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Enamel Matrix Derivative Promotes Superoxide Production and Chemotaxis, but Reduces Matrix Metalloproteinase 8 Expression by Polymorphonuclear Leukocytes

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Abstract

Background—Polymorphonuclear leukocyte (PMN) is the predominant innate immune cell type activated in acute inflammation. The aim of this study was to determine the impact of Enamel matrix derivative (EMD) on superoxide (O_2^-) generation, chemotaxis, and matrix metalloproteinase 8 (MMP 8) secretion by PMN *in vitro* to better understand the role of EMD in surgical wound healing.

Methods—PMN were isolated from healthy volunteers ($N = 14$). Superoxide generation was measured using a cytochrome-C reduction assay. Chemotaxis was measured in a modified Boyden chamber. MMP 8 secretion was analyzed by Western blotting. A relative density method was used to determine the percent of MMP 8 released from the PMN in relation to the total cellular MMP 8 content.

Results— O_2^- generation was significantly elevated when PMN were stimulated with EMD (200 μ g/ml) ($P < 0.01$). Secondary stimulation of PMN with 1 μ M fMLP triggered earlier and more sustained O_2^- generation with EMD. EMD significantly increased PMN chemotactic activity ($P < 0.05$). Combined stimulation with EMD plus formyl-methionyl-leucyl-phenylalanine (fMLP) resulted in significantly higher chemotaxis compared to fMLP alone ($P < 0.05$). Conversely, EMD did not induce MMP 8 secretion from PMN. MMP 8 secretion by PMN in response to fMLP or serum-opsonized zymosan (OZ) stimulation was significantly inhibited by EMD ($P < 0.05$).

Conclusion—EMD has specific, differential actions on PMN that suggest potential for enhancement of wound healing; bacterial and tissue debris clearance (O_2^- generation and chemotaxis) and suppress tissue damage and degradation (MMP 8). Taken together, the data suggest that EMD enhances wound healing and reduces inflammation.

Keywords

Chemotaxis; emdogain; inflammation; matrix metalloproteinase; polymorphonuclear leukocyte; superoxide

Polymorphonuclear leukocytes (PMN or neutrophils) are the primary phagocytic cells for host defense against exogenous agents¹. PMN are the numerically dominant phagocytic cells found in the peripheral circulation, comprising 50–70% of the total white blood cell population². The participation of PMN in host defense is well established. In the periodontium, PMN migrate continuously from the blood vessels through the tissues into the gingival crevice, typically making up about 90% of the cells in the gingival sulcus^{3,4}. In the sulcus, PMN presumably protect the periodontal tissues from dental plaque organisms⁵. Biofilm formation on the tooth in the gingival crevice initiates the elaboration of a variety of substances that attract PMN to the site of infection (chemotaxis). The prototype of bacterially derived chemotactic factors is N-formyl-methionyl-leucyl-phenylalanine (fMLP)³. The high affinity fMLP receptor on phagocytic cells (FPRL) interacts with bacterial fMLP like molecules and mediates chemotaxis, degranulation and superoxide (O_2^-) production⁶. O_2^- generation is a major requirement for oxidative microbial killing by PMN⁷. In addition, PMN are the main source of matrix metalloproteinase 8 (MMP 8), which is a host collagenase that mediates degradation of tissue collagen in the inflamed periodontium^{8,9}. MMP 8 is released during lysosomal degranulation during phagocytosis or from disintegrating (necrotic) PMN¹⁰. The binding of fMLP to the chemoattractant-type receptors and the ligation of the complement and Fc-receptors by serum-opsonized zymosan triggers MMP 8 release from PMN¹¹.

Enamel matrix derivative (EMD; Emdogain), in the form of a purified acid extract of proteins from porcine tooth buds, is related to important biological functions in tooth development and periodontal regeneration¹². It has been successfully developed for periodontal tissue regeneration applications. The first studies of clinical applications of EMD were published in 1997 and since then reports of the mechanism of action of EMD and the clinical potential, have appeared^{13,14,15,16}. In 2004, a Cochrane review concluded that EMD significantly improves periodontal attachment levels and reduces probing pocket depth compared to open-flap debridement¹⁷. A multitude of case reports and clinical studies have been published confirming the clinical efficacy of EMD when used in periodontal regeneration procedures. A follow-up of a series of 107 consecutive EMD cases showed stable periodontal regeneration after 5-years of observation¹⁸. Furthermore, a wide range of in vitro and in vivo experimental studies have demonstrated that EMD stimulates growth of multiple mesenchymal cell types including fibroblasts, cementoblasts, osteoblasts, and stem cells¹⁹. These data provide strong evidence that EMD supports wound healing and new periodontal tissue formation.

The impact of EMD on inflammation and wound healing has been a point of controversy. Parkar & Tonetti demonstrated that in human PDL cells, EMD down-regulates the expression of genes involved in the early inflammatory events of wound healing, whereas genes encoding growth and repair-promoting molecules were up-regulated²⁰. In another study, Bertl et al.²¹ showed that EMD at low concentrations stimulated proliferation/viability, migration, and increased gene expression of the angiogenic factor ang-2 and adhesion molecules ICAM-1 and E-selectin on endothelial cells in vitro. Petinaki et al.²² showed that EMD induced slightly enhanced proliferation of lymphocytes, restricted to the CD25 (IL-2 receptor) fraction of the CD4-positive T-lymphocytes, and a concomitant decrease of CD19-positive B-lymphocytes. Furthermore, the authors noted that EMD was

not cytotoxic. Myhre et al.²³ challenged whole blood from humans with lipopolysaccharide or peptidoglycan incubated with different concentrations of EMD or a cAMP analogue. Their results showed attenuated release of TNF- α and IL-8, while the release of IL-10 was unchanged. They concluded that EMD limits the release of pro-inflammatory cytokines induced by lipopolysaccharide or peptidoglycan in human blood. Khedmat et al.²⁴ showed that EMD did not promote TNF- α and IL-1 β secretion while it increased the phagocytic activity of monocytes. These data suggest that EMD might accelerate wound healing through enhanced clearance of the wound.

Although EMD has been shown in numerous reports to improve clinical parameters and to mediate a regenerative healing response, our understanding of the mechanism by which EMD exerts its influence on cells is incomplete and the biological mechanism is not known. The aim of the present study was to investigate in vitro the impact of EMD on PMN function.

MATERIALS AND METHODS

Preparation of Emdogain

Emdogain was supplied as a set of lyophilized EMD (30 mg) and a 1 ml solution of Propylene glycol alginate. After mixing, the EMD was diluted to 200 μ g/ml in phosphate buffered solution (PBS).

Selection of Subjects

This study was performed from January to March 2004 in Boston University, Goldman School of Dental Medicine. The Institutional Review Board at Boston University Medical Center (MA) approved this study, and informed consent was obtained from all subjects prior to evaluation. In total, the 14 healthy subjects (7 males and 7 females; mean age 46.9 ± 5.2 years), enrolled in the study were recruited from the patient pool of the Clinical Research Center at Boston University School of Dental Medicine, a unit of the General Clinical Research Center at Boston University Medical Center.

Isolation of Human Peripheral Blood PMN

Peripheral venous blood (45 ml) was obtained from each subject by venipuncture in heparinized (10 U/ml) glass tubes, and PMN were isolated using a discontinuous gradient system²⁵. Peripheral blood was layered on a mixture of MonoPoly[‡], Histopaque 1119, and Histopaque 1077[§], and the tubes were centrifuged at 500-x g for 30 min. The neutrophil-rich layer was collected, and contaminating erythrocytes were lysed with a hypotonic NH₄Cl buffer (155 mM NH₄Cl, 10 mM KHCO₃, 120 μ M EDTA, pH 7.4). Cells were washed twice with phosphate-buffered saline (PBS; 13.3 mM Na₂HPO₄, 6.7 mM KH₂PO₄, pH 7.2, 0.15 M NaCl), resuspended in 50 ml PBS, and counted with a hemocytometer. Cell preparations were routinely 99% PMN with 95% viability, as determined by trypan blue exclusion.

Superoxide Generation

O₂⁻ release was monitored spectrophotometrically at 37°C by measuring O₂⁻ dismutase-inhibitable reduction of ferricytochrome c at 550 nm²⁶. Assays were carried out in

96-well microtiter plates with flat-bottomed wells (Linbro type) ^{||}. Five separate samples were used for each condition. Control wells contained all of the components of the assay mixture plus O₂⁻ dismutase (20 U/ml) to correct for ferricytochrome c reduction by agents other than O₂⁻. Cells (1×10⁶) were suspended in PBS (200 µl/well) and stimulated by the addition of EMD (200 µg/ml final concentration) for 6 minutes and then 1 µM fMLP was added and the absorbance (optical density) recorded in a Vmax kinetic microplate reader^{||} at 550 nm and 37°C. O₂⁻ release was measured continuously under conditions of linearity with respect to time and cell number and is expressed as nmole O₂⁻/min/10⁶ PMN.

Chemotaxis

Freshly isolated PMN were resuspended at a density of 10⁶/ml in RPMI 1640 medium containing 0.3 % bovine serum albumin. Chemotaxis was assayed by using a modified Boyden chamber technique²⁷. The cell suspension was placed in the upper compartment of a modified Boyden chamber separated by a 5-µm-pore-size Micropore filter. The lower compartment contained either EMD (200 µg/ml), fMLP (1 µM), EMD plus fMLP or PGA as a control in RPMI 1640 medium containing 0.3% bovine serum albumin. The chamber was incubated at 37° C for 30 minutes. Following incubation, the chamber was disassembled and the filter was carefully removed and washed with sterile PBS. PMN that migrated through the filter were stained with a commercially available kit and counted with an oil immersion microscope objective (X400).

Release of MMP 8

PMN (3.3 × 10⁶/ml) in culture medium RPMI-1640⁸ were incubated with either 200 µg/ml EMD, 1 µM fMLP, EMD plus fMLP, 2.5 mg/ml serum-opsonized zymosan (OZ), or EMD plus OZ. The mixtures contained 1% human serum and were incubated for 15 min at 37° C. A 50 µL aliquot of the reaction mixture was removed and transferred to an Eppendorf tube placed in an ice bath to stop PMN degranulation. The tube was subsequently centrifuged (18,000 × g, 5 min, 5° C) and the supernatant was mixed with an equal volume of sample buffer (4% SDS, 0.25 M Tris, 10% glycerol and 0.01% pyronine Y, pH 6.8) and boiled for 5 min. The standards and the proteins in the samples were separated by SDS-PAGE, blotted to PVDF-membranes and detected with specific antibodies as described⁹. The antibodies for MMP 8 detection were mouse-anti-MMP 8^{||} and peroxidase-conjugated goat-anti-mouse IgG[#]. Using an enhanced chemiluminescence technique^{**}, the MMP 8 on the blotted membranes was visualized on photographic film. The optical density of the band on the film was subsequently determined in a digital scanner (GS-700)^{††} and used for quantification. Relative release of MMP 8 from human neutrophils triggered with 200 µg/ml EMD, 1 µM fMLP, EMD plus fMLP, 2.5 mg/ml OZ, or EMD plus OZ were analyzed. The relative density method was used to determine the percent of the MMP 8 released into the extracellular supernatant in relation to the total MMP 8 content of the PMN. The total content of MMP 8 was extracted from the PMN by treatment with 0.2% Triton X-100 in the presence of 1% human serum.

Statistical Analysis

Experimental results are presented as mean $\pm$ SD. Student's *t* test or analysis of variance (ANOVA) with post hoc analyses (Bonferroni correction for multiple comparisons) was used. $P < 0.05$ was considered statistically significant.

RESULTS

PMN Superoxide Generation

The kinetics of superoxide formation by unstimulated PMN and EMD (200 $\mu\text{g/ml}$) stimulated PMN are shown in Figure 1. EMD induced PMN superoxide release and the optimal stimulation of PMN by EMD was obtained at a concentration of 200 $\mu\text{g/ml}$ at 6 minutes. Superoxide production by EMD stimulated-PMN was statistically significant greater compared to unstimulated cells ($p < 0.01$). Further stimulation of EMD treated cells with 1 μM fMLP triggered an earlier and more sustained response. There was a statistically significant difference ($p < 0.001$) at 1 minute.

PMN Chemotaxis

Chemotaxis is expressed as the mean number of PMN per high-power field (PMN/HPF) that has migrated to the distal surface of a microscopic filter in the Boyden chamber. The PMN chemotactic responses to stimulation by EMD, fMLP, or EMD plus fMLP are shown in Figure 2. EMD at a concentration of 200 $\mu\text{g/ml}$ increased PMN chemotaxis significantly ($p < 0.05$) compared to control unstimulated cells. fMLP (1 μM) was a more potent chemotactic agent than EMD. The combination of EMD and fMLP resulted in significantly ($p < 0.05$) higher PMN chemotaxis compared to EMD or fMLP stimulated cells.

MMP 8 Release by PMN

Figure 3 shows the Western blot for total cellular MMP 8 in PMN incubated with 1% human serum for 15 min, in the presence of 200 $\mu\text{g/ml}$ EMD, 1 μM fMLP, EMD plus fMLP, 2.5 mg/ml opsonized zymosan (OZ), or EMD plus OZ. EMD had no impact on total MMP 8 in human peripheral PMN. Addition of EMD downregulated the expression of MMP 8 in PMN when combined with fMLP or OZ compared MMP8 expression in response to fMLP or OZ alone. OZ was used in this study for comparative purposes since it is known to be much more powerful inducer of PMN degranulation and it triggers higher MMP 8 expression and release than fMLP⁹.

Figure 4 demonstrates the relative release of MMP 8 from PMN triggered with either 200 $\mu\text{g/ml}$ EMD, 1 μM fMLP, EMD plus fMLP, 2.5 mg/ml OZ, or EMD plus OZ in relation to the total MMP 8 content of the PMN. The mean MMP 8 release for those cells incubated with fMLP was statistically significantly ($p < 0.05$) higher compared to control unstimulated cells and EMD or EMD + fMLP stimulated cells. EMD downregulated the release of PMN MMP 8 when it was combined with fMLP compared to MMP 8 release in response to fMLP only. Similar findings were observed when OZ was used to assess the MMP 8 relative release from PMN.

DISCUSSION

PMN are highly specialized leukocytes that play an important role in innate immunity. During infection or tissue injury, PMN respond to proinflammatory mediators and chemoattractants transmigration across the microvascular endothelia, extracellular matrix and mucosal epithelia to accumulate at inflammatory sites²⁸. PMN are the first cells that encounter bacteria in the gingival sulcus. In the present study, we investigated the actions of EMD in the inflammatory response of PMN. Based on our knowledge, this is the first study that examined EMD and PMN function in vitro.

The results reveal that EMD stimulated superoxide release from PMN. Superoxide production by PMN in the presence of 50, 100, and 200 µg/ml EMD was dose-dependent (data not shown). Optimal stimulation of PMN superoxide generation was observed at 200 µg/ml EMD in 6 minutes. fMLP stimulation triggered quicker and more sustained superoxide production from PMN in the presence of EMD. These results are in line with previous observations from in vitro studies showing an antimicrobial effect of EMD inhibiting the growth of three periodontal pathogens; *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia*²⁹. A similar finding was reported by Sculean et al. who also observed that EMD had an impact on the vitality of supragingival dental plaque flora. This antibacterial effect could contribute to the beneficial actions of EMD observed in wound healing studies^{30,31}.

In addition, EMD enhanced the PMN chemotactic response. The combination of EMD and fMLP further increased chemotactic response of PMNs compared to EMD or fMLP alone. It has also been reported that EMD increases chemotaxis of epithelial cells, gingival fibroblasts, PDL cells, osteoblasts, and endothelial cells¹⁹. These data suggest that EMD might accelerate wound healing through clearance of the wound.

PMN MMP 8 is one of the main collagen-degrading enzymes in GCF and saliva^{32,33}. MMP 8 is believed to be mainly responsible for collagen degradation in inflamed tissue during gingivitis and chronic periodontitis³⁴. The release and activation of MMP 8 in PMN can be induced by oral bacteria¹⁰. Release of MMP 8 is mainly observed during phagocytosis and can be attributed to either leakage of granules due to incomplete phagosome formation or induction of PMN degranulation, an active process triggered by interactions of bacterial products such as fMLP with PMNs receptors. Claesson et al. concluded that a secretion and activation of MMP 8 can be stimulated by the periodontal pathogen, *Aggregatibacter actinomycetemcomitans*, in human PMN⁹. The capacity of bacteria to stimulate MMP 8 release correlates with their leukotoxic potential. Previous studies have demonstrated increased GCF levels of MMP 8 in association with the severity of chronic periodontitis, decreasing after successful periodontal treatment with repeatedly high MMP 8 levels in unstable sites showing risk of further destruction^{35,36}. Recent generation of MMP 8 deficient mice has provided preliminary evidence for a role for MMP 8 in immune-inflammatory response modulation and protection against alveolar bone resorption in experimentally induced periodontitis³⁷.

In the present study, EMD did not induce MMP 8 expression by human PMN in vitro. EMD down-regulated MMP 8 release from PMN stimulated with fMLP or OZ. This outcome is in agreement with a previous study by Okuda et al. who found that EMD treatment during flap surgery resulted in a lower levels of MMP 8 in the gingival crevicular fluid compared to flap surgery with placebo¹⁶. Moreover, the accelerated healing response in the EMD surgery group was associated with a rapid return to baseline levels of MMP 8. The authors suggest that treatment with EMD accelerated healing at an earlier stage of wound healing following surgery. Another study by Zeldich et al.³⁸ showed that EMD stimulates tissue inhibitors of matrix metalloproteinase-3 (TIMP-3) production in human gingival fibroblasts via extracellular signal-regulated kinase, which could improve the MMP-TIMP balance in gingival tissue and curb extracellular matrix destruction. Most recently, a study by Zilm and Bartold showed that EMD contains a number of high-molecular weight compounds, which include the proteinase inhibitors, fetuin A and alpha 1-antichymotrypsin³⁹. The later study could explain a previous finding by Wennström and Lindhe, who provided evidence that EMD topically applied in instrumented periodontal pockets enhances the early healing of periodontal soft tissue wounds⁴⁰. Taken, together these observations indicate that EMD, in addition to being a stimulator of periodontal regeneration, may influence soft-tissue healing by controlling inflammation.

This study demonstrates that EMD significantly increases superoxide production and chemotaxis by human PMN and downregulates MMP 8 release. These findings suggest that EMD accelerates wound healing through clearance of the inflammatory lesion from bacteria, while inhibiting tissue damage and chronic inflammation through selective modulation of the PMN response. Further investigations are needed to confirm these observations and further characterize the actions of EMD on the acute inflammatory response.

CONCLUSION

EMD has direct actions on the PMN response promoting early superoxide production and enhancement of chemotaxis to quickly clear the wound, and suppressing MMP 8 release to prevent tissue damage and chronic inflammation. These findings may help, in part, to explain the improved clinical results observed following the use of EMD.

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Figure 1.

Kinetics of superoxide formation by unstimulated PMN and EMD (200 µg/ml) stimulated PMN. The graph shows that the optimal stimulation by EMD is obtained at 6 minutes. Further stimulation of EMD treated cells with 1 µM fMLP triggers earlier and more sustained response compared to control fMLP stimulated cells.

*Statistically significant ($p < 0.01$) compared to unstimulated cells.

†Statistically significant ($p < 0.001$) compared to 1 min fMLP stimulated cells.

Figure 2.

PMN chemotaxis in response to 200 μ g/ml EMD, 1 μ M fMLP, or EMD plus fMLP.

Chemotaxis is expressed as the mean number of PMN per high-power field (PMN/HPF) that has migrated to the distal surface of a microscopic filter in the Boyden chamber. The vertical bars indicate means $\pm$ SD.

*Statistically significant ($p < 0.05$) compared to unstimulated cells.

†Statistically significant ($p < 0.01$) compared to unstimulated cells and EMD stimulated cells.

‡Statistically significant ($p < 0.05$) compared to unstimulated cells and EMD or fMLP stimulated cells.

Figure 3.

Western blot showing the expression of MMP 8 by human PMN incubated with 1% human serum for 15 min, in the presence of 200 μ g/ml EMD, 1 μ M fMLP, EMD plus fMLP, 2.5 mg/ml OZ, or EMD plus OZ. EMD has no impact on MMP 8 expression by human peripheral PMN. EMD downregulates the expression of MMP 8 when combined with fMLP or OZ compared MMP8 expression in response to fMLP or OZ alone.

Figure 4.

Relative release of MMP 8 by human PMN triggered with 200 μ g/ml EMD, 1 μ M fMLP, EMD plus fMLP, 2.5 mg/ml OZ or EMD plus OZ. Relative density method was used to determine the percent of the MMP 8 released extracellularly in relation to the total MMP 8 content of the PMN. The total content of MMP 8 was extracted from the PMN by treatment with 0.2% Triton X-100 in the presence of 1% human serum. The vertical bars indicate means $\pm$ SD.

*Statistically significant ($p < 0.01$) compared to unstimulated and EMD or EMD plus fMLP stimulated cells.

† Statistically significant ($p < 0.01$) compared to unstimulated and EMD or EMD plus OZ stimulated cells.

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|| SpectraMax 340PC, Molecular Devices, Sunnyvale, CA, USA

¶ Calbiochem, Gibbstown, NJ, USA

Dakopatts, A/S, Glostrup, Denmark

** Pierce, Rockford, IL, USA

†† Bio-Rad Laboratories, Richmond, CA, USA

‡‡ Martha Warbington is deceased