crown morphogenesis and cytodifferentiation of odontoblasts (Jiang *et al.*, 1999). In addition, localization of runx2 has been demonstrated in the mouse dentition in later stages of crown and root development, namely in cementoblasts and PDL cells (Bronckers *et al.*, 2001).

According to Wetterwald *et al.* (1996), the antibody E11 recognizes an antigen located at the cell surfaces of late osteoblasts, pre-osteocytes, and young osteocytes. *In vivo*, the antigen was primarily found on the cytoplasmatic processes of marginal osteocytes. Zhang *et al.* (2006) suggested that at least one important function of E11 in osteocytes is the formation of cytoplasmatic processes and that it is regulated by mechanical strain both *in vivo* and *in vitro*. Schulze *et al.* (1999) suggested that the E11 antibody may be used as a marker for the late steps in the differentiation pathway of the osteoblast lineage. The finding of positive E11 immunoreactivity of only the apically located cementoblasts, but not of those cells involved in the repair process of the acellular cementum, is in agreement with the study of Tenorio *et al.* (1993) who were the first to differentiate two cementoblast subpopulations using this antibody.

With respect to the results concerned with the expression of msx2, an increase in immunopositive cells within the PDL was found but the cells reaching the root surface as reparative cells were always negative for this antibody. Msx2 has been shown to play a complex role in osteoblast differentiation. It is expressed during the proliferative phase of osteoblastic cells, but the expression level decreases with terminal osteoblast differentiation (Aubin, 1998). In the course of tooth development, msx2 has been shown to be expressed in Hertwig's epithelial root sheath cells during root formation and such expression is continued in the epithelial cell rests of Malassez (Yamashiro *et al.*, 2003). While promoting osteoprogenitor proliferation, msx2 has been demonstrated to inhibit definite osteoblast differentiation and consecutive mineralization (Ichida *et al.*, 2004). *In vitro*, msx2 is a negative regulator of osteoblast-specific gene transcription, for example OCN, through suppression of runx2 transcriptional activity (Shirakabe *et al.*, 2001). It has been demonstrated that msx2 prevents mineralization of ligament fibroblasts such as those in the PDL by repressing runx2 and in this way is responsible for the maintenance of the ligament space (Yoshizawa *et al.*, 2004).

Whether there is definitely a specific cell type with the capacity to differentiate into cementoblasts is still a matter of debate (Bosshardt, 2005). One suggestion is that a defined subfraction of PDL cells in the mature periodontium has the capacity to undergo differentiation towards an osteoblast or cementoblast phenotype (Saygin *et al.*, 2000). Other possible sources of cementoblast or osteoblast progenitors include marrow stromal and paravascular and endosteal fibroblasts (Bosshardt and Schroeder, 1996; Grzesik and Narayanan, 2002; Bosshardt, 2005). Bosshardt and Schroeder (1996) and Bosshardt (2005) have suggested that cementum-producing cells may originate from epithelial cells of Hertwig's epithelial root sheath by undergoing epithelial–mesenchymal transformation. In the present study, the cell rests of Malassez, which are remnants of Hertwig's epithelial root sheath, showed positive immunoreaction with the E11 antibody.

The existence of different cementoblast subpopulations or the biological significance of the variations of cementum types and rates of formation is not clearly understood (Bosshardt and Schroeder, 1996; Bosshardt, 2005). Cho and Garant (1989) have suggested that the cells associated with the formation of AEFC may express the cementoblast phenotype only transiently. Unlike in bone, cellular intrinsic fibre cementum, and acellular intrinsic fibre cementum, the cells producing AEFC do not deposit a typical cementoid seam (Bosshardt, 2005). These observations are in line with the present finding that following orthodontic root resorption, reparative acellular cementum-like tissue was seen to be associated mostly with cells that are hardly distinguishable from fibroblasts of the PDL. This does not necessarily imply that the AEFC-producing cells are indeed PDL fibroblasts. A comparison of the gene expression profile of cells lining the AEFC and PDL cells away from the root surface performed by D'Errico *et al.* (1997) in mice clearly showed a difference with respect to OPN, OCN, and bone sialoprotein.

Conclusions

Root resorption following orthodontic tooth movement of rat molars was followed by new formation of AEFC as early as 10 days following the release of the orthodontic force. The repair process was accompanied by a co-ordinated expression of the ECM molecules OPN, FN, and collagen type I. The cementoblasts that produced the AEFC were primarily PDL cells with a fibroblast rather than an osteoblast phenotype.

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