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Fibrin clot adhesion to dentin conditioned with protein constructs: an in vitro proof-of-principle study

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Abstract

Objectives: Periodontal regeneration is contingent on the adsorption, uninterrupted adhesion, and maturation of a fibrin clot to a periodontally compromised root surface. Clot adhesion appears vitally dependent on the formation of a resilient union between the clot and the root surface. Root surface demineralization will remove a root surface smear layer exposing dentin tubules and collagen matrix for enhanced clot adhesion. Recently, protein constructs have been introduced to condition the root during periodontal surgery. The effect of such root conditioning on clot adhesion has not been clarified. The objective of this study was to evaluate clot adhesion to protein conditioned dentin surfaces.

Methods: Human dentin blocks ($4 \times 6 \times 1$ mm) were exposed to a saturated citric acid solution (CA) or a commercial ethylenediaminetetraacetic acid (EDTA) preparation using standardized protocols. Some dentin blocks were additionally conditioned with proteins, either bovine serum albumin (BSA) or an enamel matrix protein preparation (EMP). Fresh human whole blood was applied to the blocks. The blood was allowed to clot for 20 min. in a humidified chamber. The dentin blocks were rinsed 3×5 min. in phosphate-buffered saline under standardized conditions to test clot adhesion. They were then processed for scanning electron microscopy (SEM). Two masked examiners independently evaluated the SEM images.

Results: CA removed the dentin smear layer, exposing dentin tubules and collagen. EDTA appeared less efficacious leaving smear layer residues. The BSA or EMP application resulted in a surface morphology similar to that of a smear layer. Fibrin clot adhesion was best supported by the CA-treated dentin surface. Forces produced by the rinse protocol partially removed the fibrin clot from EDTA-treated surfaces. BSA- or EMP-treated surfaces poorly retained the fibrin clot.

Conclusions: CA surface demineralization removes a dentin surface smear layer to promote adhesion of a fibrin clot. The EDTA gel appears less effective. Further conditioning of the dentin surface with protein constructs produces a surface morphology similar to that of the smear layer with poor fibrin clot retention.

Key words: periodontal regeneration; root conditioning; wound healing

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Observations from experimental studies in discriminating preclinical models suggest that periodontal regeneration is contingent on the adsorption, uninterrupted adhesion, and maturation of the fibrin clot positioned between the gingival flap and a periodontally compromised root (for a review, see Wikesjö & Selvig 1999). Blood elements imposed onto the root surface during surgery and at wound closure must establish an attachment that endures normal physiologic and other potentially rupturing forces acting on the tooth–gingival flap interface. This attachment must remain stable until such time as the interface has matured to sufficient tensile strength to offset any impact from functional or other forces (Linghorne & O'Connell 1950, Hiatt et al. 1968, Polson & Proye 1983, Wikesjö et al. 1991a, b). Observa-

tions in pre-clinical models examining limited periodontal wounds suggest that such functional integrity appears obtainable within a 14-day healing interval (Hiatt et al. 1968). Notably, the tensile strength of the tooth–gingival flap interface still appears vulnerable to functional demands following a 7-day healing interval (Hiatt et al. 1968).

Clot adhesion appears vitally dependent on the formation of a resilient union between the fibrin clot and root surface elements. The root surface becomes covered by a smear layer of instrumentation debris following routine root preparation, which is part of almost any treatment protocol for periodontal disease(s) (Jones et al. 1972, Polson et al. 1984, Wikesjö et al. 1986). In vitro studies have shown poor fibrin clot adhesion to such altered root surfaces (Baker et al. 2000). In vivo histologic studies have shown compromised wound healing at root surfaces subjected to root instrumentation as a stand-alone protocol (Nilvéus & Egelberg 1980, Polson & Proye 1983, Wikesjö et al. 1991a, b). Wound healing largely resulted in formation of an epithelial rather than a new connective tissue attachment to the instrumented root surface.

Root surface demineralization with acidic or chelating agents removes the instrumentation smear layer to expose the dentin tubules and the intra- and peri-tubular dentin collagen matrix (Héritier 1982, Polson et al. 1984, Wikesjö et al. 1986, Blomlöf & Lindskog 1995). In vitro studies have shown enhanced fibrin clot adhesion to such cleansed root surfaces (Baker et al. 2000). In vivo histologic studies have shown that wound healing is enhanced at root surfaces subjected to root instrumentation followed by root surface demineralization (Register 1973, Register & Burdick 1975, Selvig et al. 1981, Polson & Proye 1982). Studies in well-defined animal models have provided evidence of a new connective tissue attachment rather than an epithelial attachment when the affected root surfaces had been exposed to demineralization following instrumentation (Nilvéus & Egelberg 1980, Polson & Proye 1983, Wikesjö et al. 1991a, b).

Biologic constructs including synthetic peptides, extracellular matrix derivatives, purified and recombinant growth and differentiation factors have been developed, evaluated, and, for some constructs, marketed for periodontal regenerative procedures (Tarrannova & Martin 1982, Giannobile et al. 1994, Nishimura & Terranova 1996, Ripamonti & Reddi 1997, Wozney 1998, Bhatnagar et al. 1999, Gestrelius et al. 2000, Wikesjö et al. 2004). The concept behind these technologies is that the biologic mediators may enhance proliferation and migration of cells from the periodontal ligament in support of periodontal regeneration, or support differentiation of mesenchymal stem cells into periodontal ligament cell phenotype(s). The biologic compounds commonly are placed in the wound site, or onto the root surface, in a bioresorbable carrier. Some protocols also include root surface demineralization. The effect of such biologic constructs on fibrin clot adsorption and adhesion has not been clarified. The objective of this study was to evaluate fibrin clot adhesion to protein conditioned dentin surfaces.

Materials and Methods

Human dentin blocks and whole blood

Dentin blocks, approximately $4 \times 6 \times 1$ mm in size, were prepared from the roots of freshly extracted human teeth (Baker et al. 2000). The teeth had been extracted in the Temple University School of Dentistry Oral Surgery Clinic because of caries and/or periodontal disease and were predominantly represented by maxillary incisors, canines, and first molars, and mandibular canines and first molars. The dentin blocks were cut to size using water-cooled high speed-diamond burs and were then wet-planed using progressively finer grit sand paper. The prepared dentin blocks were stored in phosphate-buffered saline (PBS), pH 7.4, at 4°C until use. Fresh human whole blood (HWB) from one healthy male donor (DLB) was used.

Dentin conditioning agents

A saturated aqueous solution of citric acid (CA) or an ethylenediaminetetraacetic acid (EDTA) gel (PrefGel®; Biora, Malmö, Sweden) was used to condition the dentin block surfaces. PBS was used as control media. Following dentin conditioning with CA, EDTA, or PBS, some dentin blocks were coated with bovine serum albumin (BSA; Fraction V, Sigma Chemical Company, St. Louis, MO, USA) or an enamel matrix protein preparation (EMP; Emdogain®, Biora).

Experimental protocol

The experimental protocol generally followed the outline of our previous study (Baker et al. 2000). Dentin blocks were distributed into experimental groups in triplets (Fig. 1). The dentin blocks were conditioned for 5 min. using one of the three agents; CA (Wen et al. 1992), EDTA (following manufacturer's protocol), or PBS. The dentin blocks were then subject to three 5-min. rinses in PBS and allowed to air-dry for 20 min. After conditioning, some dentin blocks were coated with BSA or EMP (following manufacturer's protocol) and allowed to rest for 5 min. HWB was applied to the dentin blocks. The blood was allowed to clot for 20 min. in a humidified chamber.

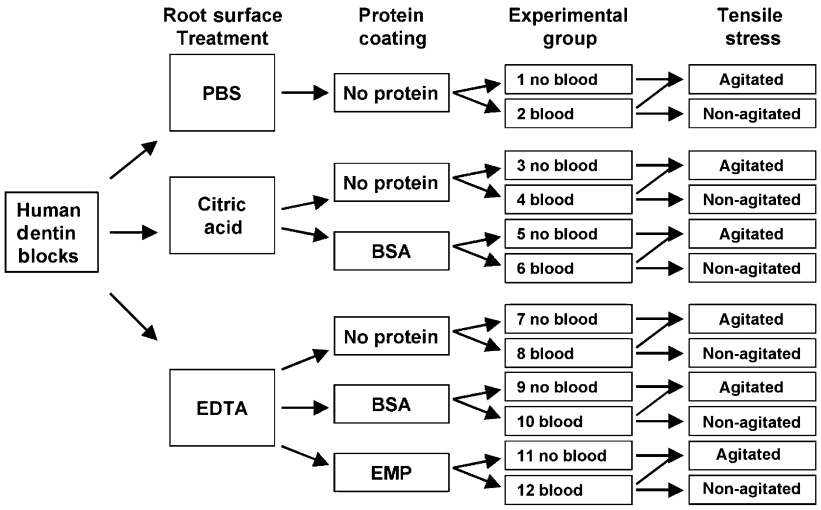

Fig. 1. Experimental design. PBS, phosphate buffered saline; BSA, bovine serum albumin; EMP, enamel matrix protein; EDTA, ethylenediaminetetraacetic acid.

Dentin blocks including surface coating with HWB were either designated as ''non-agitated'' when the dentin blocks were subject to three 5-min. rinses in PBS or ''agitated'' when the dentin blocks were subject to three 5-min. rinses in PBS on a rotary shaker table (Orbit Shaker, Lab-line Instruments Inc., Melrose Park, IL, USA) at speed #1. All steps were performed at room temperature.

Histologic preparation

Immediately after rinsing, the blocks were fixed for 30 min. in 2.5% glutaraldehyde (Tousimis Research Corp., Rockville, MD, USA). The blocks were subsequently subject to three 5-min. rinses in PBS, and 5-min. graded ethanol dehydrations (70%, 90%, and $2 \times 95\%$). Two final dehydrations were performed using hexamethyldisilazine (Tousimis Research Corp.). All fixation and dehydration steps were performed at room temperature.

Scanning electron microscopy (SEM)

The dentin blocks were mounted on aluminum stubs with colloidal graphite, sputter-coated with a thin layer of gold-palladium and stored at room temperature. SEM observations and records were performed on a JEOL JSM-1600 scanning electron microscope (JEOL USA, Ltd., Peabody, MA, USA) at 5.0 kV. Representative SEM photomicrographs for each dentin block at $\times 1500$ and $\times 3000$ magnification were produced using a Polaroid camera and POLOPAN 4000 ISO 400/27 photographic film (Polaroid Corporation, Cambridge, MA, USA).

Analysis

Two trained masked examiners (DLB & SASP) independently evaluated the SEM photomicrographs. The results represent the consensus of their independent evaluations. Only observations that were duplicated in the two evaluations are reported.

Results

Surface topography following dentin conditioning

Instrumented dentin blocks that did not receive further treatment exhibited a surface predominantly covered by a smear layer (Fig. 2). Specific dentin topographic features such as the dentinal tubules, intra- and inter-tubular collagen fibrils were not readily discernible. A similar topography was observed for dentin blocks conditioned with EDTA only, CA+BSA, EDTA+BSA, or EDTA+EMP (Fig. 2). One of three dentin blocks conditioned with EDTA only exhibited visible dentinal tubules in a small section of the overall surface. All dentin blocks conditioned with CA only exhibited a uniformly cleansed surface with wide-open dentinal tubules including exposed intra- and inter-tubular collagen fibrils (Fig. 2).

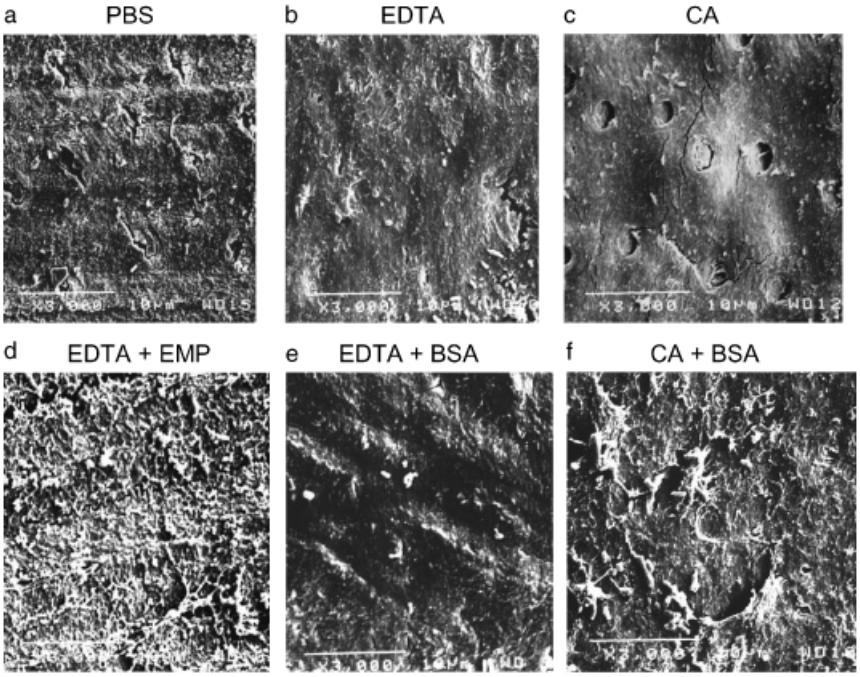

Fig. 2. Effect of surface treatment on dentin. Instrumented dentin exhibited a smear layer (a). The smear layer still obscured the dentinal tubules following conditioning with ethylenediaminetetraacetic acid (EDTA) (b). The smear layer was removed follow conditioning with citric acid (CA) exposing the dentinal tubules (c). Conditioning of CA- or EDTA-treated dentin with proteins (bovine serum albumin (BSA) or enamel matrix protein (EMP)) established a surface topography similar to that of a smear layer obscuring the dentinal tubules (d–f). The horizontal bar represents 10 μm. PBS, phosphate-buffered saline.

Blood clot adhesion to conditioned dentin

Non-agitated dentin blocks conditioned with PBS, CA+BSA, EDTA, or EDTA+BSA and covered with HWB exhibited sparsely distributed single erythrocytes or isolated clumps of erythrocytes adsorbed onto a smear-like layer without enveloping fibrin strands (Fig. 3). With one exception, all agitated dentin blocks conditioned with PBS, CA+BSA, EDTA, and EDTA+BSA and covered with HWB also exhibited sparsely distributed erythrocytes, some with shadow imprints of lost erythrocytes (Fig. 4). One dentin block exposed to EDTA and covered with HWB showed a single layer of erythrocytes entangled in fibrin strands on a small area of the block (Fig. 4). This fibrin clot appeared very thin and it was possible to observe a partially demineralized dentin surface beneath.

The majority of dentin surfaces conditioned with EDTA+EMP and covered with HWB showed no discernable blood elements. An unusual fibrin clot covered approximately 10%, 25%, and 75%, respectively, of the three non-agitated blocks, and 1%, 10%, and 50%, respectively, of the three agitated blocks. Areas of these blocks exhibiting a fibrin clot varied in appearance across a wide spectrum (Figs 3 and 4). There were isolated areas of erythrocyte conglomerates entangled in a foamy material, probably the proprietary propylene glycol alginate carrier. Some areas showed widely spaced fibrin-like strands partially creating a network. Nevertheless, generally scattered erythrocytes apparently adsorbed onto a smear-like layer were the norm. It is interesting to note that some blocks in

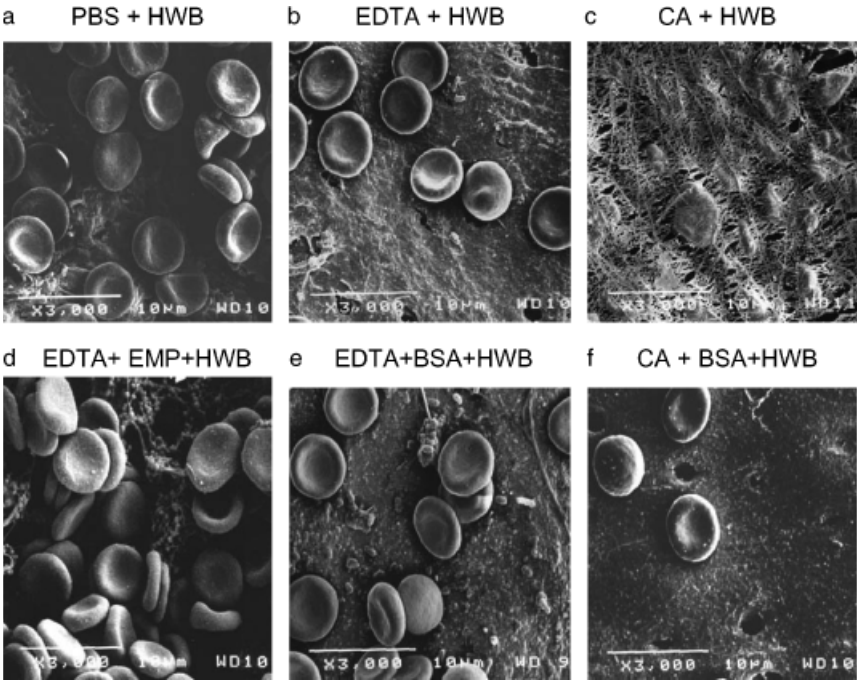

Fig. 3. Effect of surface treatment on fibrin clot formation on dentin. Only surface treatment with citric acid (CA) promoted a thick fibrin meshwork adhering to the dentin surface (c). All other surface treatments (a,b,d–f) promoted the creation of isolated groups of erythrocytes loosely scattered over the surface. The horizontal bar represents 10 μm. PBS, phosphate-buffered saline; HWB, human whole blood; EDTA, ethylenediaminetetraacetic acid; EMP, enamel matrix protein; BSA, bovine serum albumin.

this group showed erythrocytes that appeared as ''spiky spheres'' conjectured to be osmotically distressed cells (Fig. 4).

Dentin blocks conditioned with CA and covered with HWB were singular in appearance compared with all other treatments. Both non-agitated (Fig. 3) and agitated (Fig. 4) blocks showed a remarkable extent of surface coverage, nearly 100%, and apparent fibrin clot thickness. The clot was characterized by the total envelopment of erythrocytes in a fibrin mesh. Tensile or compressive forces produced by the rotary shaker did not produce a notable difference in appearance between the non-agitated and agitated specimens.

Discussion

Periodontal regeneration is contingent on the adsorption, uninterrupted adhesion, and maturation of a fibrin clot to a periodontally compromised root surface. Clot adhesion appears vitally dependent on the formation of a resilient union between the clot and the root surface. The objective of this study was to compare the effect of various root surface-conditioning protocols on the adhesion of a fibrin clot to instrumented dentin surfaces using in vitro modeling. The results show that root surface demineralization exposing the dentinal tubules and the intra- and peri-tubular dentin collagen matrix supports adhesion of the fibrin clot. Further conditioning of the root surface with protein constructs, i.e., BSA or EMP, may compromise adhesion of the fibrin clot to the dentin surface.

The in vitro modeling system (Baker et al. 2000) used in this study provides a simulation of critical steps during the earliest healing events following periodontal tissue regeneration procedures. This method allows observations of the dentin surface directly following each of the various stages of simulation, i.e.: (1) root instrumentation; (2) dentin conditioning to remove the instrumentation surface smear layer; (3) application of various proteins for root conditioning; (4) formation of the fibrin clot; and (5) subjection of the fibrin clot to mild disruptive forces. It is conceivable that those conditions that may promote or adversely affect fibrin clot adhesion in this model system may also produce similar effects in vivo.

Instrumentation of the human dentin blocks produced a surface smear layer as has been shown in previous reports (Jones et al. 1972, Polson et al. 1984, Wikesjö et al. 1986). Conditioning of the instrumented dentin with a saturated CA solution or the chelating EDTA gel, but not PBS, at least in part, removed the smear layer exposing dentinal tubules and the intra- and inter-tubular collagenous matrix. This observation is in harmony with a large number of reports evaluating the effect of acidic and chelating agents on the ultrastructure of dentin surfaces (Héritier 1982, Polson et al. 1984, Wikesjö et al. 1986, Blomlöf & Lindskog 1995, Baker et al. 2000). Interestingly, there appeared to be qualitative differences between the CA and the EDTA protocol. Whereas CA conditioning completely removed the surface smear layer, the EDTA product appeared less efficacious. The EDTA gel did not consistently produce a smear layer free dentin surface. The dentin blocks exhibited areas of exposed dentin, but not one dentin block exhibited a surface where the smear layer had been completely removed comparable with that following the CA protocol.

A vast three-dimensional array of interconnected fibrin strands, totally enmeshing the erythrocytes, was observed affixed to CA treated, but not EDTA- or PBS-treated dentin. This fibrin arrangement has also been observed at root surfaces treated with CA following surgical exposure. Polson & Proye (1983) observed the critical role of the condition of the root surface for the attachment and maturation of the fibrin clot into a connective tissue attachment by comparing instrumented and instrumented plus root surface conditioned (CA treated) teeth in a non-human primate model. They showed a fibrin network with inflammatory cells and that the fibrin network ''appeared attached to the root surface by arcade-like structures'' at surface demineralized teeth at day 1 postsurgery. At day 3, they observed healing by formation of an epithelial attachment at teeth that were only instrumented, whereas wound maturation progressed into formation of a new collagenous fiber attachment at teeth that were instrumented plus root surface conditioned. In perspective, it appears reasonable to suggest that in vitro protocols that sustain fibrin clot adhesion to dentin may support wound maturation into a connective tissue attachment in vivo. However protocols

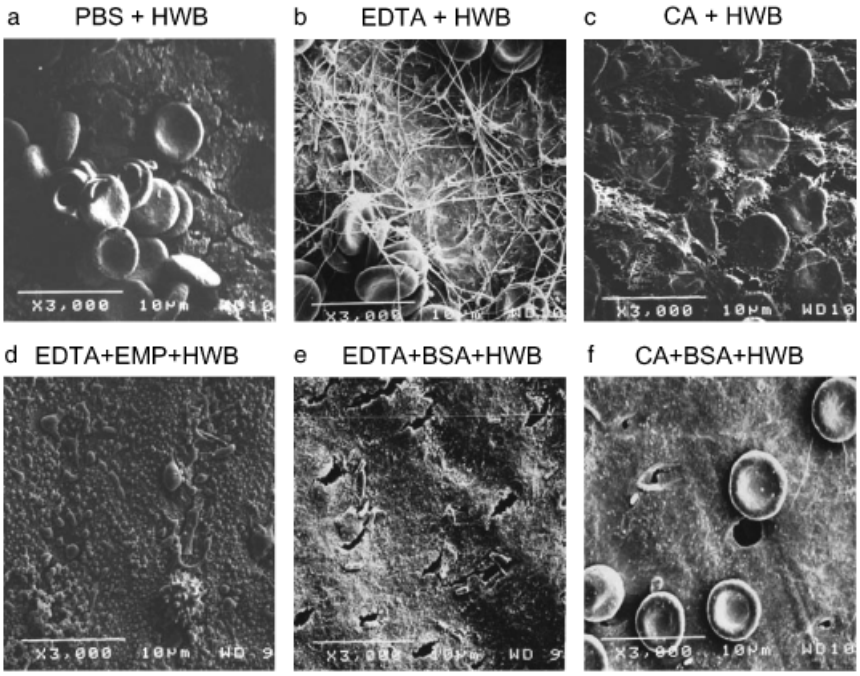

Fig. 4. Effect of surface conditioning on fibrin clot adhesion to dentin. Fibrin clots were subjected to agitation simulating tensile forces during wound healing. Only fibrin clots adhering to dentin conditioned with citric acid (CA) resisted the induced tensile forces and the fibrin clot remained adherent to the dentin surface (c). Fibrin clots on dentin following other surface treatments appeared loosely attached and easily removed by the agitation leaving few erythrocytes ((a) and (f)) or no erythrocytes ((d) and (e)). One isolated area of an ethylenediaminetetraacetic acid (EDTA)-treated specimen exhibited elements of an adherent fibrin clot (b), however the remainder of the surface showed no evidence of a fibrin clot. The horizontal bar represents 10 μm. PBS, phosphate-buffered saline; HWB, human whole blood; EMP, enamel matrix protein; BSA, bovine serum albumin.

that are less successful in vitro should not be expected to support fibrin clot adhesion in a clinical scenario.

Dentin surfaces conditioned with CA or EDTA resumed topographic features of a smear layer following application of BSA or EMP. Moreover, fibrin clot adhesion to BSA- or EMP-treated dentin appeared compromised. Extensive information exists on the behaviour of blood at natural and artificial surfaces in vitro, ex vivo, and in vivo (Leonard et al. 1987). However, the data largely concern blood contacting surfaces in dynamic intra-vascular systems or their in vitro analogues. Little is known about the behaviour of blood at solid surfaces in relatively static systems like the periodontal wound. It is well known that plasma proteins will adsorb onto solids; blood or plasma will deposit a film of fibrinogen about 50 Å thick onto a solid substrate within a 5 s exposure (Baier & Dutton 1969, Vroman & Adams 1969). However the continuous adhesion of the fibrin clot appears dependent on the wettability of the substrate. Whereas CA and to some extent EDTA conditioning removed the surface smear layer exposing dentin tubules and collagenous matrix, it likely also increased the wettability of dentin resulting in enhanced attachment of the fibrin clot imposed onto the surface. In contrast, the protein constructs produced a surface with a decreased wettability much like an instrumentation smear layer thus in turn the fibrin clot became vulnerable to the mild stress forces produced by the in vitro agitation system. Experimental observations in large periodontal defects where the root surfaces were treated with CA or CA followed by application of autologous fibronectin may be interpreted to support the observations herein (Wikesjö et al. 1988). Whereas healing at root surfaces treated with CA resulted in almost complete connective tissue repair, sites additionally treated with fibronectin exhibited only partial connective tissue repair suggesting that application of the protein construct may have compromised adhesion of the fibrin clot. Collectively, the data point to fundamental biologic principles that may govern periodontal wound healing. Successful therapy must include measures to ensure the uneventful maturation of a fibrin clot onto the root surface and in perspective further safeguard wound stability to take advantage of biologic agents applied onto the root surface with the intent to advance healing.

In conclusion, CA surface demineralization removes a dentin surface smear layer to promote adhesion of a fibrin clot. The EDTA gel appears less effective. Further conditioning of the dentin surface with protein constructs produces a surface morphology similar to that of the smear layer with poor fibrin clot retention. This effect could negatively influence the progression of wound maturation into a connective tissue attachment in vivo.

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