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Effects of enamel matrix proteins on tissue formation along the roots of human teeth

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Objective: Enamel matrix-derived proteins (EMD) are thought to trigger the formation of acellular extrinsic fibre cementum (AEFC), while other reports indicate that EMD may have osteogenic potential. The aim of the present study was to characterize the tissues developing on the root surface following application of EMD.

Methods: Twelve human periodontitis-affected teeth, scheduled for extraction, were treated with EMD. Two to 6 weeks later, the teeth were extracted, demineralized and processed for embedding in acrylic and epoxy resins. New tissue formation was analysed by light and transmission electron microscopy.

Results: New tissue formation on the root was observed in the notch and on both scaled and unscaled root surfaces distant of the notch area in six defects. The newly formed tissues on the root were thick, collagenous, devoid of extrinsic fibres, and had an irregular surface contour. The presence of electron-dense, organic material in the collagenous matrix indicated at least partial mineralization. Embedded cells were numerous and the cells on the matrix surface were very large in size. Abundant rough endoplasmic reticulum and a prominent Golgi complex were evident. The presence of a split between the treated root surfaces and the newly formed tissue was a common observation, as was the presence of bacteria and host cells in the interfacial gap.

Conclusion: Following treatment with EMD, a bone-like tissue resembling cellular intrinsic fibre cementum may develop on the root surfaces, instead of AEFC. Furthermore, EMD may both induce *de novo* formation of a mineralized connective tissue on scaled root surfaces and stimulate matrix deposition on old native cementum. Interfacial bonding appeared to be weak after 6 weeks of healing.

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Conventional periodontal therapy results primarily in the formation of a long junctional epithelium, a healing type that is referred to as periodontal repair (1–4). In contrast, regenerative periodontal therapy aims at restoring the structure and function of that part of the attachment apparatus that has been lost due to periodontitis. Root surface demineralization, barrier membranes, grafting materials, and biological mediators have been evaluated for their potential and efficacy to predictably achieve periodontal regeneration. Among the growth/differentiation factors evaluated, a derivative of enamel matrix proteins (EMD; BIORA AB, Malmö, Sweden) has been in clinical use for some years. Controlled clinical studies show that the application of EMD resulted in clinically measurable improvements in the attachment levels comparable to those when using barrier membranes (5–11), but superior to

Table 1. Summary of histological findings of human periodontal defects treated with enamel matrix-derived proteins (EMD)

Defect No.	Tooth type	Defect type	Healing period (weeks)	New tissue on the root surface in the notch	New tissue on the root surface apical to the notch on old native cementum without scaling markings	New tissue unrelated to notch and on root surface with scaling markings	New tissue unrelated to notch and on root surface without scaling markings	Mineralized tissue in the periodontal ligament
1	37	2-wall	4	–	–	+	–	+
2	12	1-wall	4	+	+	–	–	–
3	17	1–2-wall	5	–	–	–	–	–
4	27	1-wall	3	–	–	–	–	+
5	27	1–2-wall	2	–	–	–	–	+
6	26	1-wall	2	–	–	–	–	+
7	26	1–2-wall	6	–	–	–	–	–
8	46	1–2-wall	6	+	–	+	–	–
9	48	3-wall	6	+	+	+	–	–
10	27	1–2-wall	5	–	–	+	+	+
11	48	1–2-wall	6	+	–	–	–	+
12	27	1–2-wall	4	–	–	+	+	–

+, detected; –, not detected.

those obtained by conventional access flap surgery alone (12). For evaluating the efficacy of any regenerative periodontal procedure, clinical studies do not suffice. If EMD were to predictably induce the formation of acellular extrinsic fibre cementum (AEFC) (13–15), only histological studies can document this suggestion. There is, indeed, some justified concern regarding the nature of the newly formed mineralized tissue, since a cell-rich bone-like mineralized tissue was predominantly observed in some human studies (6,16). Apart from a poor characterization of the newly formed tissue, the results regarding the number of Sharpey's fibres are inconsistent, and tissue separation is a common observation in all histological studies in which EMD had been used on previously diseased root surfaces. Thus far, all histological studies investigating the effect of EMD on periodontal tissue regeneration in teeth affected by chronic periodontitis used conventional paraffin-embedded sections. Routine processing of tissues for paraffin histology does not, however, optimally preserve morphological details, and produces marked tissue shrinkage resulting in distortion artifacts (17–19). To overcome these shortcomings, the present study used tissues processed for plastic embedding. Plastic sections offer a superior quality for light microscopic analysis in terms of better resolution, less shrinkage and less distortion artifacts. Furthermore, they allow a combined light/transmission electron microscopic analysis. Most histological studies analysed the effect of EMD on periodontal healing after 4–6 months. To date, there are no data available on the early healing phases. Therefore, the present study was performed to cover the early healing phases between 2 and 6 weeks. Thus, an adequate analysis of the structural details of the tissues developing on human tooth roots affected by chronic periodontitis and treated with EMD was to be guaranteed.

Material and methods

Study population

In 10 patients (five females and five males; mean age of 50 years), 12 advanced intrabony periodontal defects were identified for treatment of the present study (Table 1). All teeth or single roots presented with chronic periodontitis and were scheduled for extraction because they were considered irrational to treat due to advanced periodontal destruction. All patients volunteered for the study, received verbal and written information about its purpose and possible risks, and were informed about the possibility to withdraw at any time. Written informed consent was obtained prior to the start of the study. The study protocol was approved by the Ethical Committee of the Semmelweis University of Medicine, Budapest, Hungary.

Surgical procedure

Full-thickness mucoperiosteal flaps were raised at both the vestibular and the lingual aspects of the teeth. After removal of granulation tissue, a notch was placed in the root surface at the apical level of the calculus present on the root surface using a small round burr (diameter 2 mm). If no calculus was present, the notch was placed at the base of the periodontal defect. Thereafter, the root surfaces were thoroughly scaled and planed by means of ultrasonic and hand instruments. Following abundant rinsing with sterile saline, the defects were treated according to the recommendations specified by the producer of the commercial EMD product (BIORA AB/Straumann, Basel, Switzerland). First, the root surfaces were conditioned for 2–3 min applying a 24% EDTA-containing gel (PrefGel®, BIORA AB) to the dried root surface to remove the smear layer, followed by copious rinsing with sterile saline. The defect sites were then dried again and filled with Emdogain® (BIORA AB). Postoperatively, the patients were adviced to rinse with 10 ml of a 0.2% chlorhexidine solution twice daily until

the end of the experimental periods. Postoperatively, no antibiotics were given. Two to 6 weeks after periodontal surgery, the teeth were gently removed under local anaesthesia. After a postsurgical healing period, the patients received complete treatment as scheduled.

Tissue processing for light and transmission electron microscopy

Immediately following extraction, the teeth were briefly rinsed under running tap water, and then fixed for 24 h at 4°C in 1% glutaraldehyde and 1% formaldehyde, buffered with 0.08 M sodium cacodylate (pH 7.3). After washing twice in 0.1 M sodium cacodylate containing 5% sucrose and 0.05% $CaCl_2$, pH 7.3, the teeth were decalcified in 4.13% ethylenediaminetetraacetic acid (EDTA) for 6–8 weeks (20) at 4°C, and extensively washed again in washbuffer solution. In order not to miss the region of interest, the roots were extensively cut into smaller tissue samples lateral, apical and coronal to the presumptive notch area on the root surface. Hence, the mesial, distal, buccal, and lingual surfaces of the partially decalcified roots were subdivided in a corono-apical direction into numerous thin segments as described elsewhere (21). This procedure resulted in a total of 228 small tissue slices. Some tissue samples were then postfixed with potassium ferrocyanide-reduced osmium tetroxide (22) and processed for embedding in Taab 812 epoxy resin (Merck, Dietikon, Switzerland), and both osmicated and unosmicated samples were processed for embedding in LR White resin (Fluka, Buchs, Switzerland).

Semithin survey sections (1-μm thick) were cut with diamond knives (Diatome, Biel, Switzerland) on a Reichert Ultracut E microtome (Leica Microsystems, Glattbrugg, Switzerland), stained with toluidine blue, and observed in a Leica Dialux 22 EB microscope. For transmission electron microscopy, selected areas were trimmed, cut (80 to 100 nm thick) with a diamond knife (Diatome), mounted on formvar- and carbon-coated nickel grids, and contrasted with uranyl acetate and lead citrate. Examination of the sections was performed in a Philips 300 transmission electron microscope operated at an accelerating voltage of 60 kV.

Results

Postoperative healing was uneventful in all cases, and no adverse tissue reactions such as root resorption or ankyloses were observed.

Light microscopy

An overview of the histological results of all defects is shown in Table 1. Of the 12 defects examined, four revealed the presence of a newly formed mineralized tissue in the notch area on the root surface. In two of these four defects, the new tissue in the notch extended apically over the native cementum. In two other defects, a localized thickening of the native cementum layer was observed at a site that was unrelated to the notch area. In five defects, new tissue formation was evident on the scaled root surface at sites unrelated to the notch area. In six defects, a mineralized tissue was observed seemingly 'free-floating' in the periodontal ligament. These mineralized tissue particles resembled mature bone and revealed necrosis.

In all tissue blocks showing new tissue formation on the root, similar observations were made. The newly formed tissue in the root notch was thick, had an irregular surface contour, was devoid of extrinsic fibres, contained embedded cells, and the cells on the matrix surface were very large (Figs 1A–C). In one defect, a prominent superficial oval-shaped matrix protrusion was observed (Figs 1A and C). The superficial thickened tissue layers that were observed apical to the notch area (Figs 1A, B and 2A) or at sites where no notch was discernible (Figs 2B and C) were clearly distinguishable from the old cementum by their lighter staining. These layers were less thick than the tissues that formed in the notch and they were found on both the root surfaces with (Fig. 2B) or without (Figs 2A and C) scaling markings. Although the morphological appearance of the interface between the treated root surfaces and the newly formed mineralized tissues varied (Figs 1A, B, D and 2A–C), a gap or split was consistently observed in the coronal-most portion of the instrumented area (Figs 2D–F). The space of the gap appeared empty (Fig. 2D) or was filled with an organic material resembling scattered (Fig. 2E) or colonies of bacteria (Fig. 2F).

Transmission electron microscopy

The matrix of the newly formed tissue on the root was collagenous (Fig. 3A). The collagen fibrils were randomly oriented and loosely packed, and a distinct mineralization front was not discernible. However, large, electron-dense matrix patches were scattered in and adjacent to the new tissue (not shown). The cells that lined the new tissue were very large and possessed abundant cisternae of rough endoplasmic reticulum and a prominent Golgi complex (Figs 3A and B). Embedded cells were enclosed in a lacuna, and a canalicular system filled with cytoplasmic processes connecting neighbouring lacunae was observed (Fig. 3C). In most cases, the coronally located gap between the new tissue and the instrumented root surface was filled with scattered (Fig. 4A) or colonies of bacteria (Fig. 4B). Red blood cells (Fig. 4C) and leukocytes (Fig. 4D) were occasionally observed in association with the bacteria.

Discussion

In the present study, the development of a new tissue on the root surface was not observed in all 12 teeth included. The teeth selected for the study had all been scheduled for extraction, because they were considered to be irrational to treat due to very advanced severe periodontal destruction. Hence, it may be speculated that the biological variability in wound healing and tissue formation may have been affected by a low regenerative potential of these teeth with periodontal lesions reaching almost to the apex. Similar observations were already made in another study using human teeth (16). Because

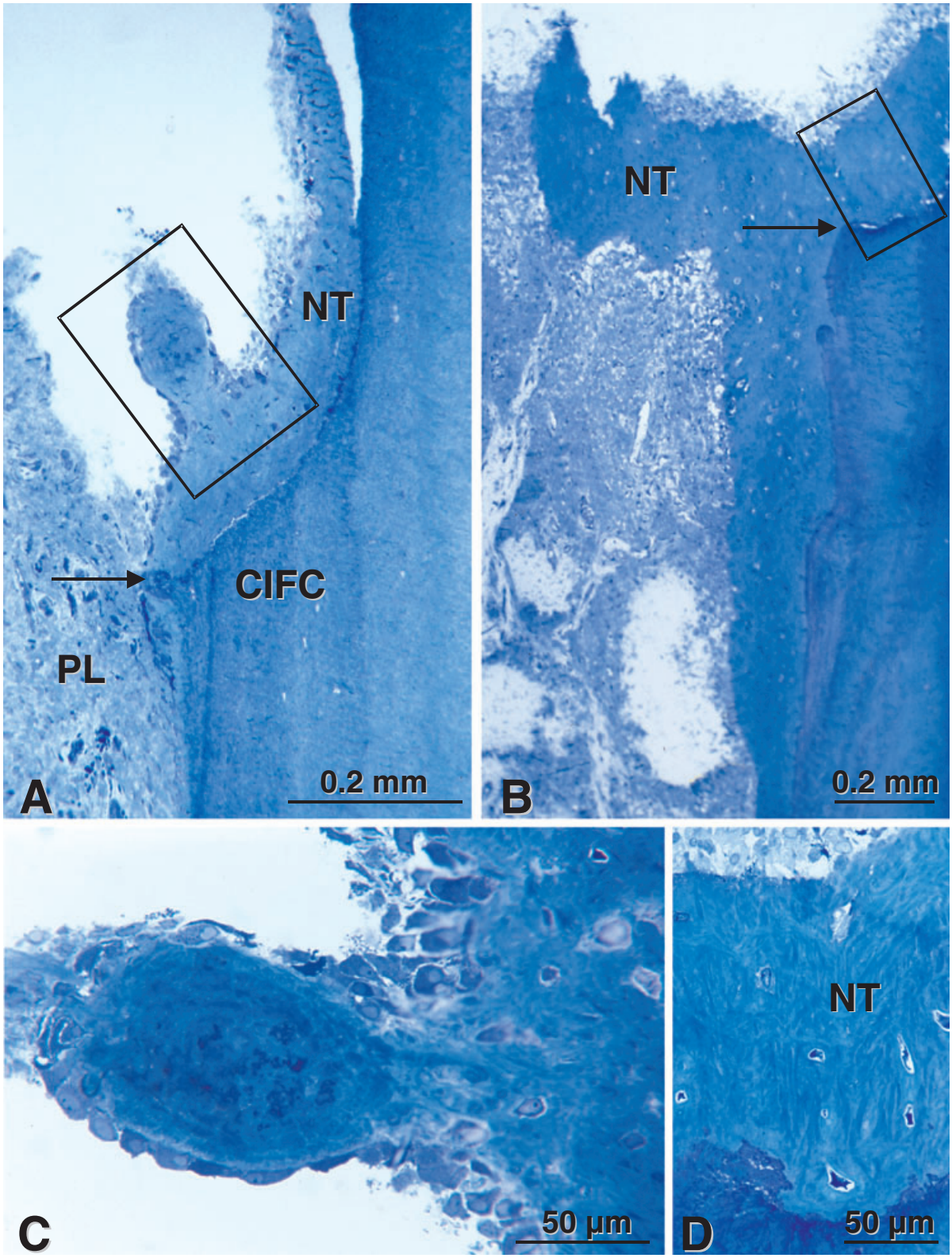

Fig. 1. Light microscopic views of the instrumented root surface 4 (A, C) and 6 weeks (B, D) following treatment with EMD. C is an enlargement of the rectangle in (A), and (D) is a higher magnification of the outlined region in (B) in a consecutive section. The new tissues (NT) that have formed in and apical to the notch areas (arrows indicate apical termination) are thick and devoid of extrinsic fibres, have an irregular surface contour, and contain numerous embedded cells. (C) shows an oval-shaped matrix protrusion lined by large cells. CIFC, cellular intrinsic fibre cementum; PL, periodontal ligament.

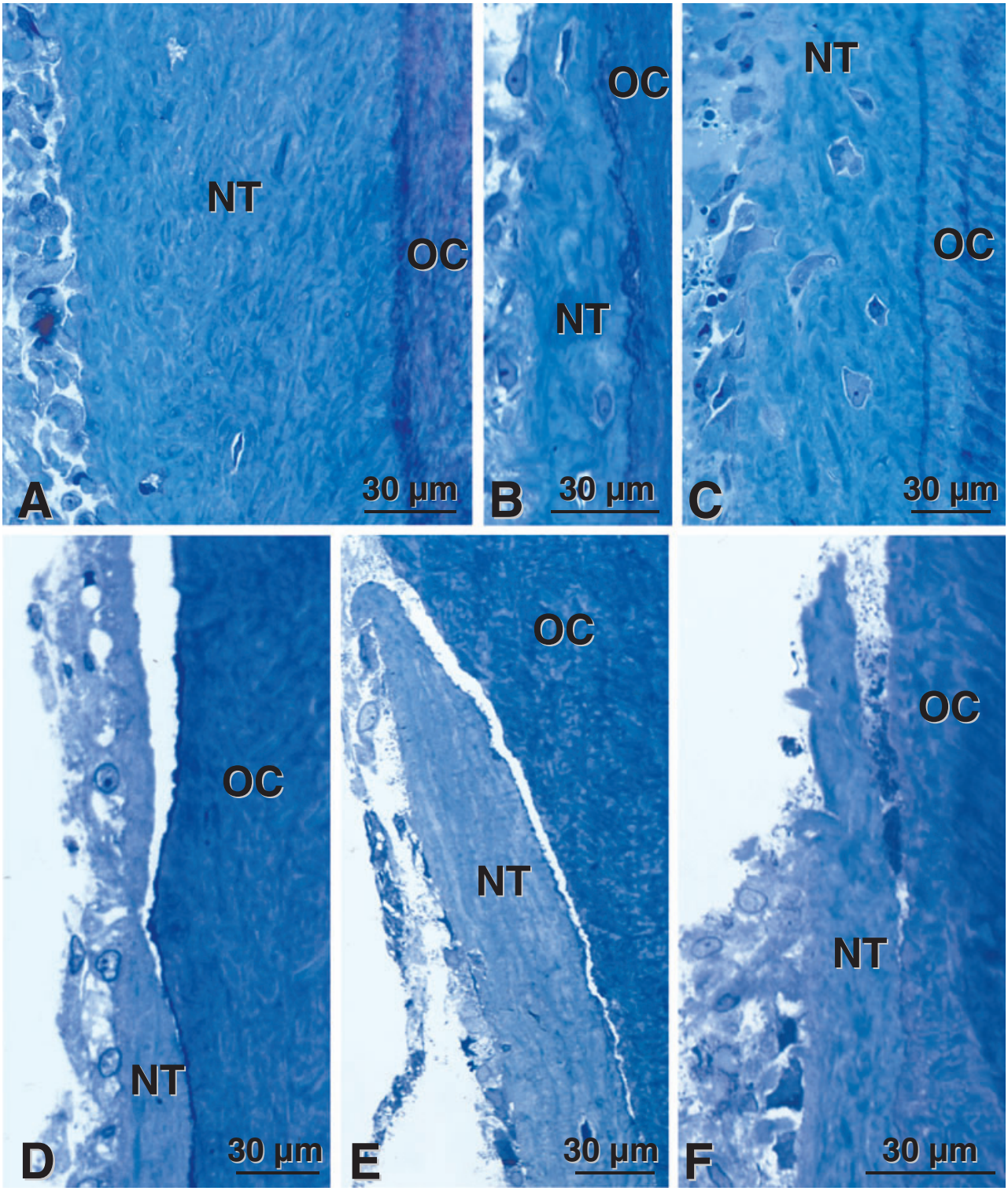

Fig. 2. High power photomicrographs showing the newly formed tissues (NT) on the tooth roots 5 (B, C, F) and 6 weeks (A, D, E) following treatment with enamel matrix-derived proteins (EMD). The newly formed tissues on the roots vary in thickness, are devoid of extrinsic fibres, and contain varying numbers of embedded cells. New tissue formation is seen apical to the notch on the native old cementum (OC) (A), and at sites unrelated to the notch on root surfaces revealing (B) or lacking (C) scaling markings. A separation of the newly formed tissues from the treated root surfaces was consistently observed, as demonstrated here in three different roots (D–F). The gap appeared empty (D) or was filled with organic matter resembling scattered (E) or colonies of bacteria (F).

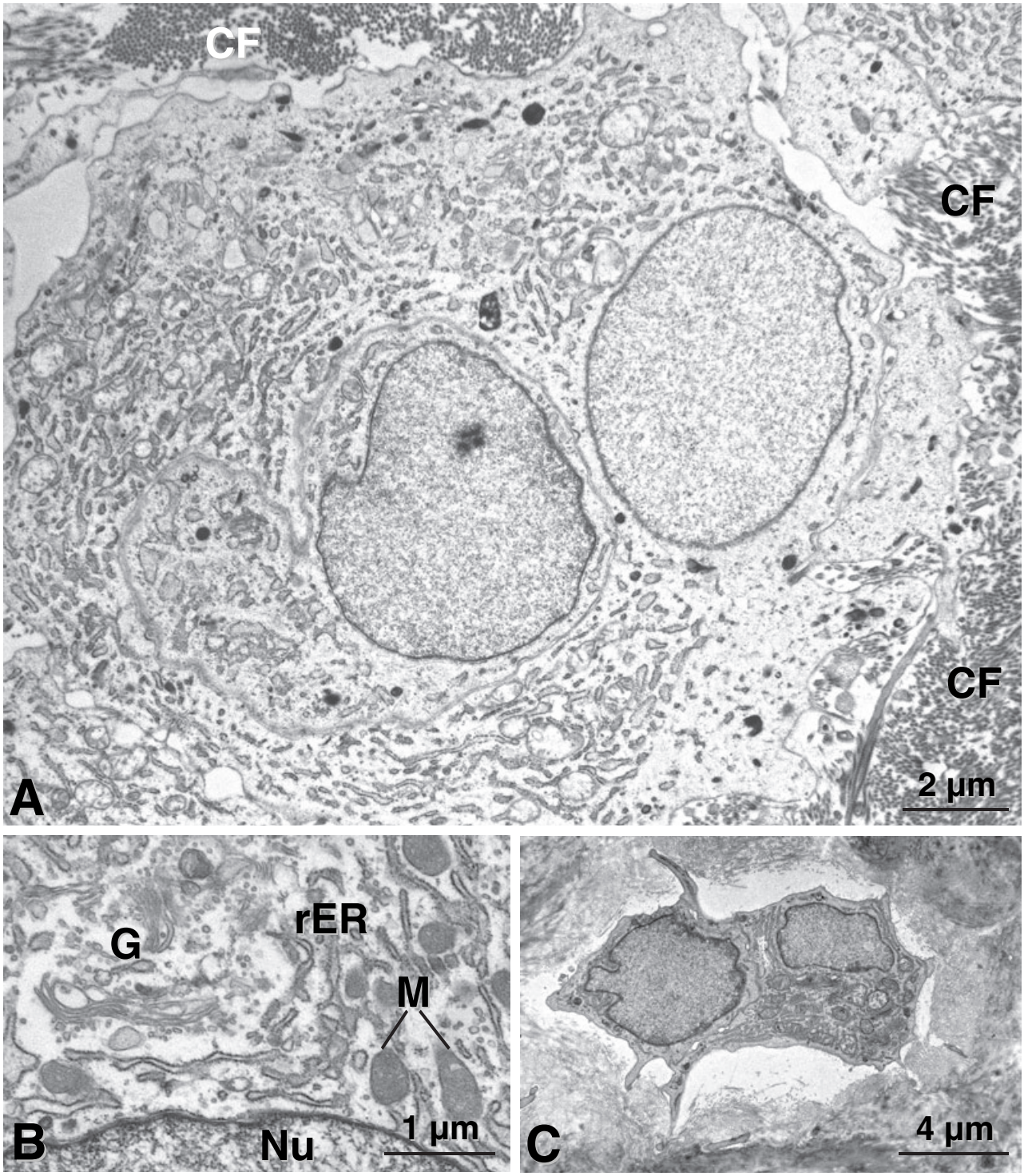

Fig. 3. Transmission electron micrographs illustrating structural details of the newly formed tissue on one root defect 4 weeks following treatment with enamel matrix-derived proteins (EMD). (A, B) The cells lining the surface of the newly formed tissue are very large in size. They possess a euchromatin-rich nucleus (Nu), abundant cisternae of rough endoplasmic reticulum (rER), many mitochondria (M), and a prominent Golgi complex (G). They abut to an extracellular matrix consisting of loosely packed collagen fibrils (CF). (C) The cytoplasm of the embedded cells is reduced in size, but still contains many cell organelles. The perilacunar matrix consists of a collagenous matrix that is obscured further away from the lacuna by an electron-dense organic material. (A) LR White section; (B, C) Epon sections.

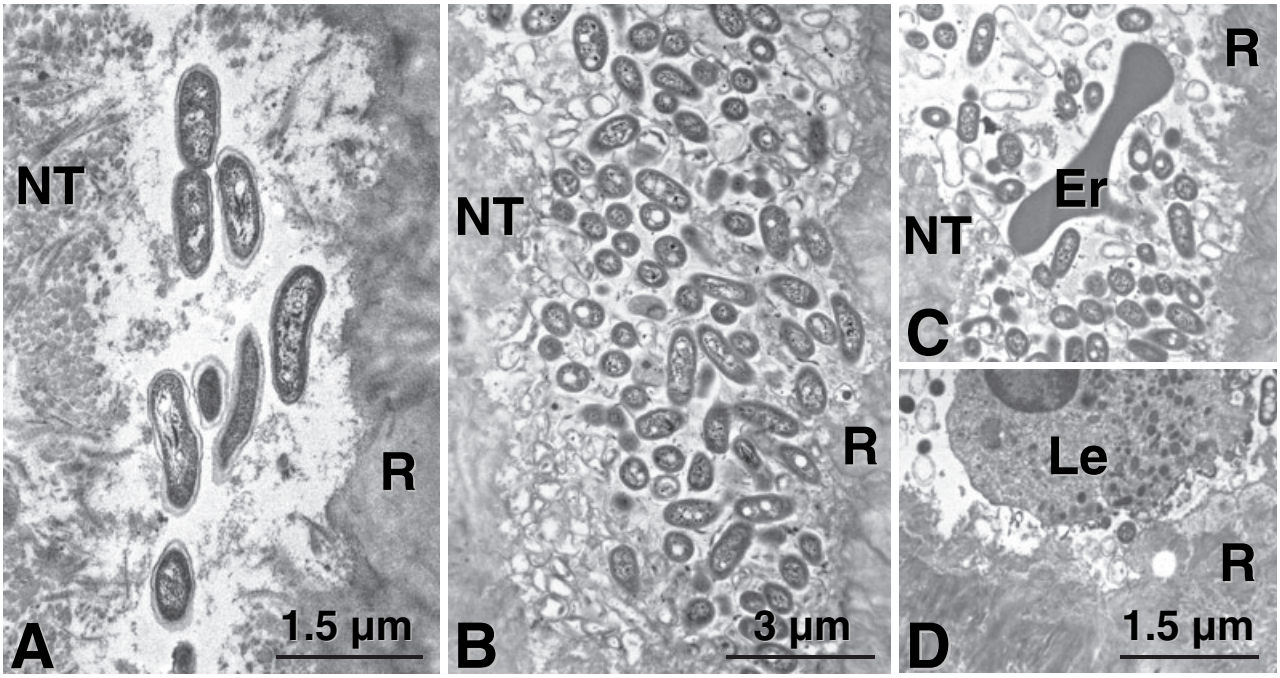

Fig. 4. Transmission electron micrographs illustrating the interfacial gap region between the instrumented root surfaces (R) and the newly formed tissues (NT) 4 (A) and 5 weeks (B–D) following treatment with enamel matrix-derived proteins (EMD). The gap space is filled with scattered (A) or colonies (B) of bacteria. Erythrocytes (Er) (C) and leukocytes (Le) (D) are found in association with these bacteria.

of ethical reasons, such histological experiments cannot be performed in patients with teeth presenting an optimal prognosis. Hence, the outcome of the present and other similar studies cannot be compared with recession or dehiscence defects where intact and healthy periodontal tissues are surgically removed before treatment with EMD (15, 23). In the present study, the chosen healing period of 2–6 weeks was not supposed to be of long enough duration to document bone healing. Therefore, extracted teeth instead of block biopsies were used for the histological analysis of the root surfaces. Owing to the fact that a notch indicated the apical extension of the defect and served as a reference point for the histological evaluation of *de novo* tissue formation on the treated root surface, no untreated control teeth were incorporated in the study. The aim of the study was, thus, a detailed structural analysis of the newly formed tissue on the roots affected by periodontitis following the application of EMD.

The rationale for using EMD for periodontal regeneration bases on the assumption that enamel matrix proteins (EMPs), synthesized and secreted by cells of the Hertwig's epithelial root sheath, trigger the differentiation of dental follicle cells into cementoblasts (13,14), representing a case of 'biomimicry' (24). Moreover, it has been suggested that EMPs, in particular amelogenins, specifically induce the formation of AEFC (13,14). The present study clearly demonstrates that the newly formed tissue on the root surfaces under the influence of EMD was not AEFC. Similar observations are already documented in a comparable human study (6), and terms like 'mixed cementum' (25) or 'cementum with inserting fibres' (11) have also been used. In the present study, the newly formed mineralized tissues on the root were clearly devoid of extrinsic fibres.

In the present study, all tissues that formed on the root surface following application of EMD had similar morphological characteristics. The presence of embedded cells suggests that the newly formed tissue on the root is either cellular intrinsic fibre cementum (CIFC) or bone. Both tissues are characterized by a mineralized collagenous matrix and lacunae filled with embedded cells. The ultrastructure of cementocytes and osteocytes is very similar. Regarding the collagenous matrix, mature bone has a higher degree of structural organization (26) as compared to CIFC (27). The collagen fibrils of woven bone, however, have no preferential orientation, a feature also found in CIFC. Furthermore, woven bone formation does not depend on the existence of a solid surface. It arises from connective tissue serving as a template. In contrast, CIFC, a mineralized tissue that may be a component of cellular mixed stratified cementum or be present as a repair tissue filling resorptive and fracture defects of the root, can only form on a pre-existing solid substrate. In this regard, the newly formed tissue may be regarded as being a more cementum-like than a bone-like tissue. On the other hand, both the irregular surface contour of the newly formed tissue and the presence of matrix protrusions resembling bone spicules (Figs 1A and C) suggest bone-like features. In the present study, it remains unclear

whether the newly formed tissues are more bone-like or more cementum-like, because of a lack of tissue markers suitable to differentiate between bone and CIFC. While evidence for a causal connection between EMPs and cementogenesis is still lacking, the bone-like appearance is in line with the chondrogenic/osteogenic activity of EMPs (28–33). Experiments with EMD also show osteogenic effects, albeit not consistently (34–46). The large amount of bone fill following application of EMD in periodontal defects also suggests an osteogenic effect (47). However, it cannot be concluded that the bone-like tissue particles found in the periodontal ligament in 50% of the teeth in the present study had formed because of the presence of EMD. They are likely bone particles that were severed from the alveolar bone during the surgical procedure, because they were mature and necrotic.

An interesting observation of the present analysis, which is in line with reports from animal studies (47,48), was that new tissue formation occurred also apical to the notch and at sites unrelated to the notch area on both scaled and unscaled older native cementum. It may be argued that the development of the newly formed tissue found on top of old native cementum at sites unrelated to the notch area is a phenomenon that was unrelated to the effect of EMD. Alternatively, the morphological similarity of the tissues formed at all these sites suggests a common biological stimulus. Thus, EMD may not only trigger *de novo* formation of a mineralized tissue, but may also stimulate matrix deposition on a pre-existing cementum layer. This conclusion is in line with the multiple effects of EMD, such as promotion of cell proliferation, attachment, differentiation, and up-regulation of extracellular matrix production (35, 36, 40, 41, 44–46, 49–54). Furthermore, gene expression profiling has shown differential expression of 121 genes in periodontal ligament cells exposed to EMD (55). Together, this multitude of effects may be held responsible for the beneficial effects on periodontal tissue regeneration. The most pronounced effect in terms of amount of new tissue formation, however, occurred in the notch region.

Common to all situations, where new tissue formation on the root surface had occurred, was the lack of a distinct mineralization front. Normal bone and CIFC formation are characterized by the presence of a clearly recognizable seam of unmineralized osteoid and cementoid, respectively. The absence of a distinct unmineralized seam may have to do with the short healing period. As this is the first study investigating early healing events after the application of EMD, a comparison to other studies cannot be made. It is, however, very unusual that the thickness of the matrix in the notch area amounted to about 120 μm (Fig. 1A) and 210 μm (Fig. 1B) after only 4 and 6 weeks, respectively. This finding suggests that EMD induced a very fast growth of a collagenous matrix. Similar observations were made in rat molars in relation to physiological tooth movement (56). The very large size of the formative cells and the existence of the full cytoplasmic armamentarium required for protein synthesis and export suggest that EMD has a profound effect on cell activity. Although light microscopy was not sufficient to detect mineralization, ultrastructural evidence of mineralization was observed in the form of mineralization foci scattered in the newly formed tissues. This observation is in line with studies showing that EMD has an effect on the mineralization process (31, 33, 41, 45, 46, 50, 52, 57).

A very common finding in experimental periodontal regeneration studies is a tissue separation between the newly formed mineralized tissue and the root surface (58–62). Such a tissue separation has consistently also been observed after the use of EMD (6, 11, 16, 47, 48, 63–66). There seem to be no major concerns about these findings, since tissue processing is held responsible for the formation of this tissue gap. Common to all those studies is, however, that paraffin sections were used. Processing of tissues for paraffin histology does not optimally preserve morphological details, and produces severe tissue shrinkage and distortion artifacts (17–19). Although tissues processed for embedding in acrylic or epoxy resins are less prone to artifacts (67), a tissue gap was consistently observed in the coronal-most portion in the present study. It may, thus, be concluded that the gap observed between the newly formed tissue and the treated root surface is not an artifact. The presence of scattered and microcolonies of bacteria in the gap supports this conclusion. Theoretically, it may happen that microorganisms grow in a buffer solution. To minimize their growth during tissue processing, antimicrobial agents such as arsenic in sodium cacodylate are widely used and most processing steps are performed at 4°C. In the material of the present study, the fact that the microorganisms in the gap resemble bacterial species commonly found in young dental plaque (68) and that erythrocytes and leukocytes are associated with the bacteria clearly indicates that the tissue separation had nothing to do with the tissue processing protocol. However, it may be possible that the gap formed during tooth extraction.

In conclusion, the present study has analysed histologically the early healing events on the root surface in human teeth after application of EMD. In all teeth where new tissue formation occurred on the root surfaces, the morphology of the newly formed tissue was identical. It may be stated that instead of the development of AEFC, a partially mineralized connective tissue formed that contained many embedded cells, but no extrinsic fibres. This tissue may thus be classified as bone-like or as a cementum-like tissue resembling CIFC. The tissue gap that was observed between the newly formed tissue and the treated root surface is not an artifactual product of tissue processing, but rather the result of a weak union between the newly formed tissue and the EMD-treated root surface.

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