

EFFECTS OF PERIODONTAL CELL GRAFTS AND ENAMEL MATRIX PROTEINS ON THE IMPLANT-CONNECTIVE TISSUE INTERFACE: A PILOT STUDY IN THE MINIPIG

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KEY WORDS

Enamel matrix proteins
 Dental implants
 Gingival connective tissue
 Periodontal ligament
 Amelogenins

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We have developed an experimental model to help identify and characterize factors necessary for periodontal connective tissue attachment formation on dental implants. In this pilot study, we report the effect of autogenous periodontal cell grafts, with and without the application of enamel matrix derivative (EMD), on the implant-connective tissue interface. Periodontal ligament (PDL) and gingival connective tissue (GCT) cultures were established from an adult minipig. Implants were placed in osteotomies prepared with exaggerated countersinks that served as recipient sites for autogenous cell grafts in bilateral edentulated posterior mandibular sextants. In addition, 1 side received an application of EMD before placement of the autogenous cell grafts. A bioabsorbable membrane covering the coronal portion of the implants was placed before closure. After 8 weeks, quantitative histomorphometric and qualitative light microscopic analyses revealed that the implants that received gelatin vehicle alone were surrounded by bone, whereas the implants that received GCT cell grafts were mostly surrounded by fibrous connective tissue. In contrast, implants that received PDL cells without the application of EMD demonstrated good bone contact, but strands of epithelium were observed in the implant-connective tissue interface. Implants that received PDL cells and EMD also had good bone contact but without evidence of epithelium. A cementum-like interface was not observed in any of the groups. Results of this pilot study suggest that EMD and the type of cell populations present in the implant wound-healing environment may alter the implant-connective tissue interface.

INTRODUCTION

Three distinct implant–connective tissue interfaces have been described depending upon the origin of the cell populations that populate the postsurgical wound-healing environment. An intimate contact of bone with the implant surface has been described as osseointegration, a term originally used by Branemark.¹ Osseointegration is dependent upon the close postsurgical approximation of the implant surface to the osteotomy site, resulting in the exclusion of nonosseous tissues such as gingival connective tissue (GCT) and epithelium. Indirect contact or fibro-osseous integration has been used to describe the presence of an intervening fibrous connective tissue between the implant surface and bone in an otherwise clinically successful dental implant.^{2–4} Fibro-osseous integration occurs when cells derived from nonosseous tissue, such as GCT, have access to the implant surface during wound healing. A third implant–connective tissue interface has been shown to be at least experimentally possible. The placement of titanium implants adjacent to retained tooth roots in a canine model resulted in the limited formation of a periodontal connective tissue attachment consisting of cementum, periodontal ligament (PDL), and alveolar bone.^{5,6} In this model, the PDL of the retained roots is thought to contribute periodontal regeneration-competent cell populations to the implant wound-healing environment.

The results of the above studies not only demonstrate that the formation of a periodontal connective tissue attachment on

dental implants is dependent upon the presence of a viable PDL but also surprisingly demonstrate that cementum can be deposited on material surfaces such as titanium. Therefore, it may be possible to bioengineer a periodontal connective tissue attachment on dental implants provided a source of periodontal regeneration-competent cells is present in the postsurgical wound-healing environment. The clinical significance of such a possibility is that, in contrast to the functional ankylosis obtained with osseointegration, the formation of a periodontal connective tissue attachment on dental implants would provide most of the advantages that a periodontal connective tissue attachment confers on the natural dentition.

With the ultimate goal of developing dental implant surfaces that can support a periodontal connective tissue attachment, we have established an experimental animal model to help identify and characterize the factors necessary for periodontal connective tissue attachment formation. The aim of the present pilot study was to test the hypothesis that primary, autogenous cell grafts obtained from different periodontal tissues with and without the application of enamel matrix proteins (EMD) would alter the type of implant–connective tissue interface obtained after wound healing.

MATERIALS AND METHODS

Materials

Tissue culture plates and other disposables were obtained from Corning Laboratories, Corning, NY. Tissue culture supplies, including culture media, proteases, antibiotics, fetal calf serum (FCS), and gelatin (tissue culture grade, type A, approximately 300

Bloom, derived from porcine skin), were obtained from Sigma Chemical, St Louis, Mo. Immune blotting supplies, secondary antibodies, and detection supplies were obtained from BioRad Laboratories, Burlingame, Calif. Enamel matrix derivative (Emdogain) (EMD) was obtained from Biora, Chicago, Ill. Bioabsorbable surgical barrier membranes (Resolut XTA4) were obtained from WL Gore, Nobel Biocare USA, Yorba Linda, Calif, and customized mini-implants and osteotomy drills were a generous gift from 3-I Implant Innovations, Palm Beach Gardens, Fla. Sutures (4/0 Vicryl braided polygalactac acid 910) and other surgical supplies were obtained from Ethicon, Syracuse, NY.

Anesthetic protocol

National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed throughout the study. The experimental protocol was reviewed and approved by the New York University Animal Welfare Committee and was administered through the University Office of Veterinary Resources. The staff of the University Office of Veterinary Resources was responsible for the anesthesia, euthanasia, and laboratory animal maintenance procedures.

A 3½-year-old, 35.0-kg, female Yucatan minipig (Sinclair Research Center Inc, Columbia, Mo) was premedicated intramuscularly with atropine (0.04 mg/kg), acepromazine (1.1 mg/kg), and ketamine (25 mg/kg) 1 hour before the following 3 surgical procedures. A transdermal fentanyl patch was also placed 24 hours before the procedure and was maintained 3 days postoperatively for analgesia. Anesthesia

was induced with thiopental (10 mg/kg intravenous) and maintained with isoflurane (1.5%–2% to effect) via an endotracheal tube during the surgical procedure. After completion of the surgical procedure, carprofen (2 mg/kg) was administered as an anti-inflammatory after recovery from anesthesia and was continued at a dosage of 1 mg/kg once daily for 3 days postoperatively.

Establishment of primary periodontal cell cultures

Routine aseptic technique was used at all times during the following surgical procedures. After anesthesia was attained, the head was draped and the mouth was isolated and prepared by the application of 0.5% chlorhexidine in 70% ethanol. Six weeks before implant placement, mandibular premolars 1 to 4 were extracted after the elevation of full-thickness buccal and lingual mucoperiosteal flaps. Three weeks before implant placement, a similar procedure was performed to extract the maxillary premolars 1 to 4. Primary cell cultures were established with the extracted maxillary and mandibular premolars. Connective tissue from the palatal mucoperiosteal flap was used to establish primary GCT cell cultures.

Primary periodontal cell cultures were established by enzymatic dissociation as previously described⁷ with minor modifications. In brief, the tissues were washed twice in culture medium (Dulbecco's Modified Eagle's Medium/Nutrient Mixture Ham's F12, 1:1 [DMEM-F12] supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin sulfate, 50 µg/mL gentamicin sulfate, 5 µg/mL amphotericin B) and then digested in culture medium containing 0.1% (wt/vol) bacterial

collagenase (type XI) and 0.125% (wt/vol) trypsin (1:250) at 37°C for 40 minutes. The cells released into the digest supernatant were collected by centrifugation, and the resulting cell pellet was washed in culture medium supplemented with 10% FCS and 50 µg/mL L-ascorbic acid. Cells were plated on 100-mm tissue culture plates and incubated in a humid atmosphere of 5% CO₂ in air at 37°C. After 24 hours of culture, the cell layer was rinsed twice with complete culture medium containing 10% FCS and 50 µg/mL L-ascorbic acid, and thereafter the culture medium was changed every 48 hours. At confluence, cells were released from the culture substrate by incubation with 0.25% (wt/vol) trypsin, 5 mM EDTA, in phosphate-buffered saline; counted by using a hemocytometer and trypan blue exclusion; and then replated 1:5 on 100-mm culture plates. Expression of a cementum-associated extracellular matrix protein, CP42, was determined by a dot blot assay as previously described.⁸ Immediately before implant placement, both PDL and GCT cells were released from culture by treatment with trypsin-EDTA and counted. The cells, at a concentration of 4×10^6 cells/mL, were transferred to the surgical site in a vehicle of 3% gelatin in DMEM-F12 medium without serum in a tuberculin syringe on ice.

Implant and autogenous cell graft placement

Bilateral, full-thickness, buccal and lingual flaps were raised in the edentulated, mandibular premolar area. A series of 10 machined 3.75- × 10-mm self-tapping implants were inserted into osteotomy sites prepared in both quadrants with exaggerated

coronal countersinks. Before the placement of the autogenous periodontal cell grafts, EMD was applied to 1 quadrant according to the manufacturer's directions. The autogenous cell grafts were then syringed into the funnel-shaped coronal area surrounding the implants. In each quadrant, 2 implants received second-passage PDL cells, 1 received fourth-passage PDL cells, 1 received GCT cells, and 1 received gelatin carrier alone. All implants were covered with a bioabsorbable surgical barrier membrane immobilized by the implant cover screws. The flaps were then repositioned to achieve primary closure and were secured with interrupted sutures.

Postsurgical phase, specimen collection, and analysis

Neither oral hygiene procedures nor debridement of the surgical sites were attempted during the postsurgical period. Doxycycline (4 mg/kg) was administered intravenously at the completion of the surgical procedure and was repeated daily for 14 days intramuscularly at a dose of 2 mg/kg. For 1-week postoperatively, regular pig chow (Ralston Purina, Evanston, III) was given presoftened by the addition of water. Sutures were removed 14 days postoperatively. After 8 weeks, the animal was euthanized by an overdose of pentobarbital (100 mg/kg). The mandibular premolar area was removed en bloc and then sectioned into blocks containing individual implants. The tissue specimens were fixed in neutral buffered 10% formalin and processed for quantitative histomorphometric and qualitative light microscopic analysis by the laboratory of Dr Michael

Rohrer, University of Minnesota School of Dentistry, as previously described.^{9,10}

RESULTS

The healing period was uneventful, and no surgical barrier membranes or cover screws were exposed 14 days postoperatively at suture removal. However, at the time of sacrifice 8 weeks after implant placement, the side not treated with EMD had 4 partially uncovered healing caps, whereas the side treated with EMD had 3 partially uncovered cover screws. Also, 1 implant that received PDL cells in second passage without EMD was lost. Upon gross examination of the recovered implant specimens, no other differences were noted between sides treated with and without EMD or between the types of autogenous cell graft placed.

By quantitative histomorphometric and qualitative light microscopic analyses of the recovered specimens, the type of implant-connective tissue interface obtained in this experimental model varied with the type of autogenous graft placed and whether EMD was present in the surgical site. The results are presented for the percent bone, connective tissue, and marrow contact for the entire implant (Table 1) and for the coronal first 4 threads (Table 2). In general, implants that received autogenous PDL cell grafts had greater percent bone contact than did implants that received autogenous GCT cell grafts (Tables 1 and 2). In addition, implants that received autogenous PDL cell grafts and EMD exhibited supra-crestal, fingerlike projections of bone into the area of the former coronal countersink (Figure 1A, C, and E). In contrast, implants that received autogenous PDL

TABLE 1

Effect of different autogenous cell grafts with or without (+/-) enamel matrix derivative (EMD) on the percent contact of bone, connective tissue, and marrow with the entire implant surface*

Group		% Contact With Implant Surface		
Type of Cell Graft	EMD	Bone	Connective Tissue	Marrow
Gelatin vehicle	-	60.8	9.4	29.8
Second-passage PDL	-	54.4	4.9	40.7
Fourth-passage PDL	-	24.9	17.2	57.9
Second-passage GCT	-	16.6	13.7	69.7
Gelatin vehicle	+	19.9	33.5	45
Second-passage PDL	+	60.8	9.3	29.8
Second-passage PDL	+	42.4	0.7	56.9
Fourth-passage PDL	+	68.9	8.5	22.6
Second-passage GCT	+	25.6	14.0	60.4

*PDL indicates periodontal ligament; GCT, gingival connective tissue.

cell grafts without EMD had thin strands of epithelium on or near the surface of the implant (Figure 1B, D, and F). The layer of epithelium did not appear to be contiguous with the oral epithelium and was confined to the coronal aspect of the implant. It was further noted that an implant-connective tissue interface morphologically consistent with a periodontal connective tissue attachment (ie, cementum formation with inserting collagen fibers) was not observed in any of the specimens.

In contrast, implants that received autogenous GCT cell grafts without the application of EMD were mostly surrounded by fibrous connective tissue (Figure

2B and D), whereas implants that received autogenous GCT cell grafts with EMD had marginally greater percent bone contact (Tables 1 and 2; Figure 2A and C) than those without EMD.

Implants that did not receive autogenous cell grafts but gelatin vehicle alone were found to have good percent bone contact on the entire implant (Table 1) or the coronal first 4 threads (Table 2) with a close apposition of bone to the implant surface in the area of the former countersink (Figure 3).

DISCUSSION

In adults, periodontal regeneration-competent cell populations

TABLE 2

Effect of different autogenous cell grafts with or without (+/-) enamel matrix derivative (EMD) on the percent contact of bone, connective tissue, and marrow with the coronal first 4 threads of the implant surface*

Group		% Contact With Implant Surface		
Type of Cell Graft	EMD	Bone	Connective Tissue	Marrow
Gelatin vehicle	-	58.8	16.5	24.6
Second-passage PDL	-	39.6	48.3	12.1
Fourth-passage PDL	-	70.7	6.6	22.7
Second-passage GCT	-	43.5	20.3	36.2
Gelatin vehicle	+	0	0	100
Second-passage PDL	+	47.7	9.3	43.1
Second-passage PDL	+	69.4	27.8	2.7
Fourth-passage PDL	+	68.8	5.9	25.2
Second-passage GCT	+	53.4	8.5	38.1

*PDL indicates periodontal ligament; GCT, gingival connective tissue.

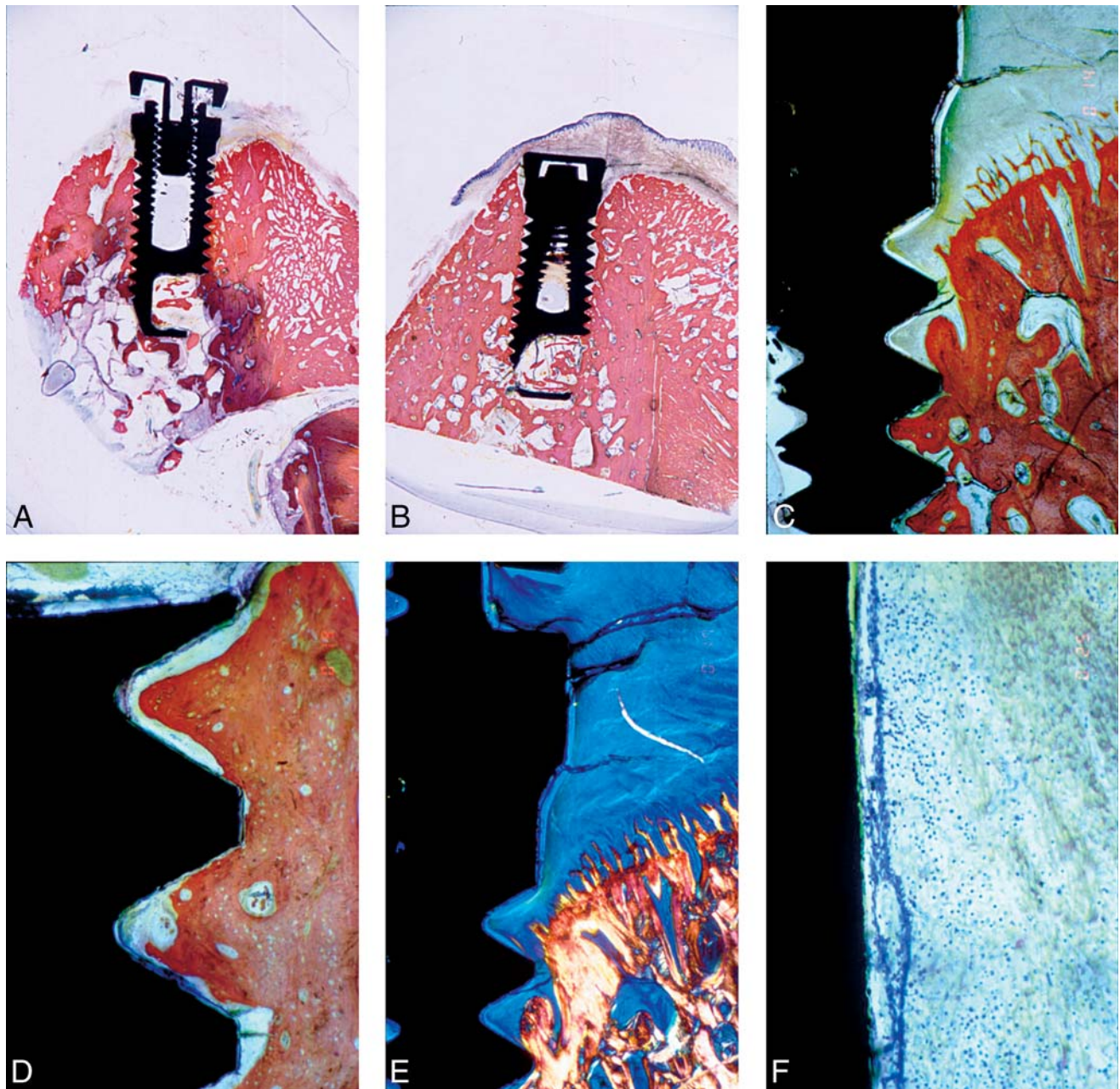


FIGURE 1. Implants with second-passage periodontal ligament autogenous cell grafts. Low-power ($\times 2.5$) photomicrograph of implants and surrounding tissues that did (A) or did not (B) receive an application of enamel matrix derivative (EMD). Higher-power view ($\times 10$) of the cervical portion of implant with EMD shows supracrestal projections of bone (C). Same implant viewed in polarized light (E). Photomicrographs of the cervical portion of an implant that did not receive an application of EMD at $\times 10$ (D) and at $\times 50$ (F) magnifications.

have been identified as residing paravascularly within the PDL¹¹ or possibly within the adjacent alveolar bone.¹² Furthermore, several reports suggest that it may be possible to expand periodontal regeneration-competent cell populations by tissue culture.

Primary PDL cell cultures have been reported to regenerate a periodontal connective tissue attachment when reimplanted onto denuded roots in vivo.¹³ In addition, primary cell cultures of both PDL and alveolar bone have demonstrated new attach-

ment formation when reimplanted in vivo on root slices¹⁴ or in periodontal defects in minipigs.¹⁵

We had previously reported that primary cell cultures of rat PDL but not GCT expressed a cementum-associated extracellu-

lar matrix protein when cultured on tissue culture plastic or titanium.⁸ However, when cultured on amalgam, calcium hydroxide, or gutta percha surfaces, primary PDL cells failed to express the cementum-associated protein.⁷ These results reflect, in part, the response of the PDL to these materials *in vivo*.^{16,17} Although controversial,¹⁸ EMD is thought to be an inductive factor for cementum formation according to results of both animal¹⁹ and clinical trials.^{20–27} We therefore asked whether EMD would induce cementum formation on surfaces that normally do not support periodontal connective tissue attachment formation. When applied to gutta percha or calcium hydroxide surfaces, EMD resulted in the expression of the cementum-associated extracellular matrix protein by primary PDL cell cultures.²⁸ In a follow-up *in vivo* study, the application of EMD to calcium hydroxide, gutta percha, or mineral trioxide aggregate resulted in the deposition of cementum and the formation of a periodontal connective tissue attachment on these materials after wound healing in an experimental model with the minipig.²⁹

On the basis of the results of the above studies, we proposed that a periodontal connective tissue attachment could be formed on dental implants provided a source of periodontal regeneration-competent cells was present in the wound-healing environment. Furthermore, the application of EMD might aid in the formation of a periodontal connective tissue attachment on dental implants. Therefore, a model was established to help identify and characterize the cells and factors associated with periodontal connective tissue attachment formation by using machined,

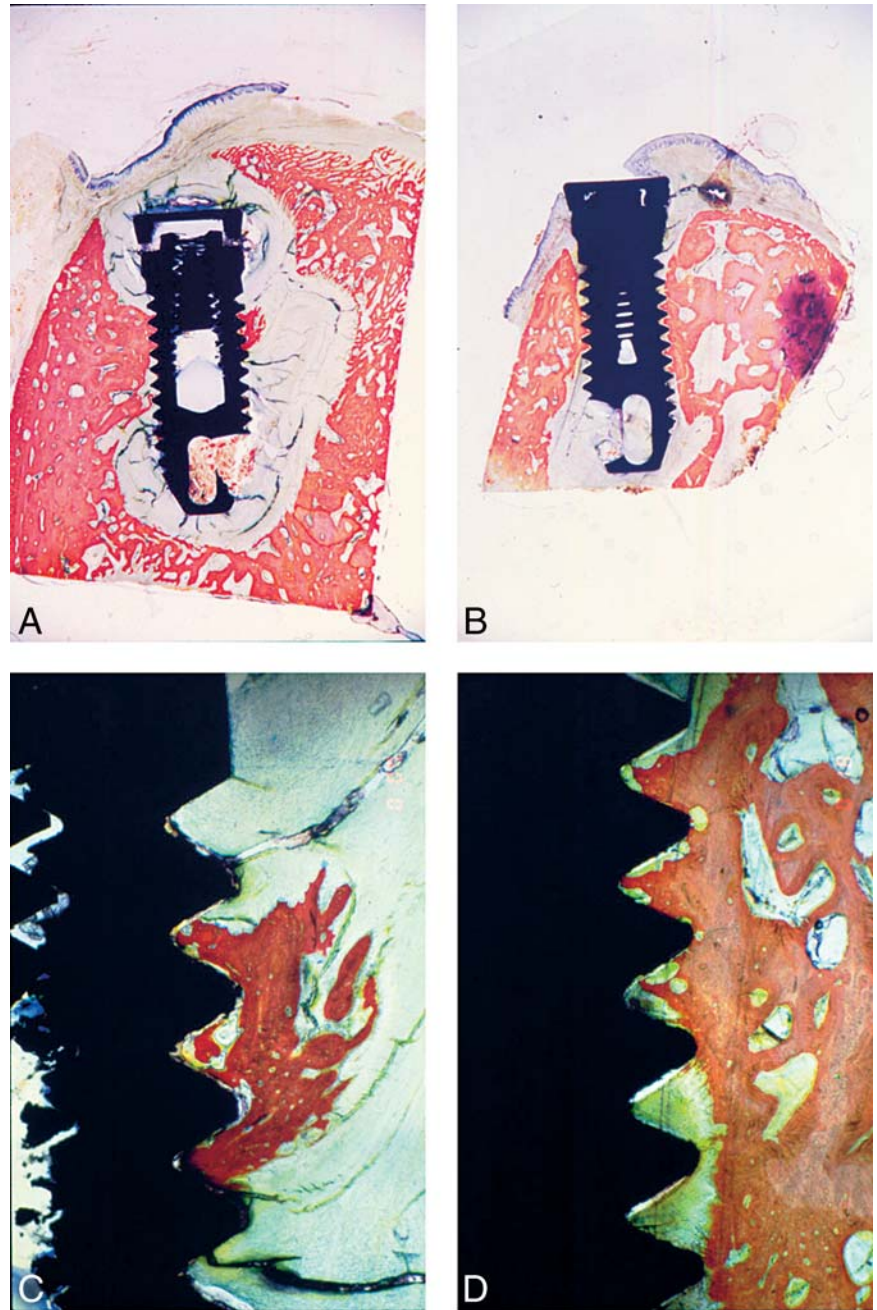


FIGURE 2. Implants with second-passage gingival connective tissue autogenous cell grafts. Low-power ($\times 2.5$) photomicrograph of implants and surrounding tissues that did (A) and did not (B) receive an application of enamel matrix derivative (EMD). Higher-power view ($\times 10$) of the cervical portion of the implant that did (C) and did not (D) receive an application of EMD.

self-tapping implants placed onto osteotomies formed with exaggerated coronal countersinks. The rationale for this experimental design was that the apical portion of the implant would integrate with the surrounding

bone both to serve as a surgical control (ie, should osseointegrate) and to stabilize the implant–autogenous cell grafting site. The coronal countersink would provide an experimental site for the placement of autogenous cell

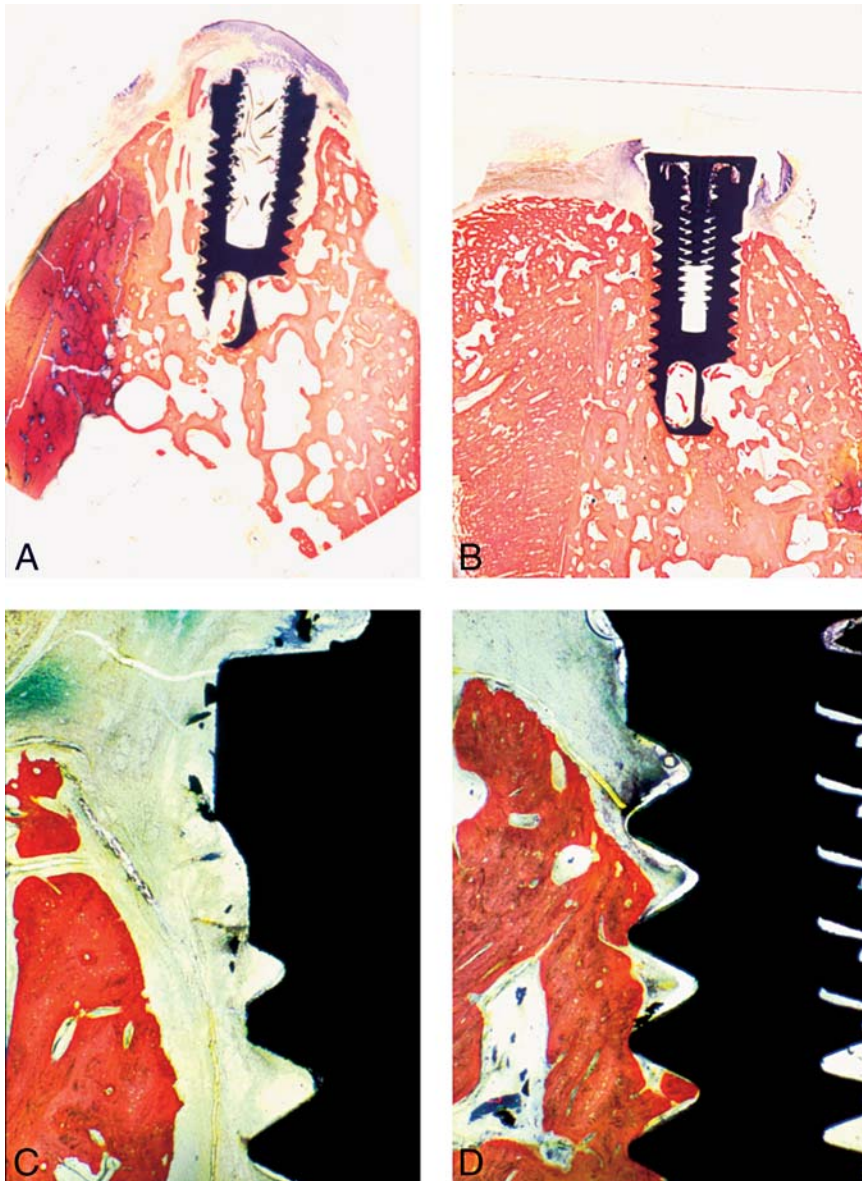


FIGURE 3. Implants with gelatin vehicle alone. Low-power ($\times 2.5$) photomicrograph of implants and surrounding tissues that did (A) and did not (B) receive an application of enamel matrix derivative (EMD). Higher-power view ($\times 10$) of the cervical portion of implants that did (C) or did not (D) receive an application of EMD.

grafts after *in vitro* expansion. Finally, a surgical membrane placed over the grafted implants would prevent cells derived from the GCT or epithelium of the flap from accessing the site. In the present study, we report the effect of autogenous PDL cell grafts, both with and without the application of EMD, on the implant-connective tissue interface in this model.

Caution should be used when interpreting the results of this study because a single animal was used and, accordingly, the study should be viewed as a pilot investigation. Nonetheless, several findings were noted.

Overall, the results of this study suggest that the type of implant-connective tissue interface obtained in this model was dependent upon the source of the

autogenous cell graft and whether EMD was applied. Autogenous PDL cell grafts or gelatin vehicle alone resulted in bone formation in the coronal aspect of the implants, whereas autogenous GCT cell grafts resulted in greater connective tissue formation, not unlike that described for fibroosseous integration.²⁻⁴ Autogenous GCT cell grafts were included in the study design because periodontal regeneration-competent cell populations have been identified as residing within the PDL¹¹ or surrounding bone¹²; therefore, GCT cell grafts would serve as a negative experimental control.

Of interest, autogenous PDL cell grafts placed without the application of EMD resulted in the formation of thin stands of epithelium in the coronal aspect of the grafted implants. It should be noted that the dissociative primary cell isolation technique used to establish primary cell cultures in this study generates a heterogeneous PDL cell population that contains both epithelial and fibroblastic cells.²⁸ Because EMD has been shown to suppress epithelial cell proliferation,^{30,31} perhaps via transforming growth factor (TGF)- β or a similar ligand,³² it is possible that the proliferation of epithelial cells observed in the autogenous PDL cell graft site without the application of EMD arose from epithelial cell proliferation from the autogenous cell graft. It may also be possible that the epithelium arose from the flap. In either case, the EMD appeared to suppress epithelial proliferation in implants where autogenous PDL cell grafts were placed with EMD. In addition, the increase in bone formation observed in implants that received autogenous PDL cell grafts or vehicle alone and EMD may have resulted

from the proanabolic, pleiotrophic properties previously reported for EMD. Earlier in vitro studies have reported EMD to increase PDL cell protein synthesis,^{31,33} proteoglycan synthesis,³⁴ total RNA,³⁵ alkaline phosphatase activity,³⁶ and mineralization.³³ Enamel matrix derivative has been reported to hasten wound closure in in vitro models^{37,38} and to increase GCT cell proliferation and extracellular matrix synthesis,³⁹ and it has been associated with increased rates of gingival wound healing in vivo.⁴⁰ The mechanism responsible for EMD stimulation of the above cellular activities has not been defined, but in cultured PDL cells EMD has been shown to increase the expression of several cytokines and growth factors, including TGF β ,^{31,34-36} insulin-like growth factor I,⁴¹ and platelet-derived growth factor.³¹ Increased expression of these cytokines may, in turn, stimulate cell proliferation and matrix synthesis via a paracrine or autocrine mechanism.

Of note, an implant-connective tissue interface morphologically consistent with a periodontal connective tissue attachment was not observed in sections from any of the implant or autogenous cell graft groups. According to studies with retained roots as a source of PDL cells, titanium oxide appears to be a surface capable of supporting cementum formation.^{5,6} We do acknowledge that it may be possible that periodontal regeneration-competent cell populations, after expansion in vitro, may not have survived reimplantation in vivo or may not have maintained a differentiated phenotype after placement into the implant graft site. However, expression of the cementum-associated extracellular matrix protein was confirmed before and after

grafting (from the culture of surplus cells not placed in the graft site, data not shown); therefore, we believe that viable PDL cells were introduced to the graft site that expressed some degree of differentiation. Furthermore, differences in the implant-connective tissue interface were observed in implants that received GCT vs PDL autogenous cell grafts. Rather, we propose that it is more likely that a factor or factors essential for cementum formation, the first of the 3 periodontal connective tissue attachment tissues formed in both development and regeneration, were not present in the postsurgical wound-healing environment. Or, it may be possible that a critical mass of autogenous PDL cells was not present to successfully compete with the osteogenic cells that would also migrate into to the graft site postsurgically. In support of this possibility, EMD has been shown to enhance the rate of trabecular bone formation⁴² but not necessarily the amount of bone contact⁴³ in implants placed in nonoral sites. Therefore, the resolution of these possibilities awaits the results of future studies using this experimental model.

In summary, an experimental model was developed to aid in the identification and characterization of the cells and factors necessary for the formation of a periodontal connective tissue attachment on dental implants. The results of this pilot study suggest that the presence of EMD and the source of autogenous cell graft placed may alter the type of implant-connective tissue attachment formed in this model.

ACKNOWLEDGMENTS

The authors acknowledge the expert assistance of Mr Damian

Bohler, Dr Gerardo Moreno, and Dr Carol Novotney of the New York University Office of Laboratory Animal Services. The authors also acknowledge the expert histologic and histomorphometric services provided by the laboratory of Dr Michael Rohrer of the University of Minnesota School of Dentistry. This study was supported by a grant from the Dr Arthur Ashman Department of Implant Dentistry, New York University College of Dentistry.

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