Experimental Regeneration of The Periodontium

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I. INTRODUCTION

The biologic possibility of regenerating periodontal tissues, especially bone, lost due to chronic inflammatory periodontal disease has fascinated researchers for over 100 years. During the past 15 years, an explosion of new knowledge in the area of periodontal physiology coupled with the application of novel techniques in cellular and molecular biology has shed new light on the biologic possibilities of regenerating periodontal tissues. Along with the breakthroughs, we have generated more challenging questions and controversies. Wound healing in general has been described in the biomedical literature using a time-scale compartmentalization (for review, see Clark[1]). Wound healing in the periodontium may best be considered as unusual combinations of biologic phenomena, typically inconsistent and incompletely understood. This article reviews the current knowledge in the area of wound repair and regeneration of periodontal tissues that have been lost due to chronic inflammatory diseases.

Regeneration of periodontal tissues requires that biologic events in the damaged tissues result in restoration of architecture and function. Wound repair, on the other hand, occurs when neither the architecture nor function is restored. Physiological regeneration (turnover) occurs when degenerated and worn-out material is replaced continuously throughout its life. Regeneration appears to be the consequence of an organized series of cellular and molecular events in the structurally and functionally impaired tissue. An understanding of these orderly events at the cellular and molecular level is crucial for a thorough understanding of this remarkable process of periodontal tissue regeneration. To put it in general terms, in order for a given tissue to be regenerated, the following biologic events appear to be necessary:

1. A pool of progenitor cells that have the phenotypic capability to synthesize the matrix of the damaged tissue must be available.
2. These progenitor cells divide in response to stimuli (e.g., growth factors — from local and systemic sources).
3. The progeny cells migrate to wanted sites in response to chemical mediators of cell migration.
4. Some or all of the progeny cells undergo amplifying divisions as needed at their new locations, differentiate into specific end-stage cells (e.g., cementoblasts), and synthesize new matrix.
5. The newly deposited matrix be reorganized to restore the architecture and function.

It is evident that each of these steps should be well regulated and that the regulatory processes in turn should be subject to some degree of therapeutic control. In order to orient the reader to this approach, we discuss the current findings under four somewhat overlapping phases:

1. Development and structural biology of periodontal tissues
2. Cells of the periodontium
3. Division and migration of cells in the periodontium
4. Behavior of cells in the periodontal tissues *in vivo* and *in vitro* observations

II. DEVELOPMENT AND STRUCTURAL BIOLOGY OF THE PERIODONTAL TISSUES

The origin of the three periodontal tissues (cementum, alveolar bone, and periodontal ligament) from the cells lining the inner layer of the dental follicle is well established.[2,3] During root development, the odontogenic epithelium extends apically as a double layer terminating as the epithelial diaphragm and is referred to as Hertwig's epithelial root sheath. As it migrates in an apical direction, Hertwig's root sheath induces differentiation of dental papilla cells into odontoblasts, leading to the formation of the outer layer of the future root dentin. Once root dentin is formed, Hertwig's root sheath loses continuity in the coronal half of the root, thereby allowing the cells of the inner layer of the dental follicle (perifollicular?) to come in *direct contact* with the root dentin. This leads to the differentiation of the first generation of dental follicle cells that come in contact with root dentin into cementoblasts. Cementogenesis is thus initiated by a complex series of apparently well-coordinated events and is the first step in the establishment of an oriented fiber system (periodontal ligament) with the ends of the fibers (Sharpey's fibers) incorporated within the matrix of cementum and bone. These inserting collagen fibrils (extrinsic matrix fibrils) are surrounded by intrinsic matrix fibrils that are part of the cementum matrix. The extrinsic and intrinsic

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matrix fibrils have different orientations. Function in the periodontal ligament (support) is made possible by the reinforcement of its fibril ends within the cementum and bone matrices. This brief summary of biologic events during development has the following important implications in regeneration: (1) biologic effects of matrix molecules during root development and (2) observations common to root morphogenesis and tissue regeneration.

A. Biologic Effects of Matrix Molecules During Root Development

First of all, the newly formed root dentin has Hertwig's root sheath adhering to it via a basal lamina.[4] Basal lamina is known to be comprised mainly of laminin and type IV collagen.[5] Laminin is the major glycoprotein of basement membranes mediating the adhesion of epithelial cells to type IV collagen.[5]

The breakup of the inner epithelial cells on the surface of dentin has been believed to be due to degeneration of Hertwig's epithelial root sheath leading to discontinuity of epithelium.[6] The timing, initiation, and sequence of cellular degeneration, if any, in Hertwig's root sheath have never been convincingly demonstrated. On the contrary, a more recent investigation by Cho and Garant[4] has led them to conclude that root sheath cells do not degenerate but are probably detached from the root dentin surface and that the underlying basal lamina is fragmented as a consequence of the directed, active migration of precementoblasts toward the dentin surface. Ultrastructural evidence has been presented to support the concept that precementoblasts develop polarity toward root dentin and exhibit major cytoplasmic processes that are oriented toward and ultimately contact dentin matrix.[4] This implies that chemoattractants for cementoblast precursors are located either in dentin matrix or the basal lamina-associated material of root sheath origin. Matrix components with biologic properties such as chemoattraction of progenitor cells have not been isolated and characterized from dentin matrix yet. That the matrix of bone contains factors with important biologic activities is known[7] and it can be expected that such biologic factors would reside in the matrices of dentin and cementum. There is no information available on the composition of the epithelial, morphologically described basal lamina seen between the root sheath and dentin. Whether structural and compositional modifications of the basal lamina occur, and if so, their role in the differentiation of precementoblasts is not known. It is also unclear if all or only some of the cells of the dental follicle are recruited to differentiate into cementoblasts.

In another recent study, Slavkin et al.,[8] using organ culture *in vitro*, have shown that Hertwig's root sheath cells from mice synthesize enamel-related proteins. Although these root sheath-derived proteins have different amino acid compositions, they share one or more epitopes with enamelin and amelogenin enamel proteins. The function of these enamel-related proteins is unknown at the present time. How the precementoblasts from the dental follicle are directed toward the developing dentin matrix is still unclear. It is possible that the basal lamina between the epithelial root sheath and the root dentin is a repository of chemoattractants and/or that the root dentin matrix itself is rich in chemoattractants. These two possibilities have been suggested by Cho and Garant[9] following their finding of ^{3}H-mannose-labeled material in these two sites. Since fibronectin and the carboxyl terminal propeptides of procollagen contain mannose, the chemoattraction of precementoblasts may be attributed to the synthesis of these and other mannose-containing matrix macromolecules.[9] Interestingly, Cho and Garant[9] found that after the onset of cementum deposition, the newly differentiated cementoblasts detach from the root surface and enter the fibroblastic compartment of the periodontal ligament.

Another recent finding has been the synthesis of tenascin by cells cultured from the adult periodontal ligament and its localization in the lamina propria of the gingiva immediately beneath the basal lamina.[10] Tenascin is a glycoprotein of the extracellular matrix that is mainly seen expressed during embryonic development and that has a very restricted temporal and spatial distribution.[11] Tenascin has been shown to be expressed in abundance during root and alveolar bone formation.[12] Although the dental follicle appears to be a tenascin-free zone, the dental papilla and the osteogenic mesenchyme of developing teeth show tenascin expression.[12] The synthesis of tenascin by adult periodontal ligament cells in culture,[10] however, suggests that tenascin expression may be a common feature of determined but relatively undifferentiated cells with the potential to form mineralizing matrices. The results of studies currently in progress in several laboratories on the possible role of tenascin in cell differentiation should provide additional interesting information about this new glycoprotein.

B. Observations Common to Root Morphogenesis and Tissue Regeneration

The findings discussed in the previous paragraphs suggest that several, as yet incompletely understood, molecular and cellular events regulate the migration and differentiation of precementoblasts. These findings have implications in the process of cementum regeneration. Knox and Aukhil[13] have shown the appearance of a bilayered, electron-dense material resembling basal lamina at the ultrastructural level, between the root dentin and newly formed cementum in healing periodontal wounds of rats. Similar observations have been reported by Listgarten[14] and Nalbandian and Frank.[15] Also, during the early stages of cementum regeneration in experimental wounds, the cells adjacent to the denuded root surfaces show numerous cell processes directed toward the root surface.[13] These cell processes appear to retract as the new cementum matrix is deposited and these sequences of events resemble those observed during embryonic root development. It is clear that this area

needs a more thorough investigation. The composition of the basal lamina-like electron-dense material seen during cementogenesis and cementum regeneration needs to be analyzed and compared. The contact of cells with the denuded root surface appears to be necessary for differentiation of the progenitor cells into cementoblasts during cementum regeneration. In a light microscopic study of new attachment formation, Aukhil et al.[16] prevented the cells migrating into experimental periodontal wounds from contacting denuded root surface by interposing a physical barrier (Nuclepore membrane, 0.1-μm pore size) between them and found no new cementum even after 3 months of healing. Interestingly, new cementum was seen at the borders of the wound where the cells were allowed to contact the denuded root dentin. Keeping in mind the limitations of such observations, the findings by Aukhil et al.[16] suggest that cell-dentin matrix interactions may be involved in cementoblast differentiation, and these events may be reminiscent of those seen during embryonic cementogenesis. The molecular aspects of embryonic root development should provide important information that may be useful in tissue regeneration. Unfortunately, this area has received very little attention by investigators in the field of tissue regeneration.

III. CELLS OF THE PERIODONTIUM

The cells associated with the periodontal ligament, cementum, and alveolar bone can be broadly classified into synthetic cells (fibroblasts, cementoblasts, and osteoblasts), resorptive cells (fibroclasts, cementoclasts, and osteoclasts), progenitor cells, and miscellaneous cells. For a detailed description of these cells, the reader is referred to specialized texts.[17,18] Of relevance to regeneration are the progenitor cell populations, their phenotypic features, and the disputed origin of synthetic cells. It has long been assumed that gingiva, alveolar bone, and periodontal ligament are structurally different and anatomically somewhat unrelated organs. If we examine the anatomical order of gingiva, bone, and periodontal ligament, the concept of individual closed compartments simply cannot be justified. No mechanism(s) or structural barriers have been shown to exist to prevent the migration of cells from one tissue compartment to the other. For example, there is no evidence that progenitor cells from the periodontal ligament would not migrate into the lamina propria of the gingiva. This leads us to the more logical concept of an open compartment[19] where the three connective tissues, gingiva, alveolar bone, and periodontal ligament, are intercommunicating. If these anatomical zones are intercommunicating, it is then reasonable to expect an unrestricted (or limited as the case may be) migration of cells from one compartment to another (Figure 1). It is in this context that discussion on the progenitor cells, phenotypic features of cells, and origin of synthetic cells will be focused. Certain matrix components such as type I collagen, proteoglycans, and fibronectin are common to all three tissues — gingiva, alveolar bone, and periodontal ligament. Similarly, mineralized matrices are either part of or form anatomical borders of lamina propria of gingiva, alveolar bone, periodontal ligament, and cementum. This would then imply that cells with similar phenotypic features could constitute, at least in part, the cellular population in all the three zones. Several recent studies lend support to this concept. McCulloch et al.[19] have shown, via continuous labeling, that mesenchymal cells from bone stromal compartment migrate into the periodontal ligament via vascular channels. This finding suggests that the progenitor cells and in turn the synthetic cells may originate from the bone stromal compartment. In addition to autoradiographic data on cell kinetics, the consistent observation of increased thickness of cementum on roots in areas opposite to openings of endosteal spaces suggests the origin of progenitor cells from bone stromal zone.[19] A more fundamental question that remains to be answered is whether the three synthetic cell types (osteoblasts, cementoblasts, and fibroblasts) have a common progenitor cell (or does each have its own subpopulation of progenitor cells?). Preliminary information from cloned periodontal ligament cells suggests that the periodontal ligament contains distinct populations of fibroblast-like cells.[20]

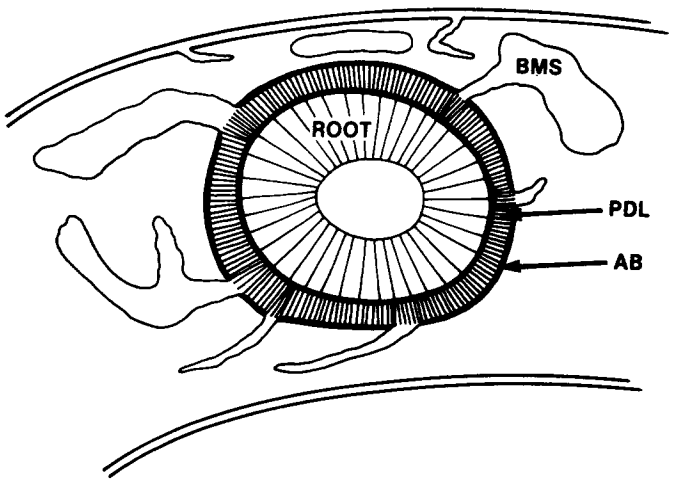


FIGURE 1. Diagrammatic illustration of the concept of open compartmentalization in the periodontium presented by McCulloch et al.[19] The illustration represents a transverse section through the alveolar process to show bone marrow spaces (BMS) containing the progenitor cell population opening into the periodontal ligament (PDL) space. The BMS would then serve as a reservoir of progenitor cells with the capacity to form cementum, alveolar bone (AB), and periodontal ligament.

Another elegant approach to study the interrelationships of gingival, alveolar bone, and periodontal ligament compartments is to characterize *in vitro* the cells derived from these sources. A preliminary study has shown that some cells derived from alveolar bone and periodontal ligament exhibit biochemical characteristics consistent with the osteoblast-like phenotype.[21] Bone and periodontal ligament-derived cells show a significant increase in PTH-stimulated c-AMP and high basal

levels of alkaline phosphatase.[21] In fact, elevated levels of alkaline morphatase activity, a feature thought to be suggestive of osteoblast-like phenotype, have been observed in cultured periodontal ligament cells by several investigators.[22,23] In contrast, cells cultured from gingiva and one periodontal ligament cell line were observed to have characteristics consistent with a fibroblast phenotype.[21] Although these studies suggest possible differences in cell phenotypes, it should be remembered that heterogeneous populations of cells are being studied and that some clonal selection of cells can occur late in culture due to the presence of some rapidly dividing subpopulation(s). Also, expression of different phenotypes does not necessarily mean that the cells originate from different sources. Expression of a given phenotype may depend on many environmental factors. The search for markers — biochemical or structural, specific for cell types (gingival, periodontal ligament, or alveolar bone derived) — has not provided any information that can be useful in identifying these cell populations. In a recent study, Yamauchi et al.[24] have purified a new pepsin-solubilized low-molecular-weight collagenous component (M_r = 30,000) from bovine periodontal ligament. This new collagenous component may be part of an insoluble highly cross-linked larger-molecular-weight collagen and it appears to be unique to the periodontal ligament.[24] If this collagen is confirmed to be unique to the periodontal ligament, it may serve as a marker for identifying cell populations.

Information on progenitor cell populations in the connective tissues of the gingiva is very limited. Using ^3H labeling (flash labeling), Pender et al.[25] have identified two progenitor cell populations in the lamina propria of gingiva — one in contact with cementum and junctional epithelium and the second lying in the body of the papilla at its most apical level. These two populations appear to extend coronally from the level of the crest of the interdental septum, suggesting that their origin may be from the periodontal ligament and/or alveolar bone. No information is available on the relationship of these two progenitor cell populations in the gingiva to blood vessels. Fibroblast substrain heterogeneity has been shown for cells cultured from gingival connective tissue also.[26]

In the past, morphologic studies have been relied on to assess the potentials of cells originating from the periodontal ligament and alveolar bone to form periodontal tissues (for review, see Aukhil et al.[27]). The data available are conflicting. Nonetheless, studies in animal models have suggested that the ability to form cementum and periodontal ligament is more a feature of progenitor cells in the periodontal ligament (reviewed by Iglhaut et al.[28]). At least one study has shown that *in vitro* only cells derived from embryonic rat calvaria have the capacity to form a cementum-like material.[29] There are no studies demonstrating the ability of gingival cells, either *in vivo* or *in vitro*, to form periodontal tissues such as cementum and bone. Although attempts have been made,[30] the results are inconclusive on the ability of cells derived from gingiva. All the studies using animal wound models should be interpreted with caution because (1) the origin of cells presumably synthesizing the new matrix has not been identified; (2) different wound models have been used; and (3) no biochemical/histochemical data are available to support the morphologic observations. It is not unreasonable to expect cells expressing different phenotypes to migrate into healing wounds of the periodontium. Studies comparing fibroblastic subpopulations in wounded and normal oral mucosa from rabbits have clearly shown differences in glycosaminoglycan production, response to interleukin-1, and secretion of interleukin-1 activity into culture medium.[31] To what extent such biochemical factors affect expression of cell phenotype in periodontal wounds has not been investigated.

IV. DIVISION AND MIGRATION OF CELLS IN THE PERIODONTIUM

The periodontium is considered to be an area of continuous cellular turnover. More specifically, the periodontal ligament shows continuous turnover of cells and matrix remodeling. What factors regulate the division of cells under homeostatic conditions? What cells in the periodontium divide in response to wounding and how is this regulated? Answers to these important questions may provide some clues for understanding the biology of periodontal tissue regeneration. Virtually nothing is known about factors regulating the cellular homeostasis in periodontal tissues. It is known that continuously dividing progenitor cells are located paravascularly in the periodontal ligament[32] and in the vascular channels of alveolar bone opening into the periodontal ligament.[19] Also, the finding of cells in the periodontal ligament expressing a high number of binding sites for epidermal growth factor (EGF) suggests the existence of progenitor cells.[33] That the newly divided daughter cells migrate toward the cementum and bone ends from their central, paravascular zone is also known.[34] Using ^3H-Tdr labeling, McCulloch and Melcher[35] have shown that the labeling index is inversely related to cell density in the periodontal ligament. Also, the labeling index was significantly higher in the middle of the ligament than in the zones adjacent to bone and cementum.[34] It has also been suggested that progenitor cell proliferation takes place in the apical zone "and cells migrate from the apical zone to middle and cervical zones, but maintaining a decreasing degree of proliferative activity as they migrate."[36] Taken together, these findings suggest that the paravascular zone of progenitor cells, presumably originating from bone stromal tissue and eventually populating the periodontal ligament and lamina propria of gingiva, is a continuously dividing population and their dividing capacity increases in an apical direction under physiologic conditions.

Regeneration of periodontal tissues during repair of the periodontium is subject to the orderly influx of progenitor cell population(s) into the lesion. It should be noted that unlike cell

division and migration under homeostatic conditions, regeneration demands cell mobility within the context of a rapidly changing environment.

Under pathologic conditions such as wound healing, no zonal restrictions are seen. Gould et al.[37] have shown that regardless of the level of wounding, a very narrow zone of adjacent periodontal ligament provides the progenitor cells to populate the wound. Based on the concept proposed by Melcher[38] that the progenitor cells for formation of new periodontal ligament and cementum are located in the remaining healthy periodontal ligament, Nyman et al.[39] developed a wound model that facilitates population of curetted root surfaces by cells derived from periodontal ligament. Several studies in animal models have since shown that by using physical barriers to exclude gingival epithelial cells and gingival flap connective tissue from healing periodontal wounds, the chances of obtaining new cementum and periodontal ligament are enhanced (reviewed in Reference 28). This principle has been called guided tissue regeneration, presumably to imply guiding of the coronal migration of progenitor cells from the adjacent periodontal ligament by the physical barrier, usually a biocompatible membrane. This principle of guided tissue regeneration assumes that only cells from the periodontal ligament migrate into the wound. In a recent study using the guided tissue regeneration model, Iglhaut et al.[28] have shown that in response to wounding, both the periodontal ligament and bone compartments provide progenitor cells that populate curetted root surfaces. This study[28] questions the claim that only periodontal ligament-derived cells can form cementum and supports the concept that progenitor cells originate from bone. A second study,[28] employing the guided tissue regeneration model, showed that a very limited zone of adjacent periodontal ligament (approximately 200 μm) provides the progenitor cells and this is in agreement with the findings by other investigators.[37] Taken together, these two studies suggest that during so-called guided tissue regenerations:

1. Both bone and periodontal ligament zones provide progenitor cells in response to wounding.
2. A very limited area (approximately 200 μm) of these two zones provides the cells.
3. These zones pump one generation of progenitor cells into the wound and then become quiescent.
4. The cells pumped into the wound compartment from bone and periodontal ligament (PDL) must undergo amplifying divisions.
5. The outcome of the wound healing depends on the rates of amplifying divisions in the subpopulations of cells with apparently different phenotypic features.

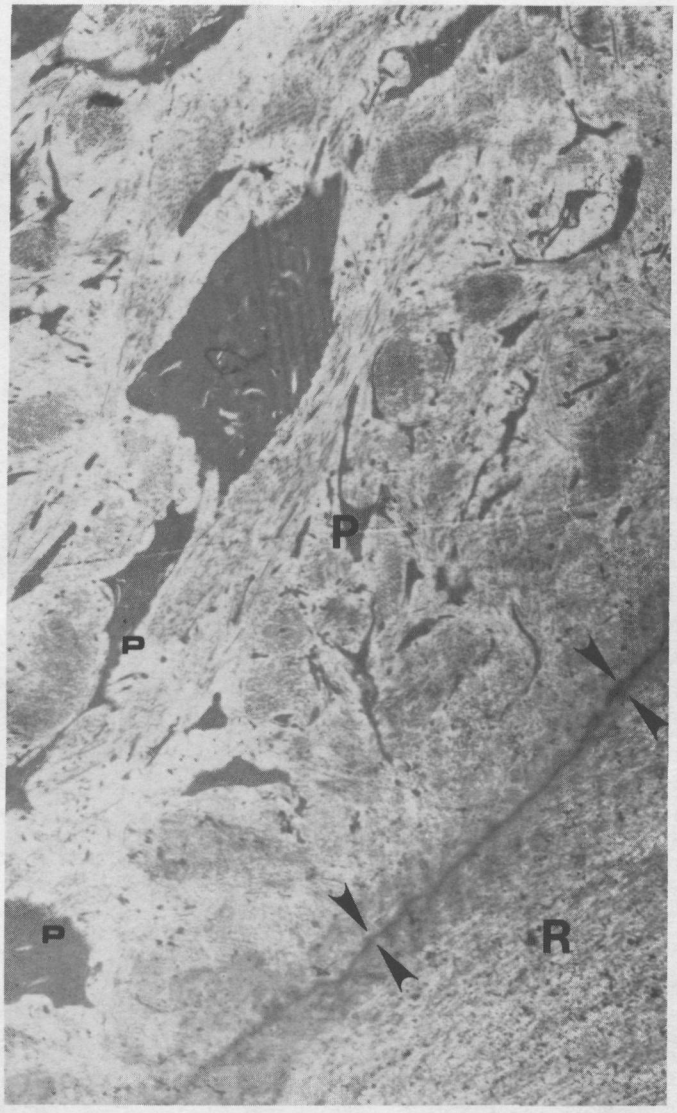
According to this concept, regeneration of cementum, bone, and PDL is the result of coordinated amplifications of the appropriate subpopulations of progenitor cells and it should be biologically possible to regenerate the periodontal tissues even if only a very small zone of the normal periodontal ligament and alveolar bone is remaining. Unusual healing such as ankylosis may be the result of uncontrolled amplification of the subpopulation of cells with the phenotype for bone formation. In order to test this hypothesis, molecular biology techniques have to be applied to study heterogeneity of cell populations in healing wounds.

V. BEHAVIOR OF CELLS IN THE PERIODONTAL TISSUES — *IN VIVO* AND *IN VITRO* OBSERVATIONS

Once the progenitor cells have divided and migrated to their respective domains, how do the cells differentiate, synthesize, and deposit matrix components? During root formation and cementogenesis, precementoblasts become hypertrophied during the directed migration and develop cytoplasmic polarity toward the dentin.[4] The precementoblasts appear to project several processes toward the root surface, leading to the degradation of the basal lamina.[4] It is interesting to note that while the components of the basal lamina may be responsible for the directed migration of precementoblasts, its degradation may be part of the cellular differentiation process. Once differentiated, the cementoblasts show a large euchromatic nucleus, prominent RER, cytoplasmic polarity, and cellular processes that are smaller than those seen prior to differentiation. It can be speculated that the complex network of cellular processes seen during initial cementogenesis may have some significance in cell-cell and cell-matrix interactions.

The behavior of cells (morphologic changes, regulation of phenotype expression, etc.) in regenerating periodontal wounds is incompletely understood. During the early stages of healing, a bilayered electron-dense material resembling basal lamina is seen at the ultrastructural level on the denuded root surfaces (Figures 2A and 2B).[13-15] In the normal dentin-cementum junction, no such electron-dense, bilayered zone has been described (Figure 3A). A complex network of cellular processes is also seen around cells immediately adjacent to the root surface.[13] Compared to cementogenesis during root formation, the electron-dense layer remains intact at least during the periods observed in cementum regeneration. Cementoid matrix and oriented bundles of collagen fibrils (presumably ligament fibers) are seen deposited over the electron-dense layer during cementum regeneration (Figures 3B and 4).[13] In their study on the formation of cementum-like tissue by bone cells cultured from calvaria of rats, Melcher et al.[29] also describe "osmiophilic incremental lines" between the curetted roots and the new matrix. This osmiophilic structure resembles the electron-dense layer described by others.[13-15]

The behavior of periodontal ligament and gingival fibroblasts cultured against biologic substrata such as root dentin



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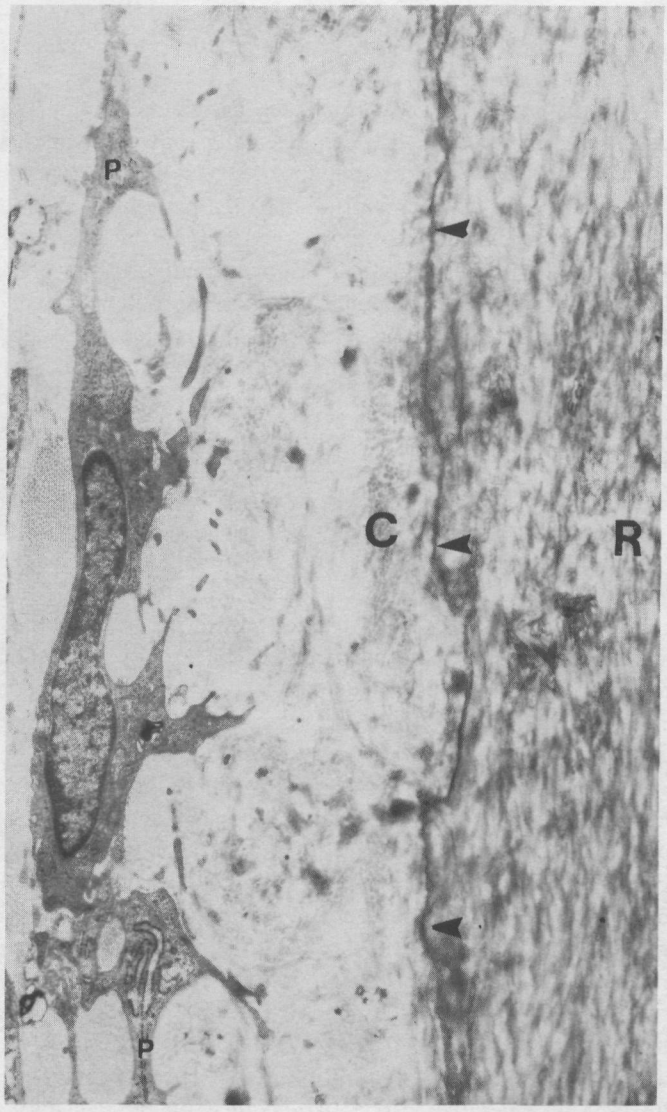

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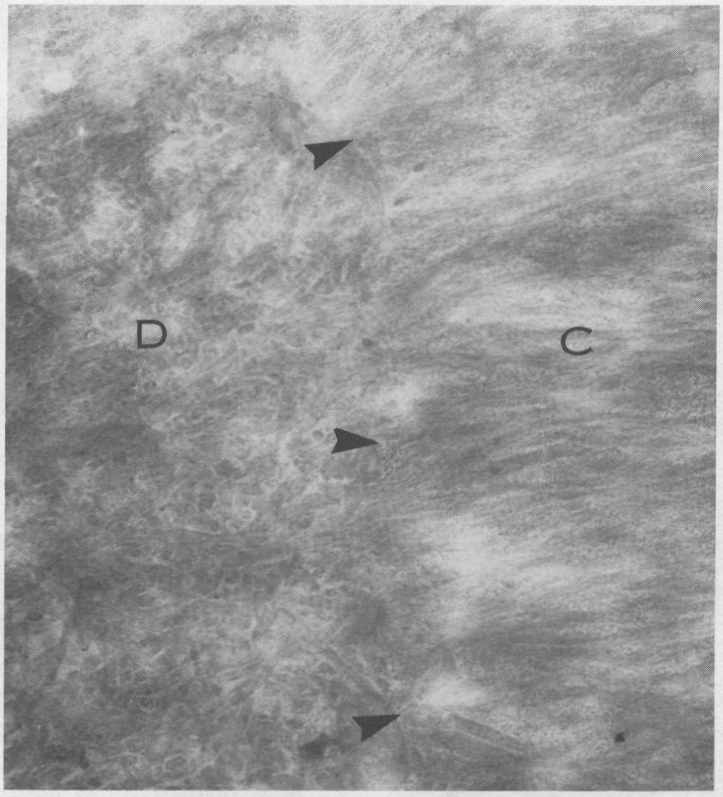
FIGURE 2. (A) A low-power electron micrograph of the denuded root surface in a fenestration wound model in rat. This is a 2-week-old specimen to show the extensive network of cellular processes cut in cross-section (P) adjacent to the denuded root (R). Note the electron-dense material (between arrowheads) between the denuded root surface and newly synthesized matrix. (B) Electron micrograph of a healing periodontal wound in monkeys to show formation of the electron-dense zone (arrows) between the denuded root (R) and the newly synthesized cementum matrix (C). Note the cellular processes (P) surrounded by bundles of collagen fibrils.

and bone *in vitro* has also been investigated. Caution should be exercised in interpreting the literature in this area because of differences in cell populations, culture methods, preparation of biologic substrata, observation periods, etc. Nonetheless, *in vitro* observations have at least supported some speculations. For example, cultured fibroblasts from gingiva have been shown to attach better, spread, and synthesize proteins on partially demineralized and fibronectin-coated surfaces.[41] Cells have been shown to prefer partially demineralized over nondemineralized substrata for attachment and migration.[41-43] This preference may be attributed to the affinity of fibronectin binding to exposed and denatured collagen seen as a result of demineralization.[44] The concept of superficially demineralizing denuded root surfaces to enhance new connective tissue attachment formation has been tested *in vivo* as well (reviewed by Cole et al.).

At the ultrastructural level, cultured cells can attach directly to the partially demineralized root surface *in vitro* or the attachment may be mediated via newly synthesized matrix components (Figures 5 and 6).[46-48] Unfortunately, most of the studies on the behavior of cells cultured against root and bone surfaces *in vitro* have concentrated on reestablishment of collagen fibril linkage (reattachment) and very little information



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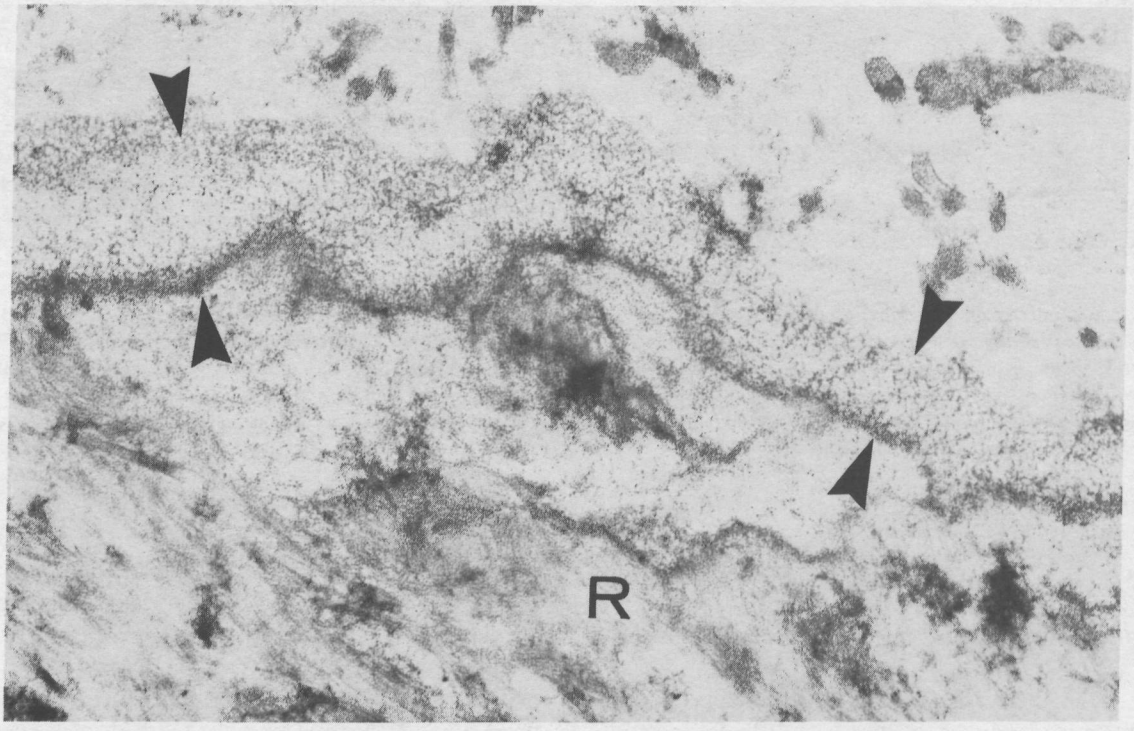

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FIGURE 3. (A) Electron micrograph of a normal dentin-cemental junction (D-C-J). Note that the junction between matrices of dentin and cementum (arrowheads) is not clearly defined and the obvious difference is in the orientation of matrix fibrils. (B) An electron micrograph depicting the granular, electron-dense material (between arrowheads) coating the denuded root surface (R) in experimental wounds of rats. The zone is bilayered with the more dense layer on the root side and the wider but granular and less dense layer on the periodontal side.

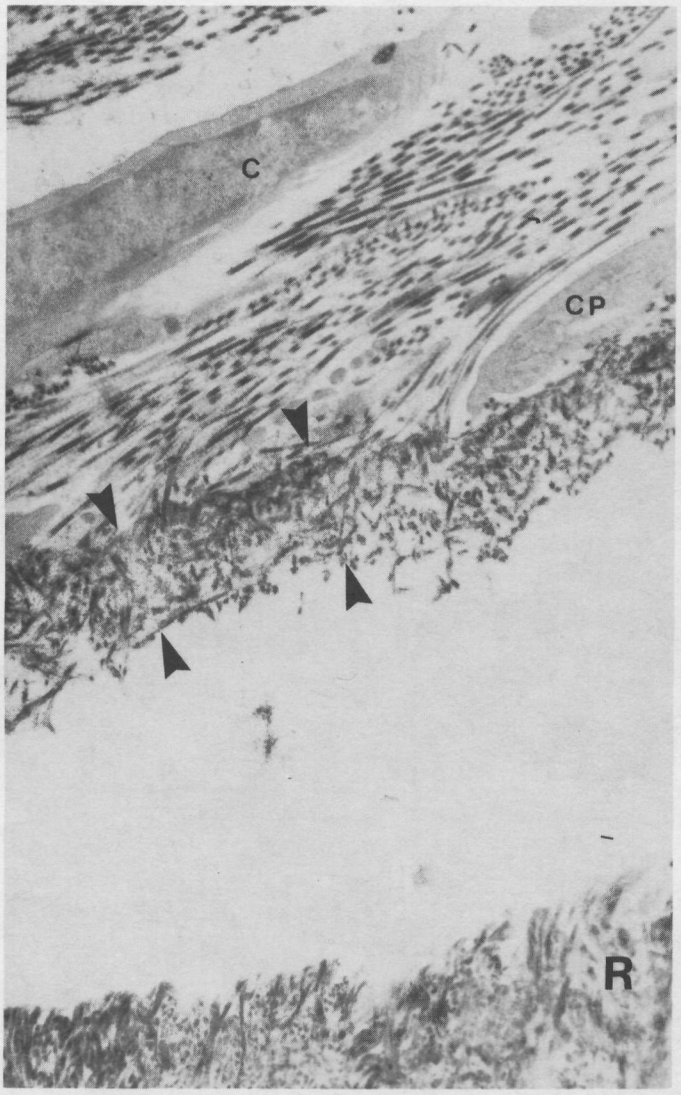

FIGURE 4. An electron micrograph representing a 2-week-old regenerating fenestration wound in the periodontal ligament of rat. The new cementoid matrix is artifactually separated from the root surface (R). The electron-dense zone (between arrowheads) has fibrillar material and some fibrils are continuous with the bundles of collagen fibrils oriented toward the periodontal ligament side. C and CP are cells and cellular processes, respectively.

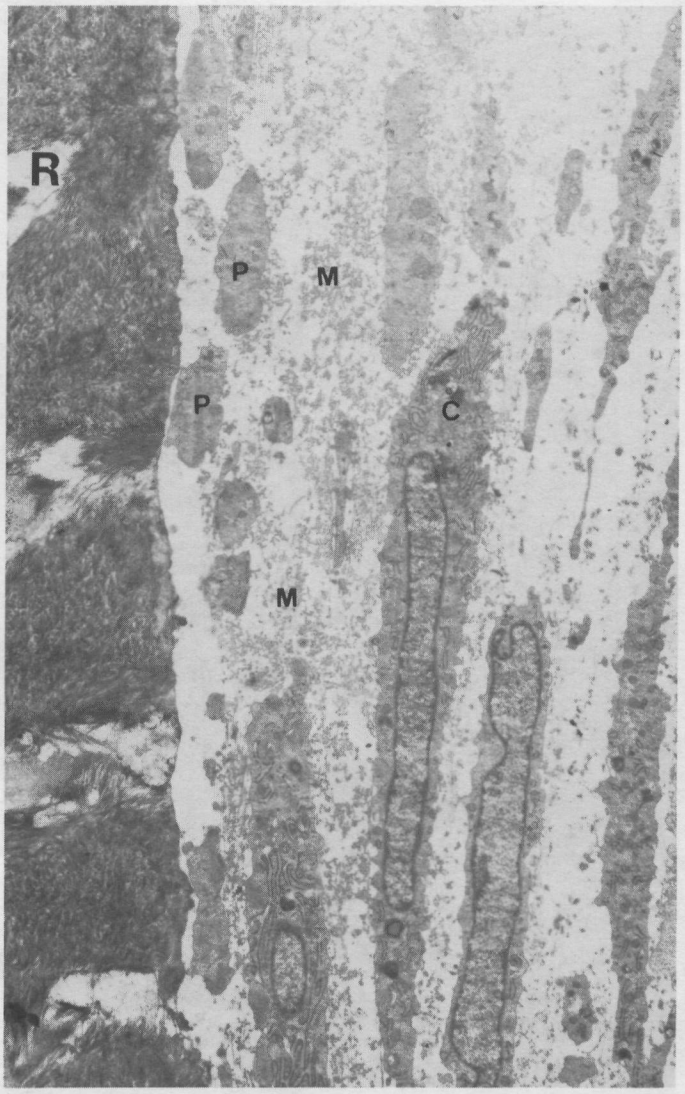

FIGURE 5. Electron micrograph of the vertical surface of a partially demineralized root slice (600-μm-thick, forming the walls of a simulated periodontal space) cultured with human gingival fibroblasts. The cultures were maintained for 30 d in medium supplemented with 50 μg/ml of ascorbic acid. Numerous cells (C) and their processes (P) are seen oriented parallel to the root surface (R) along with newly synthesized matrix fibrils (M). The orientation of cells and the scattered patches of new matrix fibrils do not resemble new attachment formation seen on roots *in vivo*.

is available on the structural changes in the cells.[46-49] However, there is general agreement that cells grown against the vertical surfaces of roots and bone *in vitro* are elongated and oriented parallel to the substrata surface. Although these flattened cells can be surrounded by patches of newly synthesized collagen fibrils,[46-49] the lack of cementum formation and actual reattachment suggests that the *in vitro* models may be inappropriate in this present form for studying regeneration. The absence of a three-dimensional environment, some growth factors, and other cell-cell interactions in the *in vitro* environment may contribute to the negative results observed. Since the cells experience a three-dimensional matrix in the presence of other cell types *in vivo*, it may be important to simulate these conditions *in vitro*. No information is available on experiments involving coculture of different cell types within reconstituted matrices. Upon long-term cultures, however, the cells form a three-dimensional matrix in the vertical spaces between closely spaced biologic substrata.[46-49] The spatial organization of the matrix synthesized in the spaces *in vitro* simulates fibers in tension (such as some periodontal and gingival fibers).[42] How-

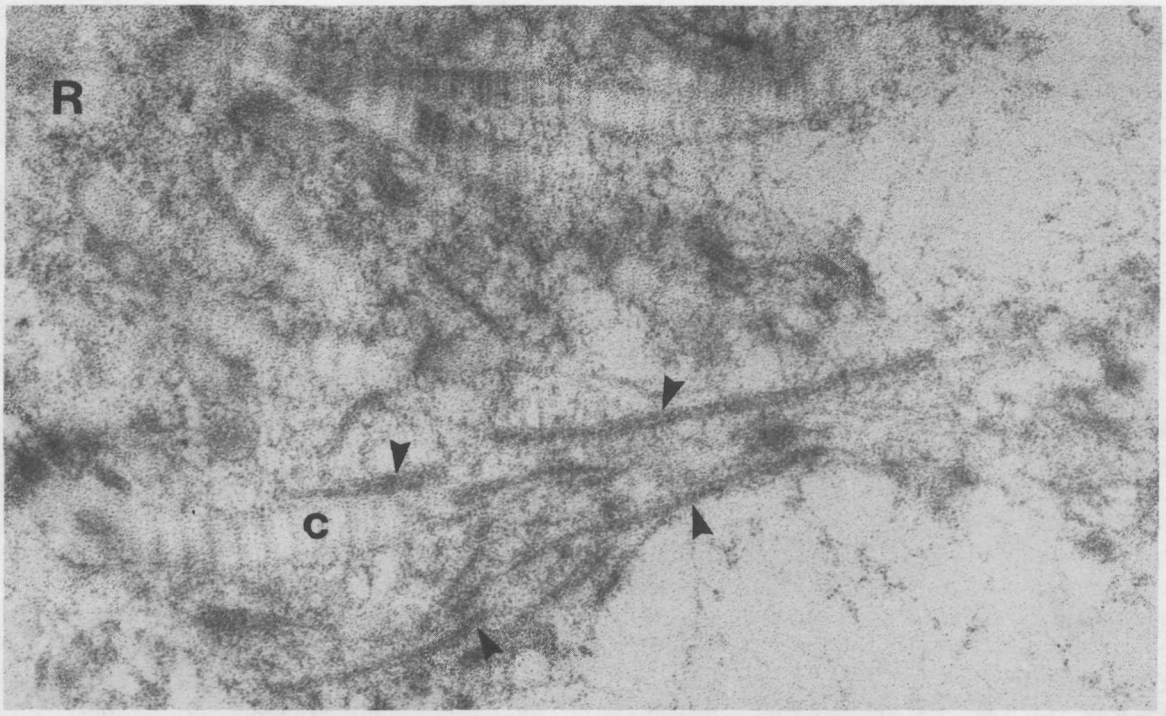

FIGURE 6. Electron micrograph of the vertical surface of a partially demineralized root slice (R) that was cultured over monolayers of human gingival fibroblasts for 30 d. At this magnification, fibrillar material (arrowheads) that is smaller in diameter compared with the collagen fibrils of the dentin matrix (C, along with periodicity of banding) appears to be closely associated with the dentin collagen fibrils.

ever, synthesis and organization of such a matrix by cultured fibroblasts are also seen in spaces formed by nonbiologic substrata such as Plexiglass®,[50] suggesting that this may be a generalized ability of fibroblasts. Using immunohistochemical methods, Qwarnstrom and Page[47] have analyzed the formation of extracellular matrix by cultured human gingival fibroblasts in an *in vitro* model for the periodontal tissues. They observed that the matrix initially contained hyaluronic acid, chondroitin sulfate, dermatan sulfate, and fibronectin followed by collagen fibrils with collagen types I, III, and V eventually dominating the matrix. They suggest that this model could be used to study the various aspects of regeneration of extracellular matrix. None of the *in vitro* studies have provided morphologic (cell shape, matrix relationship) or biochemical evidence of cell differentiation. All the ultrastructural studies on cell behavior against root surfaces *in vitro* have observed elongation of cells along the root surface and this is different from the *in vivo* situation where the cells along the root surface (cementoblasts) are more rounded. There is some suggestion that cell shape can affect gene expression at the level of transcription as well as translation.[51]

Another major event in the regeneration of connective tissue attachment is the formation of oriented fibers that provide the physical properties to ligaments. Since the establishment of oriented fiber bundles with their ends incorporated into new cementum and bone is crucial, lack of it does not constitute regeneration. Whether the cells actually deposit the matrix fibrils in the defined orientation or they initially deposit the fibrils randomly and later organize them is unclear. The latter possibility appears more reasonable because (1) the cells are usually not oriented in the defined pattern of fibril bundles; (2) orientation of matrix deposition is usually random in the early stages; and (3) the cells maintain very close contact with the fibrils along with active microfilaments in their cytoplasm, suggesting the generation of contractile forces.[46-49] *In vitro*, the concept of tractional structuring, whereby cells exert tractional forces on the substrata they are in contact with, leading to morphogenetic rearrangement of matrix components, appears to be very attractive.[52] Several *in vitro* models for periodontal ligament have shown such matrix reorganization by cells.[46-50,53] It should be noted that for tractional forces to result in morphogenetic patterns, and in our case ligament bundles, attachment of cells to substrata is as critical as the generation of forces. There is no direct evidence that tractional structuring occurs in periodontal ligament wounds *in vivo*, but some limited, preliminary information suggests so.[53,54]

VI. REGENERATION OF CONNECTIVE TISSUE ATTACHMENT TO PREVIOUSLY DISEASED ROOT SURFACES

Whether new cementum and bone with inserting, oriented fibers of the regenerated periodontal ligament form against previously diseased root surfaces has long been debated. The literature is replete with reports of so-called "new attachment", "reattachment", and "bone regeneration". These reports have relied on either clinical, radiographic, or light microscopic data to support the claims.

Chronic inflammatory periodontal disease results in loss of connective tissue attachment with the consequent apical migration/proliferation of oral epithelium. Structurally, potential spaces are created between the gingiva and the root surface, clinically referred to as pockets. Periodontal pockets are usually inhabited by a variety of aerobic and anaerobic microbes. Wound healing in the periodontium is unique in the sense that soft connective tissues, oral epithelium, calcified tissues such as bone and cementum, and a complex microflora are all involved in the healing process.

Conventional treatment for chronic inflammatory disease includes the removal of tissue irritants such as bacterial plaque and calculus, curettage of the inflamed tissues, and planing of root surfaces to remove surface cementum/dentin that may harbor bacterial endotoxins. Usually following instrumentation, the gingival tissues are closely adapted against the curetted root surfaces and the blood clot helps retention of adapted tissues. Adjacent cells, epithelial and connective tissue in origin, begin to migrate into the wound along with a complex cascade of cellular and molecular events (for a general review on wound healing, see Clark[1]).

Following conventional treatment, the junction between the curetted root surfaces and the soft tissues is usually an adhesion of junctional epithelium for the most part and a very limited zone of connective tissue attachment at the most apical part.[56] The so-called "long junctional epithelium" is the mode of attachment even in periodontal tissues where the pocket depths appear to reduce significantly and clinical attachment levels show substantial gains. Such an epithelial attachment is also seen between new bone and the root surface.[57] Several observations in the healing process following conventional therapy have important biologic implications. These include the tendency for apical migration of oral epithelium along the root surface, the extent of tissue removal during instrumentation, and cellular kinetics.

Why the oral epithelial cells tend to migrate apically along the root surface is unclear. It should be noted that the epithelial cells possess special adhesion structures on substrata such as hemidesmosomes.[58] The directionality for epithelial cells (in our case, the persistent direction being apical along the root surface) can be partly explained by the "free-edge effect" property of epithelial cells.[59] In the case of wounds involving epithelium, the marginal cells show lamellipodia into the defect within a few minutes.[59] Wound healing studies have shown that initial migration of epithelial cells starts from the lowest cell layers of the stratum spinosum or basale.[60] The marginal cells lose desmosomal and hemidesmosomal contacts; definition of basement membrane zone is lost and cells appear loosely attached. A synchronized locomotion of cells in the sheet is suggested by the observed direct correlation between the density of gap junctions and concentration of contractile proteins.[60] Epithelial cells at the leading edge become actively phagocytic and the availability of fibronectin can enhance epithelial cell phagocytosis.[60] Epithelial cell migration continues until movement is inhibited by contact with opposing epithelial sheet. In the case of apically migrating junctional epithelium, there is no epithelium from the opposite side and migration is presumably stopped by the presence of collagen fibers of the intact periodontal ligament and/or other connective tissues cells and matrix components. No information is available at the ultrastructural level on how the apical migration of the junctional epithelium is contained. Once cell migration stops, the epithelial cells regain the cytologic features of stationary cells. The formation of desmosomes is necessary for the end of migration[60] and the regulation of desmosome/hemidesmosome formation is not well understood.

The apical migration of oral epithelium along the root surface would imply adhesion to the root surface via basal lamina in addition to the basal lamina on the lamina propria side. The recent concept of adhesion of epithelial cells to type IV collagen via laminin[5] is attractive. However, evidence to the possible applied aspects of this effect is not very convincing. Cultured epidermal cells have been shown to attach better if plates are coated with fibronectin.[61] Epidermal cells have been found to adhere and spread as well on fibronectin as on types I, III, and IV collagen and laminin.[62] So, it appears that no one substrate protein is absolutely necessary and they all support adhesion and spreading of epithelial cells to about the same degree.[60] Thus, the proposed therapeutic use (localized) of extracellular matrix proteins to direct specific cell types in healing periodontal wounds[5] appears unrealistic at least for the present.

The retardation or prevention of the apical migration of oral epithelium along the curetted root surface can theoretically enhance the chances of obtaining new connective tissue attachment by allowing the progenitor cells to populate the curetted root surfaces. Several experimental approaches have been adapted to this effect. First of all, it was speculated that by superficially demineralizing the curetted root surfaces, the apical migration of oral epithelium could be retarded.[63] Several studies in animal models reported increased amounts of new connective tissue attachment following use of citric acid to condition root surfaces (for a review, see Selvig et al.[64]) initially. The mechanisms used to explain the initially impressive results included early establishment of a fibrin-linkage,[65] interdigitation of new collagen fibrils from flap connective tissue

with the collagen fibrils on the root surface exposed by citric acid demineralization,[64,66] and preferential binding of fibronectin to demineralized root surfaces.[44] However, long-term observations showed that the apparently regenerated cementum with inserting fibers was subjected to delayed resorption of roots.[67] It should be noted that these experimental surgical procedures using citric acid conditioning of roots recommend additional surgical incisions and moving of the flaps coronally before suturing. It is possible that this coronal displacement of flaps in itself can retard the apical migration of epithelium. This approach is unrealistic because coronal movement of surgical flaps is not always possible in humans. The second problem with this approach is the delayed root resorption seen in animal models.[67] The reasons why superficially demineralized root surfaces are resorbed have not been investigated. It can be speculated that since acid conditioning of root surfaces (pH 1) denatures collagen, and this leads to preferential binding of fibronectin, nonspecific chemotaxis of cells may be significantly elevated. This in turn can lead to ankylosis and, eventually, resorption of roots. This explanation is supported by the observation of focal areas of ankylosis in acid-conditioned roots, implanted in surgically created sites, detectable only by a careful analysis of step-serial sections.[27,68] Clinical and histologic studies have reported conflicting results on the beneficial effects of citric acid conditioning. The concept of early fibrin linkage to retard apical migration of oral epithelium[65] has not been proven convincingly. Similarly, that the theory of interdigitation between the exposed old (root) and new (flap) collagen fibrils actually occurs has been based solely on morphologic criteria. Selvig et al.[64,66] have recently published some ultrastructural findings that suggest that there may be interdigitation of new collagen fibrils with the denuded root matrix collagen. Differences in diameter between the "old and new" collagen fibrils and continuity of fibrils based on appearances in electron micrographs have been some of the main criteria used to interpret the findings. Keeping in mind the limitations of observations made upon sections of fixed tissues, the authors emphasize that such close approximations of fibrils are no evidence of reattachment providing resistance to mechanical faces. It is to be noted also that there is no reconstruction using serial sections (at the EM level) to support the concept of "interdigitation". In the absence of electron microscopic findings from such wounds representing the time point zero (i.e., immediately after wounding) and complications such as limited resorption of root surfaces, it is difficult to draw definite conclusions. There are no radioautographic studies at the ultrastructural level to confirm this. The fate of the superficial zone of demineralized roots is not known. Whether the superficially demineralized zone remineralizes or is resorbed during the healing process has not been addressed in detail. Although the concept of partially demineralizing the exposed root surfaces is interesting, the above-mentioned gaps in our knowledge have cautioned the use of this approach.

A second approach to retard apical migration of oral epithelium is the application of collagen solution to the denuded roots during periodontal surgery.[68-70] Preliminary studies at the light microscopic level report retardation of epithelial migration apically,[68-70] and the concentration of collagen in the solution does not appear to be important.[70] Interesting as this approach may be, no information is available on the actual binding of collagen solution, which incidentally gels at body temperature, to the root surface and its fate. Since complete periodontal therapy usually involves more than one area of surgery, the possibilities of subjects developing host responses following repeated use of collagen need consideration. One important aspect of this approach is that collagen-fibronectin matrices serve important functions of enhancing adhesion and migration of fibroblasts.[71] The problem of apical migration of oral epithelium has also been addressed by researchers in the field of implantology (for review, see Brunette[72]). The phenomenon of "contact guidance" has been explained by the shape of the substratum imposing certain mechanical restrictions on the formation of linear bundles of microfilaments involved in cell locomotion.[72-74] Thus, it appears that the direction of cellular outgrowth from a source (e.g., tissue explants) can be guided by the topology of the substratum and this has been demonstrated with grooved titanium surfaces.[73] Preliminary studies using percutaneous implants have shown that grooving the surface can decrease the length and rate of epithelial migration.[73-75] Although the research in this area is revealing important information, very little is known about the effects of changing surface topography of substrata on the proliferation and matrix synthetic profiles of cells.

The third approach to preventing/retarding apical migration of oral epithelium is the concept of guided tissue regeneration mentioned earlier. In this procedure, biocompatible physical barriers are used to create a "periodontal space" and to exclude the undesirable cells such as epithelium and flap connective tissues from populating the curetted root surfaces. It has been presumed that this facilitates population of the denuded root surfaces by "cells originating from the periodontal ligament". Numerous wound healing studies in animal models and humans, utilizing clinical and light microscopic criteria, have presented data showing apparent enhancement of connective tissue attachment with the use of physical barriers.[39,56,76-83] The extent of new attachment formation reported varies and it appears that predictability is yet to be established.[56] The reasons for variations in the healing pattern can be attributed to our lack of understanding of the healing mechanisms at the cellular and molecular levels. For example, it has not been proven that placement of physical barriers in wounds actually favors the coronal migration of cells originating from the remaining periodontal ligament. How long these physical barriers should be left undisturbed in their implanted sites is unclear, although cell division in the adjacent, ostensible feeder compartments (periodontal ligament and bone) is complete within 21 d.[28] It

appears that the outcome of the healing depends mainly on the ability of the small population of cells pumped into the wound from adjacent sources to undergo amplifying divisions.[40] Additional factors critical to the outcome include the rates at which the newly divided cells migrate and then differentiate into specific end-stage cells. No information is available to this effect.

Since citric acid conditioning of roots is believed to enhance new attachment formation, the combination of root conditioning and guided tissue regeneration has also been investigated. Studies in animal models[84,85] have shown that root conditioning has no additional advantage when combined with the use of physical barriers. Root resorption has been reported as an accompanying feature of the healing process with the use of physical barriers and citric acid conditioning.[77,82,84] The significance of the self-limiting, surface resorption reported during regeneration of connective tissue attachment in animal models[28,56,77,82,84] is not known. In addition to these studies in animal models providing circumstantial evidence in support of the concept of guided tissue regeneration, Boyko et al.[86] have shown formation of a periodontal ligament-like structure when roots bearing cultured periodontal ligament cells (autologous) were implanted in surgically created sites. However, direct interpretations cannot be made since the implanted cells were not labeled.[86] Other investigations have used variant forms of related wound models in animals to suggest that new attachment is formed by coronal migration of cells originating from the periodontal ligament.[87-89] None of the data presented in the literature confirms the theory of guided tissue regeneration. We want to emphasize that although circumstantial evidence to support the concept of guided tissue regeneration exists, it remains to be shown unequivocally that the capacity to form new cementum is restricted to cells from the periodontal ligament. Studies in animal models comparing the abilities of bone and gingival cells to form cementum[90-93] are to be interpreted very cautiously. No identification of cell types is possible and the wound models themselves are likely to induce resorption of roots. Unless the biochemical nature of the tissues surrounding the roots implanted at surgically created sites, as used in these studies,[90-93] is identical to that of gingiva and bone seen adjacent to root surfaces, such comparisons on the phenotypic capacities of cells are not justified. That the matrix environment of healing wounds is dynamic is known and how it affects healing in these types of wound models is unclear. Since there are no histochemical studies to show that the tissues surrounding implanted roots (as in the models used in References 90 to 94) are similar to normal gingiva, it is possible that the lack of cementum formation may be the result of inappropriate wound models. Future wound healing studies should utilize immunohistochemistry to support their observations.

The use of topical application of cell adhesion molecules has also been suggested in the literature to enhance new attachment formation. Studies have been reported where topical application of fibronectin is used to augment adhesion of cells to citric acid-conditioned root surfaces.[41,94,95] Since the local concentrations of fibronectin increase significantly in wounds,[96] the clinical benefits of exogenously provided fibronectin have not been demonstrated. In a recent study, Pearson et al.[97] have shown that 1 μg of fibronectin can saturate approximately 1 mg of demineralized bone or tooth powder. As serum contains nearly 300 μg of fibronectin per milliliter, the bleeding that occurs at the surgical site should be more than adequate for cell adhesion.[97] In addition, recent wound healing studies have shown no additional benefits of exogenous fibronectin in periodontal wounds.[7,98,99]

In addition to surface demineralization of roots with acids,[41,100] and use of exogenous matrix factors,[101] reports also exist in the literature on the use of antibiotics such as tetracycline hydrochloride to enhance cell attachment to root surfaces.[102-105] Most of the studies on cell attachment to root surfaces *in vitro* have relied on trypsin treatment to detach cells for counting. It has now been well established that such trypsin treatment of roots does not detach all cells from root surfaces.[105] Using a new assay that utilizes labeling of cells with ^{51}Cr, Lowenberg et al.[105] reexamined the attachment of cells to demineralized and nondemineralized roots and found no significant differences in the number of cells attached to the two groups of roots. Since other factors such as increased rates of cell division, migration, and chemotaxis can all affect the number of cells, adhesion alone should not be construed as the dominant event. Thus, the data from *in vitro* studies on cell adhesion to partially demineralized roots need to be reexamined.

VII. CONCLUSION

The fascination of being able to regenerate periodontal tissues lost to chronic inflammatory disease is closer to reality than ever before. Recent research findings in this area point at some very significant clues that, if further explored with cellular and molecular biology techniques, can lead to the development of therapeutic procedures that will result in predictable regeneration of periodontal tissues. Although circumstantial in nature, evidence now exists that identifies progenitor cell populations in alveolar bone and periodontal ligament compartments. Also, experimental therapeutic procedures have been developed that presumably facilitate population of instrumented root surfaces by cells representing these progenitor populations. Morphologic data exist to show increased tissue regeneration following these procedures. As expected, these bright sides of research findings also have some dark sides. For example, the problem of root resorption seen during experimental regeneration in animal models has not been further explored. Whether surface resorption of roots is a prerequisite for new attachment formation is unclear.

Angiogenesis is an essential part of wound healing and tissue regeneration. Although of transient nature, angiogenesis is a

dynamic process in wound healing and regeneration. A number of different factors can regulate angiogenesis *in vivo*.[106] Virtually nothing is known about neovascularization (angiogenesis) in healing periodontal wounds. This is particularly important because progenitor cells are guided into dead spaces such as the periodontal ligament space created by the nonvital root surface on one side and the biocompatible physical barrier on the other. The need for thorough understanding of neovascularization in healing periodontal wounds is unquestionable.

Growth factors and matrix molecules available in pure forms need to be utilized in a *rational way* to enhance the cellular events. As an example, coating of biocompatible materials such as implants used in periodontal therapy with these growth factors and cell-adhesion molecules should prove beneficial. However, it is emphasized that much more information is needed on the phenotypic capabilities of cells in the periodontium before these therapeutic manipulations can be rationalized.

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