

Healing of the root surface-associated periodontium: an immunohistochemical study of orthodontic root resorption in man

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SUMMARY The purpose of the present investigation was to study resorption and regeneration of periodontal tissues incident to orthodontic tooth movement, in particular cells resorbing the root surface and the subsequent regeneration of the periodontal epithelial network and forming reparative cementum. The study was carried out using a select number of immunohistochemical markers on extracted human teeth which had been treated orthodontically.

The most striking finding in the resorbing areas was the presence of what appeared to be two populations of KP 1⁺ mononuclear cells located at a distance of 50–100 µm from the root surface and multinucleated cells in resorption lacunae in close contact with the root surface. KP 1⁺ has previously not been reported for odontoclasts. The mononuclear KP 1⁺ cells in the periodontal ligament may represent either precursors to odontoclasts or phagocytic scavenger cells of the macrophage lineage.

The subsequent healing of the resorption lacunae was characterized by re-establishment of nervous, vascular and epithelial tissues as evidenced by S-100⁺ filamentous delicate structures, factor VIII⁺ vessels and cytokeratin⁺ clusters of cells, respectively. However, cytokeratin⁺ single cells in close contact with the unresorbed cementum did not re-appear within the healing period. Although the present results are not quantitative in nature, cementoblasts located in the vicinity of resorption lacunae, especially healing ones, appeared to show an up-regulation of epidermal growth factor (EGF) receptors. It may be suggested the intense positive staining for EGF receptors may be an expression of an auto- or paracrine stimulatory pathway increasing the rate of reparative cementum formation.

Introduction

The periodontium consists of a complex mixture of mineralized and non-mineralized tissues derived from the ectoderm and mesoderm. It develops as a single phylogenetic unit (Ten Cate *et al.*, 1971; Yoshikawa and Kollar, 1981; Palmer and Lumsden, 1987; Lumsden, 1988; MacNeil and Thomas, 1993), a fact which is reflected in periodontal healing mechanisms.

Initial healing of wounds in the periodontal ligament (PDL) does not differ from the healing of other types of connective tissue wounds. It begins with the formation of granulation tissue subsequent to necrosis and blood clot formation regardless of type of periodontal challenge. Organization of the granulation tissue follows,

during which vascular and nervous components enter the area (Parlange and Sims, 1993) as well as new periodontal connective tissue including fibroblasts, collagenous fibres (Melcher, 1970, 1976; Line *et al.*, 1974; Caton and Nyman, 1980; Caton *et al.*, 1980; Nyman *et al.*, 1982; Harrison and Juronsky, 1991; Wikesjö *et al.*, 1992) and the junctional epithelium (Taylor and Cambell, 1972; Wirthlin *et al.*, 1980). Although evidence also indicates the possibility of the epithelial rests of Malassez regenerating (Brice *et al.*, 1991), their role in periodontal healing remains obscure (Spouge, 1980).

PDL healing and the expression of different periodontal mesenchymal phenotypes is intimately associated with formation of mineralize

tissues, either root surface-associated or associated with the alveolar bone (Lindskog and Blomlöf, 1992). In this respect, the PDL shares not only morphological similarities with the periosteum, with which it interlaces at the alveolar crest, but also functional similarities such as a mineralized tissue-forming capacity evidenced by its intense activity of non-specific alkaline phosphatases (Lilja *et al.*, 1984; Arceo *et al.*, 1991; Lindskog and Blomlöf, 1994).

It has been shown that two different types of reparative cementum may result after re-population of a damaged root surface areas by mesenchymal cells expressing different phenotypes (Lindskog *et al.*, 1983; Melcher *et al.*, 1987; McCulloch and Bordin, 1991; Lindskog and Blomlöf, 1992, 1994; Tenorio *et al.*, 1993; Blomlöf and Lindskog, 1994), either a cementoblast phenotype (root resorption) or an osteoblast phenotype (instrumented root surfaces). Expression of the cementoblast phenotype may only be possible after a superficial resorption of the dentine surface. However, this can not be the only factor which promotes formation of attached reparative cementum since resorption has also been shown to precede non-attached bone-like reparative cementum formation (Lindskog and Blomlöf, 1992; Blomlöf and Lindskog, 1994). It is likely that additional factors, such as the potency of the source of undifferentiated mesenchymal cells (most pronounced in young teeth with incomplete root closure) also determine which type of root surface healing will occur (Blomlöf *et al.*, 1992; Lindskog and Blomlöf, 1992).

Orthodontic root resorption is preceded by an aseptic necrosis in the pressure zones of the PDL (Lilja *et al.*, 1983, 1984). Upon release of the force and removal of the necrotic area, the resorbed dentine surface heals with adhering reparative cementum (Langford and Sims, 1982; Hammarström and Lindskog, 1985; Vardimon *et al.*, 1993). However, during the past decade other components, such as the nervous and vascular supply of the regenerating periodontium have been devoted only little attention, and thus the relationship between regenerating components of the PDL remains largely unexplored.

The purpose of the present investigation was to study resorption and regeneration of the root surface-associated periodontal tissues incident to orthodontic tooth movement, using a select

number of immunohistochemical markers, for the demonstration of resorbing cells and cells participating in the subsequent healing of the periodontal epithelial network and forming reparative cementum. Antibodies directed against the following human antigens were used: lysosome-associated glycoprotein CD68 (KP 1), cytokeratins nos. 10, 17 and 18, von Willebrand (coagulation) factor VIII and epidermal growth factor (EGF) receptor. In addition, antibodies directed against protein S-100 from cow brain, which also reacts with the human sequence of the protein (Stefansson *et al.*, 1982) was used.

Materials and methods

Patients

A total of 10 premolars in five patients were selected for the study. The patients were scheduled for maxillary expansion with fixed appliances and bilateral extraction of premolars. The patients were selected on a voluntary basis and appropriate ethical approval was granted prior to instigation of the study. The age of the patients ranged from 11–14 years (mean 13.3 years) at the start of the treatment.

Orthodontic appliance

Quad-helix fixed appliances were used. The appliances were made with 0.9 mm wire (Feder-Hart, Remanium, Dentaurem, Germany), ensuring contact with the lingual aspects of the premolars selected for the study. Immediately prior to cementing the appliances they were activated corresponding to half the buccolingual width of the premolars. This corresponded to a force of 0.8 N.

Experimental outline

Both premolars in each patient which were scheduled for extraction were subjected to an orthodontic force in a buccal direction for a mean of 52 days (SD 37 days, Table 1). On the day the force was released, one premolar in each patient was randomly chosen for extraction (resorbing teeth). The other premolar was kept in place without retention and not exposed to any orthodontic force for periods of 13–114 days (Table 1) in order to allow repair of any root resorption to take place (healing teeth), before extraction. Both premolars in each patient were extracted as gently as possible in order to preserve as much root surface-associated PDL as possible.

Table 1 Details of the experimental material. On the day the force was released, one premolar in each patient was randomly chosen for extraction to study root resorption (resorbing teeth). The other premolar was kept in place not exposed to any orthodontic force for varying periods in order to allow repair to take place (healing teeth).

Patient	Tooth	Force application time (days)	Healing time (days)
1	Resorbing	27	0
	Healing	27	114
2	Resorbing	31	0
	Healing	31	29
3	Resorbing	31	0
	Healing	31	79
4	Resorbing	62	0
	Healing	62	44
5	Resorbing	114	0
	Healing	114	13

Preparation of tissue

The teeth were immersed in 4% formaldehyde in a phosphate buffer (pH 7.4) for at least 48 hours. The buccal root was separated from the rest of the tooth and decalcified in 24% EDTA (pH 7.4) at room temperature for about 4 weeks. After demineralization the roots were embedded in paraffin. Longitudinal sections of the buccolingual aspect of the roots were cut at 4 µm, taken up on silane-coated slides and processed for indirect immunohistochemistry (see below).

Between 20–30 longitudinal central sections from each buccal root were evaluated making a total of in excess of 250 sections. Incubation for demonstration of the different antigens was performed on alternating sections throughout the roots. The description below is based on

consistent representative staining patterns observed throughout the experimental material.

Antibodies

All primary antisera, anti-KP 1, anti-cytokeratin, anti-Factor VIII, anti-EGF-receptor and anti-S-100 (Table 2) were diluted in 4% normal serum in a 0.5 M Tris-saline buffer, pH 7.6 (TRIS). The antibody solution immuno-reactive towards EGF receptor also contained 0.3% Triton X-100. The secondary antibodies were biotinylated rabbit anti-mouse or goat anti-rabbit (Dako) diluted 1:200 or 1:300 in TRIS. Staining specificity was assessed by omission of the primary or secondary antisera or by incubation of the primary antiserum previously absorbed with the antigen.

Immunohistochemistry

Sections were, after deparaffination, processed with the avidin–biotin–peroxidase complex (ABC) method (Hsu *et al.*, 1981). In brief, the sections were incubated with the primary antisera followed by the secondary biotinylated antibody and the ABC complex (Dako). The incubations were performed at room temperature for 30 minutes or at 4°C overnight (EGF receptor antibody only). The immunoreactions were visualized by a chromogen substrate solution consisting of 0.6 mg/ml diaminobenzidine and 0.01% hydrogen peroxidase in TRIS for 5 minutes. Non-specific endogenous peroxidase staining was reduced by section immersion in hydrogen peroxidase before the antibody incubations. The sections were thoroughly rinsed in TRIS before and after incubations, counterstained with Harris haematoxylin and were mounted in Eukitt after dehydration. For observation and photography a Leitz

Table 2 Specifications for the primary antibodies.

Antibody against	Specificity	Supplier*	Dilution	Species	Reference
KP 1	Human CD 68	Dako	1:100	Mouse	Pulford <i>et al.</i> , 1989
Cytokeratin	Human cytokeratins nos. 10, 17 and 18	Dako	1:50	Mouse	Moll <i>et al.</i> , 1982
Factor VIII	Human von Willebrand factor	Dako	1:200	Rabbit	Bukh <i>et al.</i> , 1986
EGF receptor	Human EGF receptor peptide corresponding to residues 1005–1016	SDS	1:50	Rabbit	Hunter <i>et al.</i> , 1984
Protein S-100	Cow proteins S-100 A and B	Dako	1:400	Rabbit	Kindblom <i>et al.</i> , 1984

* Antibodies from Dako and SDS were purchased from Dako A/S, Glostrup, Denmark and Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA respectively.
EGF=epidermal growth factor.

Aristoplan microscope equipped with image digitizing facilities was used.

Results

KP 1

Intensely KP 1⁺ cells were present in what appeared to be two populations in the resorbing teeth. The majority of cells were mononuclear cells located at a distance of 50–100 µm from the root surface (Figure 1). They appeared in clusters of several cells with a uniform staining for KP 1 throughout their cytoplasm. In the vicinity of these clusters, intensely KP 1⁺ multinucleated cells were seen in close contact with the dentine surface in resorption lacunae (Figure 1). KP 1 staining in these cells was preferentially located to the part of the cell closest to the dentine surface. The remaining cells in the adhering PDL were void of KP 1 staining.

Cytokeratin

Intense staining for cytokeratin was seen in two locations in both resorbing and healing teeth: tightly adherent clusters of cells at a distance of approximately 50 µm from the intact cementum surface and in single cells in contact with the non-resorbed cementum surface (Figure 2). The cytokeratin⁺ clusters of cells appeared to be more frequent in the apical part of the roots,

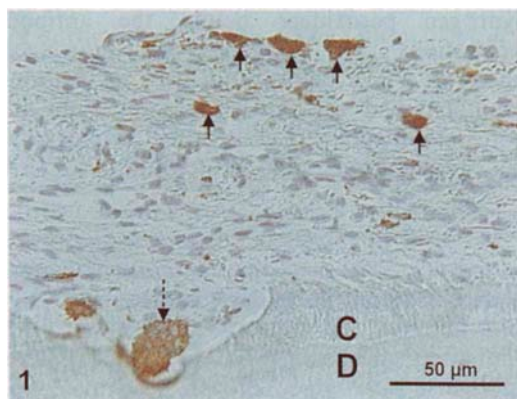


Figure 1 Intensely KP 1⁺ mononuclear cells located at a distance of 50–100 µm from the root surface (solid arrows) as well as a resorbing multinucleated KP 1⁺ cell in close contact with the dentine surface in a resorption lacuna (broken arrow) with staining preferentially located to the part of the cell closest to the dentine surface. D=dentine; C=cementum; patient 4 (Table 1). Bar = 50 µm.

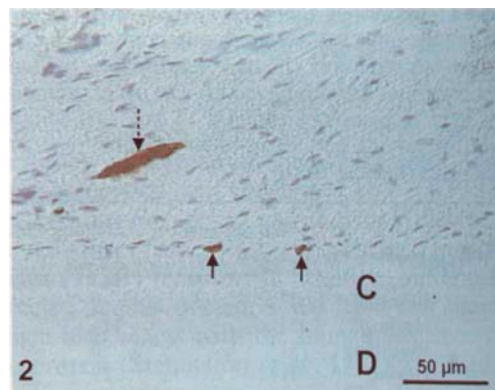


Figure 2 Intense positive staining for cytokeratin in tightly adherent clusters of cells at a distance of approximately 50 µm from the intact cementum surface (broken arrow) and in single cells in contact with the non-resorbed cementum surface (solid arrows). D, dentine; C, cementum; patient 2 (Table 1); bar = 50 µm.

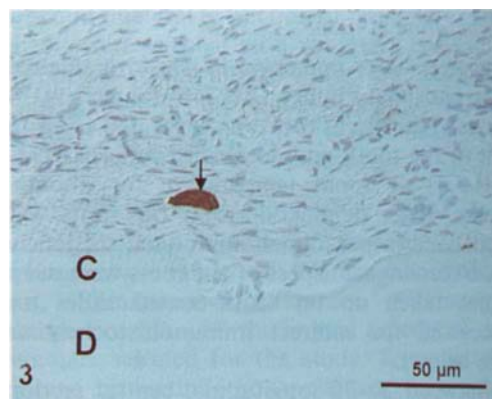


Figure 3 Small cluster of cytokeratin⁺ cells in a healing resorption lacuna (solid arrow). D=dentine; C = cementum; patient 1 (Table 1). Bar = 50 µm.

where they were also seen as elongated strands of cells. Such formations, although smaller were also observed in the healing but not in the active resorption lacunae (Figure 3). However, no single cytokeratin⁺ cells were seen in contact with the healing cementum surfaces.

Factor VIII

Intense staining for factor VIII was seen in the walls of vessels throughout the adhering PDL and in the pulps in all teeth. In the resorbing teeth, no preferential localization to active resorption areas could be observed, although these areas were not void of factor VIII⁺ vessels. In the healing teeth factor VIII⁺ vessels were

present in the healing resorption bays but always at a distance from the reparative cementum surface (Figure 4).

S-100

S-100⁺ delicate filamentous structures as well as thicker nerve-like bundles were consistently found in the vicinity of the non-resorbed cementum surfaces in both resorbing and healing teeth. In the active resorption lacunae, no S-100⁺ structures were observed, while the healing lacunae consistently displayed S-100⁺ delicate filamentous structures (Figure 5).

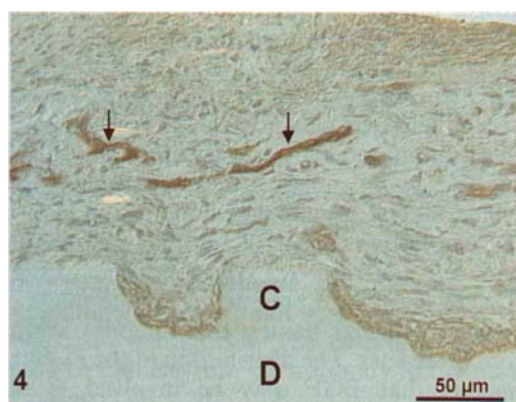


Figure 4 Intense staining for factor VIII in endothelial cells in a healing resorption lacunae at a distance from the reparative cementum surface (solid arrows). D=dentine; C=cementum; patient 3 (Table 1). Bar=50 μm.

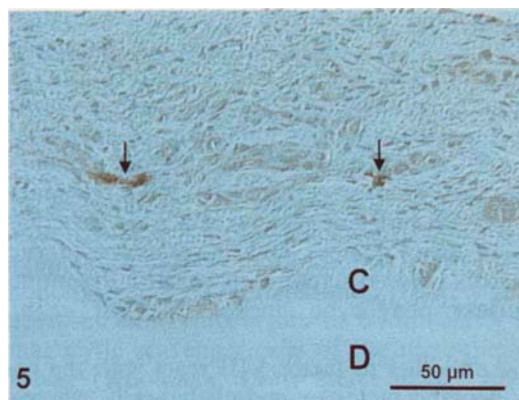


Figure 5 S-100⁺ delicate filamentous structures in a healing resorption lacunae (solid arrows). D=dentine; C=cementum; patient 3 (Table 1). Bar=50 μm.

EGF receptor

A uniform weak to moderate EGF receptor positive staining was observed in mesenchymal cells throughout the PDL in both resorbing and healing teeth. Some epithelial cell rests of Malassez, especially those close to healing resorption lacunae were intensely EGF receptor⁺ (Figure 6). Cementoblasts preferentially located to healing resorption lacuna also displayed an intense staining for EGF receptors (Figure 7), similar to that seen in odontoblasts and endothelial cells (Figure 8).

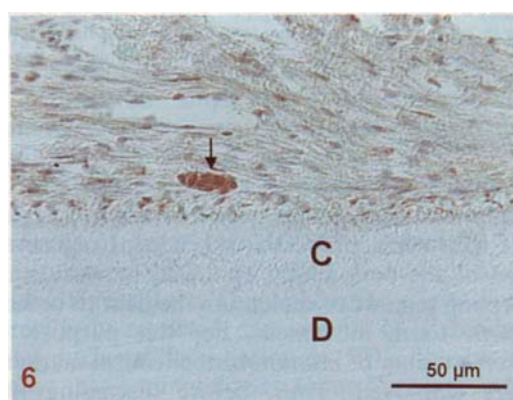


Figure 6 Epidermal growth factor (EGF) receptor⁺ staining in an epithelial cell rest of Malassez (solid arrow) close to an intact cementum surface in a resorbing tooth with active resorption lacunae. D=dentine; C=cementum; patient 1 (Table 1). Bar=50 μm.

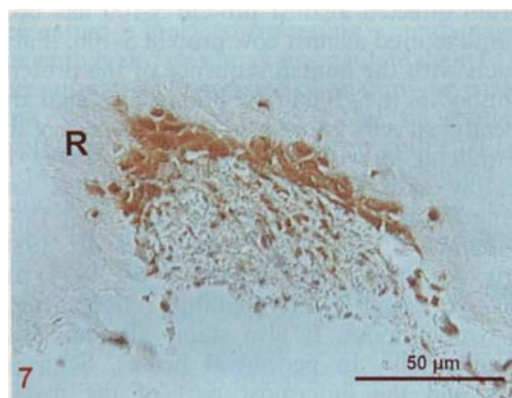


Figure 7 Epidermal growth factor (EGF) receptor⁺ staining in cementoblasts and cementocytes located in a healing resorption lacunae. R=reparative cementum; patient 2 (Table 1). Bar=50 μm.

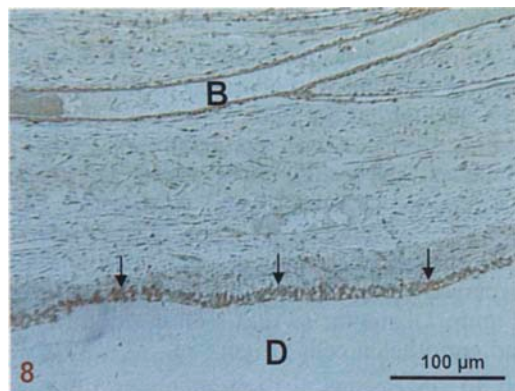


Figure 8 Epidermal growth factor (EGF) receptor⁺ staining in odontoblasts (solid arrows) and in endothelial cells. D = dentine; B = blood vessel; patient 5 (Table 1). Bar = 100 μ m.

Discussion

The purpose of the present investigation was to study the relationship between cells resorbing the root surface and the subsequent regeneration of the periodontal epithelial network and forming reparative cementum incident to orthodontic tooth movement. For this purpose, a select number of immunohistochemical markers were employed. Thus, before discussing the particular findings some methodological issues need to be addressed.

Immunohistochemistry uses antibodies to detect specific substances in tissue sections. The antibodies anti-KP 1, anti-cytokeratin, anti-factor VIII and anti-EGF receptor are directed against human antigens. Although the antiserum directed against protein S-100 has been manufactured against cow protein S-100, it also reacts with the human sequence of the protein. Antibodies to S-100 in the brain stains glial and ependymal cells. Moreover, Schwann cells of the peripheral nervous system are positive. In healthy skin and mucosa, melanocytes and Langerhans cells (Cocchia *et al.*, 1981; Nakajima *et al.*, 1982; Charbit *et al.*, 1986; Cruchley *et al.*, 1989; Barrett *et al.*, 1994) are positive, as well as interdigitating reticulum cells in lymph nodes. It also stains all the above tumour cells. In peripheral nerves, Schwann cells and the outermost part of the myelin sheath are positive; but not axons. In all other organs, Schwann cells, of both myelinated and unmyelinated peripheral nerves, and satellite cells of ganglia are positive (Stefansson *et al.*,

1982). Human odontoblasts from deciduous tooth germs taken from 12–18 week old foetuses may be S-100⁺ (Lombardi *et al.*, 1992), a finding supported by Carbone *et al.* (1987) in normal and teratomatous tooth germs. The S-100⁺ delicate filamentous structures seen in the regenerating PDL, thus indirectly indicates regenerating peripheral myelinated nerves.

The antibody directed against the EGF receptor reacts specifically with the 170 kDa EGF receptor protein in human cells by Western blotting analysis. It can also be used for immune complex kinase assays and it reacts with EGF-receptor in formaline-fixed, paraffin-embedded tissue sections. EGF mediates its effects on cell growth through its interaction with a 170 kDa cell surface glycoprotein designated the EGF receptor (Hunter, 1984). EGF receptors have been identified on basal cells of stratified squamous epithelia and skin adnexa (Gustavson *et al.*, 1984). Immunoreactivity was detected in palatal gingiva, buccal gingiva, soft palate, lateral tongue, dorsal tongue and floor of the mouth. The connective tissues of the PDL and dental pulp were non-reactive (Whitcomb *et al.*, 1993). EGF receptors are present on a wide range of normal epithelial tissues and tumours arising from those sites. The distribution of the receptor suggests that EGF may be involved in the control of proliferation and possibly differentiation of surface epithelia (Gustavson *et al.*, 1984).

Antibodies to KP 1 stain monocytes/macrophages in a wide variety of human tissues including Kupffer cells and macrophages in the red pulp of the spleen, in the lamina propria of the gut, in lung alveoli and in bone marrow (Pulford *et al.*, 1989). Peripheral blood monocytes are also positive with a granular staining pattern. All mast cell subtypes, that are normal and reactive and also malignant or neoplastic, exhibit strong consistent cytoplasmic immunoreactivity (Horny *et al.*, 1990). KP 1 recognizes a fixation-resistant epitope in a wide variety of tissue macrophages and in granulocyte precursors, thus reacting with cells of the mononuclear phagocytic lineage (Pulford *et al.*, 1989).

Antibodies to factor VIII react with the von Willebrand factor on endothelial cells, where it shows a granular pattern of reactivity and can also be detected within the megakaryocytes in sections of human bone marrow. Detection of

factor VIII is of value for delineating endothelial cells in human tissue (Mukai *et al.*, 1980). Tumours arising from endothelial cells or megakaryocytes may be identified by staining for this antibody (Hruban *et al.*, 1987). Blast cells in acute megakaryocytic leukaemia are strongly positive for factor VIII (Hruban *et al.*, 1987).

Antibodies to cytokeratins show a broad pattern of reactivity with human epithelial tissues from simple glandular to stratified squamous epithelium, which include epidermis, eccrine sweat gland, mammary gland ducts, tracheal epithelium and amniotic epithelium (Moll *et al.*, 1982). Epithelial cells are labelled whether they are ectodermal or endodermal in origin (Moll *et al.*, 1982).

Antibody specificity has been ascertained through immunoelectrophoresis, immunoabsorbant columns, enzyme-linked immunosorbent assay (ELISA), control tissue staining or Western blotting analysis by the supplying companies. However, cross-reactivity with peptides and proteins, present in the tissue section, with a chemical composition similar to the antigen cannot be excluded. Furthermore, negative staining results do not necessarily prove the absence of the antigen. The detectable epitope could be hidden from the antibody in the tissue situation.

The most striking finding in the resorbing areas was the presence of what appeared to be two populations of KP 1⁺ mononuclear cells located at a distance of 50 to 100 µm from the root surface and multinucleated cells in resorption lacunae in close contact with the root surface. The distribution of KP 1 staining differed between these two types of cells. In the multinucleated cells, it was preferentially located to the part of the cell closest to the dentine surface, while the mononuclear cell displayed a uniform cytoplasmic staining. KP 1⁺ has previously not been reported for odontoclasts only for osteoclasts (Athanasou *et al.*, 1991), although there is strong evidence to indicate a common origin for both of these cells. The mononuclear KP 1⁺ cells in the PDL may thus represent either precursors to odontoclasts or phagocytic scavenger cells of the macrophage lineage (Roodman, 1991; Pierce *et al.*, 1991; Brudvik and Rygh, 1993; Jäger *et al.*, 1993).

The subsequent healing of the resorption lacunae was characterized by re-establishment of nervous, vascular and epithelial tissues in the

resorption lacunae as evidenced by S-100⁺ filamentous delicate structures, factor VIII⁺ vessels and cytokeratin⁺ clusters of cells, respectively, in accordance with previous morphological (Parlange and Sims, 1993) and histochemical (Kvinnslund *et al.*, 1991; Saito *et al.*, 1993) studies. However, the cytokeratin⁺ single cells in close contact with the unresorbed cementum did not re-appear within the healing period. The function of these cells as well as the cytokeratin⁺ clusters of cells in the PDL remains obscure, although the literature is not lacking in suggestions. These range from an involvement in maintaining the width of the periodontal space (Lindskog *et al.*, 1988; Wallace and Vergona, 1990) to an active role in cementogenesis (Brice *et al.*, 1991). The lack of cytokeratin⁺ single cells in close contact with reparative cementum may, however, cast some doubt on the latter suggestion.

Although the present results are not quantitative in nature, cementoblasts located in the vicinity of resorption lacunae, especially the healing type appeared to show an up-regulation of EGF receptors. This appears to contradict a previously reported decrease in EGF binding sites during differentiation of 'PDL fibroblastic cells into mineralized tissue-forming cells' (Matsuda *et al.*, 1993) or of cementoblasts (Cho *et al.*, 1991). However, these studies were performed with radioactively-labelled EGF without directly assessing the presence of EGF receptors. Furthermore, it is reasonable to assume that cementoblasts which produce reparative cementum at a higher rate compared with the continuous slow deposition of cementum on surrounding undamaged cementum surfaces, may up-regulate their EGF receptors as an expression of an auto- or paracrine stimulatory pathway. The intermittent expression of EGF receptors on epithelial cell rests of Malassez is in accordance with previous data on an intense binding of EGF to these cells (Thesleff, 1987). However, the functional implications remain unclear.

In conclusion, the data presented in this study indicates that other tissue components of the PDL as well as connective tissue such as nerves, epithelial cell rests of Malassez and blood vessels are capable of regeneration following orthodontic root resorption. Furthermore, an up-regulation of EGF receptors on cementoblasts may be involved in the increased formation of cementum repairing the resorbed areas.

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