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Anti-inflammatory properties of enamel matrix derivative in human blood

A. E. Myhre[1], S. P. Lyngstadaas[2], M. K. Dahle[1], J. F. Stuestøl[1], S. J. Foster[3], C. Thiemermann[4], P. Lilleaasen[1], J. E. Wang[1], A. O. Aasen[1]

[1]University of Oslo, Faculty Division Rikshospitalet Institute for Surgical Research, Oslo, Norway, [2]University of Oslo, Faculty of Dentistry, Clinical Research Laboratory, Institute of Clinical Dentistry, Oslo, Norway, [3]Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, UK, [4]Department of Experimental Medicine & Nephrology, The William Harvey Research Institute, London, UK

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Background and Objective: Enamel matrix derivative (EMD), extracted from porcine tooth buds, has been shown to promote periodontal healing in patients with severe periodontitis. This involves modulation of the inflammatory response followed by the onset of periodontal regeneration. Based on these observations, we examined the ability of EMD to modulate the release of a pro-inflammatory cytokine [tumor necrosis factor (TNF)-α], an anti-inflammatory cytokine (interleukin-10) and a chemokine (interleukin- 8) in whole human blood challenged by bacterial cell wall components.

Material and Methods: Whole blood from healthy donors was challenged by lipopolysaccharide or peptidoglycan and incubated with different concentrations of EMD or a cAMP analogue 8-(4-chlorophenyl)thio-cAMP (8-CPT-cAMP). TNF-α, interleukin-8 and interleukin-10 were analysed from plasma by enzyme-linked immunosorbent assay (ELISA) while cAMP levels of peripheral blood mononuclear cell lysates were analysed by enzyme immunoassay (EIA).

Results: We found that EMD attenuated the release of TNF-α and interleukin-8 in whole blood from healthy donors challenged by lipopolysaccharide or peptidoglycan, while the release of interleukin-10 was unchanged. Enamel matrix derivative also produced a four-fold increase in the cAMP levels of peripheral blood mononuclear cell lysates. Like EMD, 8-CPT-cAMP attenuated the formation of TNF-α, but not of interleukin-10, in blood challenged by lipopolysaccharide.

Conclusion: Enamel matrix derivative limits the release of pro-inflammatory cytokines induced by lipopolysaccharide or peptidoglycan in human blood, suggesting that it has anti-inflammatory properties. We propose that this effect of EMD is, at least partly, secondary to an increase in the intracellular levels of cAMP in peripheral blood mononuclear cells.

Anders E. Myhre, Institute for Surgical Research, Rikshospitalet University Hospital, Sognsvannsveien 20, N-0027 Oslo, Norway
Tel: +47 23073520
Fax: +47 23073530
email: A.E.Myhre@studmed.uio.no

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There is substantial evidence that enamel matrix derivative (EMD) improves the clinical outcome of periodontal treatment in patients with severe periodontitis (1,2). This effect involves a local increase in the synthesis and secretion of growth factors and cytokines, the enhancement of periodontal ligament and gingival fibroblast proliferation, and the promotion of vessel formation, thereby promoting healing of soft tissue wounds and attenuating gingival inflammation (2–7). EMD is extracted from developing porcine tooth buds and contains primarily amelogenins, which are the major proteins of developing tooth enamel matrix and predominantly involved in the formation of enamel and dental cementum. The available literature suggests that EMD has immunomodulatory properties, but the potential effects of EMD on inflammatory responses, such as cytokine formation, remain mostly unexplored.

Bacterial cell wall components have the ability to induce a burst of

pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), from peripheral blood mononuclear cells and tissue macrophages. Production of the chemokine, interleukin-8, is partly triggered by TNF-α (8), and serves to recruit phagocytes to an infectious site. The anti-inflammatory cytokine, interleukin-10, inhibits the production of TNF-α and interleukin-8 *in vitro* (9,10), and interleukin-10 has been shown to be a functional repressor of monocyte activation in blood from patients with sepsis (11). In the early stages of sepsis, the levels of pro-inflammatory cytokines become highly elevated, leading to aberrant regulation of host defense systems followed by profound hemodynamic disturbances, making sepsis with multiple organ dysfunction syndrome (MODS) the major cause of death in the surgical intensive care unit (12). Modulation of cellular inflammatory processes is one possible approach to improve survival and outcome in sepsis patients.

To investigate whether EMD might modulate inflammatory responses in general, we assessed the significance of EMD in regulating the release of TNF-α, interleukin-8 and interleukin-10 in whole human blood stimulated with either lipopolysaccharide or peptidoglycan (PepG) derived from the cell walls of gram-negative and gram-positive bacteria, respectively.

Material and methods

Reagents

One vial containing 10 mg of sterile lyophilised porcine enamel matrix protein (Biora AB, Malmö, Sweden), containing, according to the manufacturer, 80% amelogenin, small amounts of residual water, calcium phosphate and acetic acid, was dissolved to 20 mg/ml using 0.1% acetic acid and then further diluted to working strength dilutions using neutral phosphate-buffered saline (PBS; Cambrex Bio Science, Verviers, Belgium). *Staphylococcus aureus* PepG was isolated from bacterial cell walls, as previously described for *Bacillus subtilis* (13). The PepG preparations were analysed for the presence of lipopolysaccharide by using the Limulus amebocyte lysate test (COAMATIC Chromo-LAL; Chromogenix, Falmouth, MA, USA), and were shown to contain ≈ 35 pg of lipopolysaccharide/mg of PepG. *Escherichia coli* (serotype O26:B6) lipopolysaccharide was purchased from Difco Laboratories (Detroit, MI, USA). Additional reagents were purchased from Sigma unless specified otherwise.

Whole-blood experiments

The human whole-blood model was used, as previously described (14), with some modifications. In brief, venous blood from healthy volunteers was anticoagulated with heparin (30 IU/ml of blood; Leo, Ballerup, Denmark) and incubated in 0.5-ml thin-wall tubes (ABgene House, Epsom, UK) at 37°C with slow rotation in the absence or presence of EMD (1, 10, 100 or 300 μg/ml) and *S. aureus* PepG (10 μg/ml) or lipopolysaccharide (10 ng/ml). Samples were removed for analysis after 6 and 24 h. In some experiments, different doses of the 3′,5′ cyclic adenosine monophosphate (cAMP) analogue, 8-(4-chlorophenyl)thio-cAMP (8-CPT-cAMP) (10, 25, 50 or 100 μM), were added. In other experiments, the blood was given either pre- or post-treatment, by administering EMD (100 μg/ml) 2 h before lipopolysaccharide, at the same time as lipopolysaccharide, or 1 h after lipopolysaccharide. In experiments aimed at studying effects on cAMP levels, whole blood was incubated in the presence or absence of EMD (100 μg/ml), as previously described, and put on ice after 5 or 10 min for the isolation of peripheral blood mononuclear cells.

Isolation of peripheral blood mononuclear cells

The blood was diluted 1 : 1 (v/v) in ice-cold PBS, and peripheral blood mononuclear cells were isolated on a Lymphoprep (Axis-Shield, Oslo, Norway) gradient. The cells were washed twice by centrifugation (1000 *g*, 5 min, 4°C) and resuspended in cold PBS, before being lysed in 0.1 M hydrochloric acid and stored at −20°C for later measurements of cAMP.

Enzyme-linked immunosorbent assays/enzyme immunoassays

Plasma was removed by centrifugation at 2500 *g* for 5 min and stored at −20°C in 96-well microplates (Greiner Bio-One, Frieckenhausen, Germany) for later analyses by enzyme-linked immunosorbent assay (ELISA) specific for TNF-α, interleukin-8 (R & D, Minneapolis, MN, USA) and interleukin-10 (CLB, Amsterdam, the Netherlands). cAMP was measured by a competitive enzyme immunoassay (EIA; Cayman Chemical Company, Ann Arbor, MI, USA). All analyses were performed in accordance with the manufacturers' instructions.

Statistical evaluation

Data are presented as mean values ± standard error of the mean (SEM). Differences between study groups were analysed using one-way analysis of variance (ANOVA) with Tukey's post hoc assessment or Student's *t*-test analysis. A *p*-value of < 0.05 was considered significant.

Results

Effects of EMD on cytokine release in whole human blood

As shown in Fig. 1(A), EMD reduced the production of TNF-α, stimulated by lipopolysaccharide (10 ng/ml) or PepG (10 μg/ml), in a dose-dependent manner. The greatest reduction was obtained by 300 μg/ml of EMD, attenuating the lipopolysaccharide- and PepG-induced release of TNF-α by 54% and 69%, respectively ($p < 0.05$). EMD also reduced the release of interleukin-8 induced by PepG ($p < 0.05$, Fig. 1B). In contrast, the release of interleukin-10 was not significantly influenced by EMD (Fig. 1C). EMD had no effects on cytokine release when administered alone.

Effect of incubation time

In order to assess whether EMD was more effective as pre- or post-treatment,

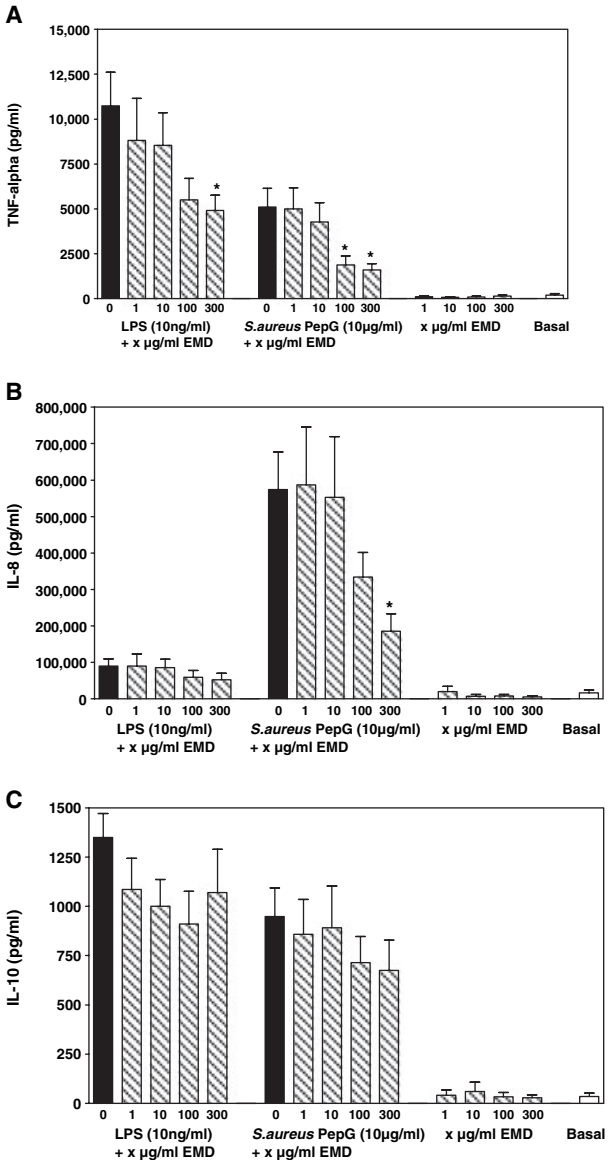

Fig. 1. Effects of enamel matrix derivative (EMD) on the release of tumour necrosis factor-α (TNF-α) (Fig. 1A), interleukin-8 (Fig. 1B) and interleukin-10 (Fig. 1C) in whole human blood. Fresh venous blood was added to different concentrations of EMD and spiked with lipopolysaccharide (LPS; 10 ng/ml) or peptidoglycan (PepG, 10 µg/ml). Plasma was isolated and analysed for TNF-α (after 6 h), interleukin-8 and interleukin-10 (after 18 h) by enzyme-linked immunosorbent assay (ELISA). Mean values ± standard error of the mean (SEM) of eight donors are shown. *Significantly lower ($p < 0.05$) than levels obtained by lipopolysaccharide/PepG alone.

EMD (100 µg/ml) was given (i) 2 h prior to stimulation with lipopolysaccharide, (ii) at the same time as lipopolysaccharide, or (iii) 1 h after stimulation with lipopolysaccharide. Figure 2 shows that TNF-α release was significantly reduced in blood that was pretreated with EMD, while administration of EMD, together with, or even after, lipopolysaccharide, was not significantly effective.

Regulation of cAMP and effects on cytokine release

We have previously shown that cAMP is elevated in periodontal ligament and epithelial cells after exposure to EMD (5). Thus, we wished to establish whether EMD increases cAMP formation in peripheral blood mononuclear cells in the whole-blood model, elucidating one possible mechanism for the prevention of TNF-α release caused by EMD. As shown in Fig. 3(A), the exposure of whole blood to EMD (100 µg/ml) caused a rapid (within 5 min) fourfold increase of cAMP in peripheral blood mononuclear cells. The amount of cAMP was still more than twofold increased compared with baseline levels 10 min after the addition of EMD. To establish whether prevention of the formation of pro-inflammatory cytokines by EMD could be secondary to increased cAMP levels, we studied the effects of cAMP on cytokine release in whole human blood. Like EMD (Fig. 1), the cAMP analogue, 8-CPT-cAMP (10, 25, 50 or 100 µM), attenuated the TNF-α formation caused by lipopolysaccharide in a dose-dependent manner ($p < 0.01$), but did not affect the release of interleukin-10 (Fig. 3B).

Discussion

This study is the first to report that EMD modulates the formation of cytokines produced in response to bacterial toxins in human blood. Specifically, we show that EMD differentially modulates cytokine release induced by wall components from different pathogens, attenuating the release of a pro-inflammatory cytokine (TNF-α) and a chemokine (interleukin-8), while the release of the anti-inflammatory cytokine, interleukin-10, is preserved. The effect of EMD on TNF-α release was most pronounced when EMD was given as a pretreatment.

The immunological impact of EMD has been studied only minimally. One study has shown that EMD has low stimulatory effects on human blood lymphocytes *in vitro*, reporting a slight increase in the proliferation of $CD25^+$ T cells and a decrease in $CD19^+$ B cells (15). Furthermore, a report from the same group concludes that EMD does not alter lymphocyte function in patients treated with EMD after periodontal surgery (16). Our

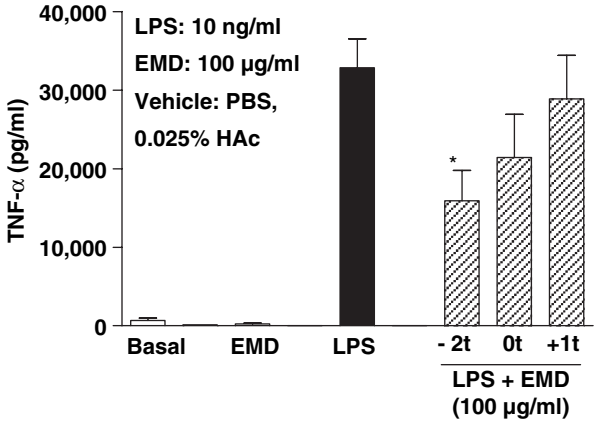

Fig. 2. Effect of pretreatment vs. post-treatment with enamel matrix derivative (EMD) on the release of tumour necrosis factor-α (TNF-α) in whole human blood. EMD was added to fresh venous blood 2 h before (– 2t), at the same time (0t), or 1 h after (+1t) the addition of lipopolysaccharide (LPS; 10 ng/ml). Six hours after the addition of lipopolysaccharide, plasma was isolated and analysed for TNF-α by enzyme-linked immunosorbent assay (ELISA). Mean values ± standard error of the mean (SEM) of six donors are shown. *Significantly lower ($p < 0.05$) than levels obtained by lipopolysaccharide alone. HAc, acetic acid, PBS, phosphate-buffered saline.

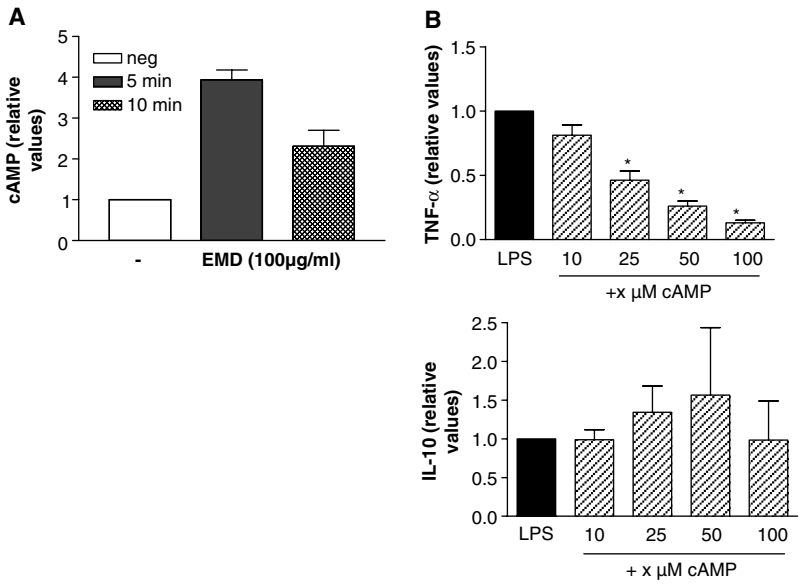

Fig. 3. cAMP accumulation in peripheral blood mononuclear cells after stimulation of whole human blood with enamel matrix derivative (EMD), and regulation of cytokine release by cAMP. EMD was added to fresh venous blood and peripheral blood mononuclear cells were isolated after 5 and 10 min, lysed and analysed for cAMP by enzyme immunoassay (EIA) (Fig. 3A). In other experiments, the blood was added to different concentrations of 8-(4-chlorophenyl)thio-cAMP (8-CPT-cAMP) (8-CPT-cAMP) and spiked with lipopolysaccharide (10 ng/ml). Plasma was isolated and analysed for tumour necrosis factor-α (TNF-α) (after 6 h) and interleukin-10 (after 18 h) by enzyme-linked immunosorbent assay (ELISA) (Fig. 3B). Mean values ± standard error of the mean (SEM) of six donors are shown. *Significantly lower ($p < 0.01$) than values induced by lipopolysaccharide alone.

focus has been on the innate immune system, which is strongly responsible for exaggerated cellular and inflammatory responses to infectious agents, leading to the high mortality rate reported as a result of sepsis. During sepsis, bacteria or their cell wall components (such as lipopolysaccharide or PepG) are released into the bloodstream. Studies of patients with sepsis, and of animal models, have shown that these toxins activate circulating leukocytes and tissue macrophages, leading to a massive inflammatory response in the blood compartment as well as in different tissues (17–19). The pro-inflammatory cytokine, TNF-α, is elevated in the early stages of sepsis, and is the central mediator of a number of inflammatory responses (20,21). Production of the chemokine, interleukin-8, is partly triggered by TNF-α (8). Interleukin-8 activates neutrophils, inducing chemotaxis, exocytosis and the respiratory burst (22). In *in vitro* experiments, interleukin-10 has been found to inhibit the production of TNF-α and interleukin-8 by monocytes/macrophages (9,10), and it is a functional repressor of monocyte activation in blood from sepsis patients (11). Hence, new means to modulate the aberrant cytokine response in patients with sepsis may have therapeutic potentials. However, the above-mentioned cytokines are important components of our immune system, and are pivotal for an effective host defense to infections. Blockade of single mediators, such as TNF-α, has been attempted in patients with sepsis, without much success (23). Arthur E. Baue explained to us that there are no 'magic bullets', suggesting that the deleterious inflammatory response in sepsis cannot be prevented by blocking a single effector (24). Instead, means to limit excessive inflammation, or prevent these effects by modulating the response of the macrophage and the reticulo-endothelial system to bacterial components, should be addressed. Our results do indeed show that EMD may influence the cytokine profile in human blood in a beneficial manner, implicating a role for EMD as an immunomodulatory agent.

Hatakeyama *et al.* have demonstrated that amelogenin-null mice exhibit increased activation of the osteoclastogenic pathway initiated by receptor activator of nuclear factor-κB (RANK) ligand (25). RANK is reported to initiate a broad range of signaling events associated with inflammatory cellular

responses (26), indicating a role for amelogenin in modulating cellular inflammation in monocyte-derived cells. However, the cellular mechanisms by which amelogenin products, such as EMD, may modulate inflammation are mostly unknown. We have previously shown that cAMP is elevated in periodontal ligament and epithelial cells after exposure to EMD (5). In the present study, we report that EMD also raises cAMP levels in peripheral blood mononuclear cells. In addition, we show that cAMP attenuates the TNF-α response in whole blood, as opposed to the interleukin-10 response, which is preserved, a similar pattern to that obtained by EMD administration in our studies. This suggests that the immunomodulatory effects of EMD are mediated by a cAMP-dependent pathway.

The monocyte/macrophage-driven cytokine burst is, under normal circumstances, sequentially down-regulated by anti-inflammatory cytokines as well as by several factors that initiate intracellular formation of cAMP (10,11,27,28). The second messenger cAMP is known to inhibit activation of nuclear factor-κB and, particularly, the release of TNF-α, as well as regulating several other mechanisms of innate and adaptive immunity (29–32). Previous studies have aimed to influence immune function in sepsis by manipulating cAMP-dependent pathways; however, the conclusions were unclear (27,33,34). It is, nevertheless, pertinent to highlight that the effects of cAMP are crucially dependent on the subcellular localization and hence the mechanisms by which it is activated. EMD probably has multiple functions on cell activation, and should, at this point, not be reduced to merely a cAMP elevating factor.

Amelogenins account for more than 80% of EMD, and when separating them by gel electrophoresis a complex mixture of gene splice products and degradation products are revealed (35). These exhibit differences in solubility and biological effects (36,37), and probably only some are responsible for the described impact on innate immune responses. Veis *et al.* studied the significance of specific amelogenin splice products in inducing signaling and gene expression during amelogenesis (37,38), and similar steps might be taken to elucidate the roles of specific low-molecular-weight amelogenins in modulating innate immune responses.

In this study we demonstrate that EMD limits the release of pro-inflammatory cytokines induced by lipopolysaccharide or PepG in human blood, suggesting that it has anti-inflammatory potentials. Based on this, we hypothesize that EMD may ameliorate pathology caused by septic challenges.

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