2013

Amelogenin Peptide Extract Increases Differentiation and Angiogenic and Local Factor Production and Inhibits Apoptosis in Human Osteoblasts

Rene Olivares-Navarrete
Virginia Commonwealth University, ronavarrete@vcu.edu

Sharon L. Hyzy
Virginia Commonwealth University

Argelia Almaguer-Flores
Universidad Nacional Autonoma de Mexico

See next page for additional authors

Authors

Rene Olivares-Navarrete, Sharon L. Hyzy, Argelia Almaguer-Flores, Corinna Mauth, Anja C. Gemperli, Barbara D. Boyan, and Zvi Schwartz

Research Article

Amelogenin Peptide Extract Increases Differentiation and Angiogenic and Local Factor Production and Inhibits Apoptosis in Human Osteoblasts

Rene Olivares-Navarrete,[1] Sharon L. Hyzy,[1] Argelia Almaguer-Flores,[2] Corinna Mauth,[3] Anja C. Gemperli,[3] Barbara D. Boyan,[1] and Zvi Schwartz[1,4]

[1] *School of Engineering, Virginia Commonwealth University, 601 West Main Street, Suite 331, Richmond, VA 23284-3068, USA*
[2] *Facultad de Odontologia, Universidad Nacional Autonoma de Mexico, Ciudad Universitaria, Coyoacán, 04510 DF, Mexico*
[3] *Institut Straumann AG, Nauenstrasse, 4052 Basel, Switzerland*
[4] *Department of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA*

Correspondence should be addressed to Barbara D. Boyan; bboyan@vcu.edu

Enamel matrix derivative (EMD), a decellularized porcine extracellular matrix (ECM), is used clinically in periodontal tissue regeneration. Amelogenin, EMD's principal component, spontaneously assembles into nanospheres *in vivo*, forming an ECM complex that releases proteolytically cleaved peptides. However, the role of amelogenin or amelogenin peptides in mediating osteoblast response to EMD is not clear. Human MG63 osteoblast-like cells or normal human osteoblasts were treated with recombinant human amelogenin or a 5 kDa tyrosine-rich amelogenin peptide (TRAP) isolated from EMD and the effect on osteogenesis, local factor production, and apoptosis assessed. Treated MG63 cells increased alkaline phosphatase specific activity and levels of osteocalcin, osteoprotegerin, prostaglandin E_2, and active/latent TGF-β1, an effect sensitive to the effector and concentration. Primary osteoblasts exhibited similar, but less robust, effects. TRAP-rich 5 kDa peptides yielded more mineralization than rhAmelogenin in osteoblasts *in vitro*. Both amelogenin and 5 kDa peptides protected MG63s from chelerythrine-induced apoptosis. The data suggest that the 5 kDa TRAP-rich sequence is an active amelogenin peptide that regulates osteoblast differentiation and local factor production and prevents osteoblast apoptosis.

1. Introduction

Enamel matrix derivative (EMD) is a decellularized extracellular matrix (ECM) isolated from porcine tooth germs and has been used clinically in a carrier as Emdogain (Institut Straumann AG, Basel, Switzerland) to promote periodontal tissue regeneration, including periodontal ligament, alveolar bone, and cementum [1–3]. It has been suggested that EMD induces periodontal tissue regeneration by mimicking events in normal periodontal tissue development [4]. During tooth formation, enamel matrix proteins are secreted by ameloblasts and Hertwig's epithelial root sheath. In addition to providing the structural matrix for the developing enamel, these proteins also act as mediators at the epithelial/mesenchymal interface, resulting in formation of periodontal ligament, alveolar bone, and dental cementum [5–7].

EMD not only functions as a scaffolding for cell migration and clot organization, but one or more of its constituents also have biological activity associated with wound repair. In addition to its effects on periodontal bone formation, EMD has been applied to long bone defects, increasing *de novo* trabecular bone formation [8]. It has also been used to heal acute and chronic skin wounds, increasing the amount of granulation tissue and reepithelialization, causing healing to progress twice as fast as untreated wounds [9]. In addition, amelogenin, a component of EMD [10], has been applied to treat difficult-to-heal venous ulcers, decreasing ulcer area, pain, and exudates [11, 12].

EMD is mainly composed of amelogenins (~90%) [10], a highly hydrophobic protein family that shares high homology across species [13]. The remaining protein portion of EMD

is composed of extracellular proteins and enzymes such as enamelin, ameloblastin, and proteases [14–16]. Amelogenins self-assemble into hydrophobic nanosphere aggregates [17, 18] that show high affinity to hydroxyapatite crystals and collagen [19]. These assembled structures undergo a slow, progressive proteolytic degradation that results in several polypeptide fragments that are released to the matrix [17, 20, 21]. These peptides as well as isoforms and splice variants of amelogenin can activate diverse functions in adjacent cells or tissues [22–27]. Two smaller amelogenin peptides, leucine-rich amelogenin peptide (LRAP) and tyrosine-rich amelogenin peptide (TRAP) [28], have been proposed to be the functional part of the intact amelogenin.

As is the case with other decellularized matrices, the specific roles of individual EMD components or subsets of components in tissue regeneration are not well understood. It is unclear whether the therapeutic effect of EMD is due to the full-length amelogenin protein, due to the splice variants, or to a combination of both. Three subfractions of EMD have been isolated by high-performance liquid chromatography: one containing mainly a 20 kDa peptide, one represented by two peptides of 12 kDa and 9 kDa, and one fraction identified as a single band at 5 kDa by SDS-PAGE analysis [29]. The 20 kDa peptide corresponds to the full-length amelogenin protein and the 5 kDa peptide corresponds to a portion of the N-terminus of the protein that includes peptides with the TRAP and LRAP sequences.

In vitro studies indicate that EMD has a differential effect on osteoblast proliferation and differentiation depending on the maturation state of the cells, increasing cell numbers in less mature cells and increasing differentiation in more mature cells, including increased alkaline phosphatase activity, osteocalcin, bone sialoprotein, and mineralized nodule formation [30–33]. EMD treatment results in an increase in proliferation of other cell types as well [34–37]. Studies using DNA microarray technology indicate that EMD regulates expression of genes involved in cell cycle, proliferation, and apoptosis [38]. Whereas genes that induce apoptosis such as MADD and TNF-α were upregulated, genes that inhibit apoptosis and increase cell survival such as MCL1 were upregulated as well. These conflicting observations suggest that EMD has pleiotropic effects in part via the actions of different constituents and in part due to differences in the responding cell populations. Therefore, the aim of the present study was to elucidate the contribution of the 5 kDa peptides, specifically the TRAP sequences, to the osteogenic potential of EMD by examining the responses of osteoblasts to these peptides in comparison to recombinant human amelogenin.

2. Materials and Methods

2.1. Ethics Statement. Human osteoblasts (HOBs) were isolated from bone obtained from a 16-year-old male patient at Children's Healthcare of Atlanta under Institutional Review Board approval from the Georgia Institute of Technology and Children's Healthcare of Atlanta. Written informed consent was obtained from the patient's guardians on behalf of the minor participant.

2.2. Cell Culture. Human MG63 osteoblast-like cells were obtained from the American Type Culture Collection (Rockville, MD, USA). HOBs were isolated from bone chips. First, bone chips were washed in Dulbecco's modification of Eagle's medium (DMEM, Mediatech, Manassas, VA, USA) containing 3% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), followed by incubation in 0.25% trypsin-EDTA (Invitrogen) for 1 hour. The bone was then cut into 1 mm^2 pieces and cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Mediatech) and 1% penicillin-streptomycin for two weeks to allow immature osteoblasts to migrate into the culture surface. To validate osteoblast phenotype, isolated cells were treated for 24 hours with 10^{-8} M 1α, 25(OH)$_2$D$_3$, and alkaline phosphatase-specific activity (an early marker of osteoblast differentiation) and osteocalcin production (a later marker of osteoblast differentiation) measured (data not shown). For experiments, MG63 or first passage HOB cells were plated at a seeding density of 10,000 cells/cm^2 and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin until confluent, when they were treated as described in the following.

2.3. Proteins. Recombinant human amelogenin (rhAmelogenin) and the 5 kDa peptides (Fraction C) were extracted and purified by Institut Straumann AG using a modification of previously described methods [29, 39]. Briefly, Fraction C was extracted from EMD via size exclusion high-performance liquid chromatography (TSKgel SW3000, Tosoh Bioscience GmbH, Stuttgart, Germany) in 30% acetonitrile containing 0.9 mM NaCl resulting in one peak around 5 kDa. This peak was then subjected to preparative reverse phase high-performance liquid chromatography (XBridge C8, Waters Corporation, Milford, MA, USA) leading to only two peaks (with very small shoulders), which were identified by liquid chromatography-mass spectrometry as two TRAP species, one 43 amino acids and the other 45 amino acids in length (Figure 1). The peaks were eluted using a gradient of mobile phase A (Milli-Q water containing 0.1% trifluoroacetic acid (TFA)) and mobile phase B (ACN containing 0.1% TFA) from 5% to 70% B during 7 column volumes. The two TRAP species were identified by sequence analysis performed at BASF SE (Ludwigshafen, Germany).

Lyophilized proteins were reconstituted in 0.1% acetic acid and sterile filtered with a low binding protein filter (Millex-GV Filter Unit, Millipore, Billerica, MA, USA) to produce a 1 mg/mL stock solution. Further dilutions of proteins (0.01–100 μg/mL) were performed in culture media.

2.4. Osteoblast Differentiation Assays. Confluent cultures of MG63 and HOB cells were treated with either vehicle (0.01% acetic acid) or protein (rhAmelogenin, Fraction C) for 24 hours. After 24 hours, conditioned media were collected and assayed. Osteocalcin was measured using a radioimmunoassay (Biomedical Technologies, Inc., Stoughton, MA, USA). Levels of osteoprotegerin (OPG), active and latent transforming growth factor beta-1 (TGF-β1), vascular endothelial growth factor-A (VEGF-A), and fibroblast growth factor 2 (FGF-2) were measured using ELISA (DuoSet, R&D Systems,
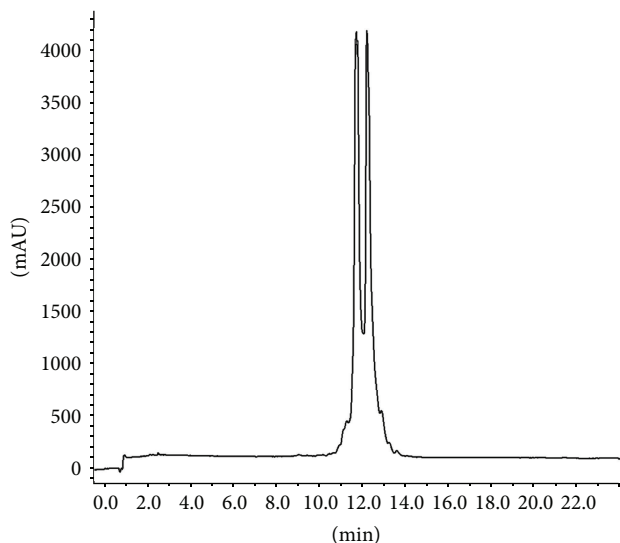
Figure 1: Preparative reverse phase high-performance liquid chromatography spectrum of Fraction C. HPLC spectrum of Fraction C, demonstrating two peaks corresponding to 43 and 45 amino acid tyrosine-rich amelogenin peptide sequences.

Minneapolis, MN, USA). To differentiate between active and latent TGF-β1, an aliquot of conditioned medium was acidified and used to calculate total TGF-β1. Active TGF-β1 was measured in a second, nonacidified aliquot. Latent TGF-β1 was calculated by subtracting the active TGF-β1 from the total. The amount of prostaglandin E_2 (PGE$_2$) in the conditioned medium was measured using radioimmunoassay (Perkin Elmer, Waltham, MA, USA) as described previously [40].

Cell monolayers were rinsed twice with PBS and lysed in 0.05% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Total DNA was quantified using PicoGreen (Quant-iT PicoGreen dsDNA kit, Invitrogen) following the manufacturer's instructions. Briefly, lysates were incubated with 0.5 μL PicoGreen for 5 min and fluorescence intensity measured on a fluorescent plate reader (Beckman Coulter, Brea, CA, USA) using excitation at 480 nm and emission at 520 nm. Concentration was calculated using a DNA standard. Alkaline phosphatase was assayed in the cell lysates by measuring the release of p-nitrophenol from p-nitrophenylphosphate at pH 10.2 [41] and normalized to total protein (Pierce BCA Protein Assay, Thermo Fisher, Rockford, IL, USA).

2.5. Alizarin Red Staining. HOBs were plated in 24-well plates at 10,000 cells/cm^2. Cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin and treated with 1 μg/mL rhAmelogenin or Fraction C for 14 days. Monolayers were assayed for Alizarin red staining using a quantitative method [42]. Briefly, monolayers were fixed in 10% neutral buffered formalin and stained with 40 mM Alizarin red solution. Monolayers were solubilized in 10% (v/v) acetic acid, heated to 85°C, and neutralized with 10% (v/v) ammonium hydroxide. 100 μL aliquots were read at

405 nm and quantities extrapolated to initial stain uptake using known dilutions of Alizarin red.

2.6. Apoptosis Assays. In all assays, confluent cultures were pretreated with 10 μM chelerythrine (EMD Chemicals, Gibbstown, NJ, USA) for 30 minutes to induce apoptosis [43, 44]. Cells were then incubated with rhAmelogenin or Fraction C. Cell viability was determined using MTT assay after 24 hours of treatment. Cultures were incubated with 5 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid) (Sigma-Aldrich) for 4 hours. After incubation, cell layers were rinsed with PBS and dissolved in dimethyl sulfoxide (Sigma Aldrich) and absorbance measured at 570 nm. Apoptosis was assessed using DNA fragmentation. After 20 hours of treatment, cultures were incubated with 1 μCi/mL ^{3}H-thymidine for 4 hours. Cells were trypsinized and lysed and fragmented DNA separated by ultracentrifugation. Percent of fragmented DNA was determined by measuring intact and fragmented DNA by liquid scintillation counter. Caspase-3 activity was measured in cell lysates 6 hours after treatment using a Caspase-3 Colorimetric Assay (R&D Systems) and normalized to total protein content. Phosphorylated p53 was measured in cell lysates 6 hours after treatment using a commercially available ELISA following manufacturer's instructions (R&D Systems).

2.7. Statistical Analysis. Data presented are from one representative example of two independent experiments with similar results. Data are the mean ± SEM of six independent cultures per variable. Data were analyzed using ANOVA, and significance between groups was determined using Bonferroni's modification of Student's t-test. $P < 0.05$ was considered significant.

3. Results

3.1. Osteogenic Response of MG63 Cells. Treatment with rhAmelogenin or Fraction C had no effect on DNA content of MG63 cells, regardless of the protein concentration used (Figure 2(a)). rhAmelogenin increased alkaline phosphatase-specific activity (Figure 2(b)). MG63 cells treated with Fraction C had higher alkaline phosphatase-specific activity than untreated cells, with the greatest effect seen in cultures treated with 1 μg/mL.

Treatment with rhAmelogenin increased levels of osteocalcin over control levels at all doses (Figure 2(c)). Effects were greatest in cultures treated with 1 μg/mL protein. Fraction C had a comparable effect on osteocalcin at doses up to 1 μg/mL, but at higher concentrations the peptide was less stimulatory than rhAmelogenin, and at the highest concentration osteocalcin levels were comparable to those of control cultures.

rhAmelogenin also increased OPG in the conditioned medium, with the greatest effect seen in cultures treated with 1 μg/mL (Figure 2(d)). Fraction C had a robust stimulatory effect on OPG at lower concentrations. Peak effects were seen in cultures treated with 1 μg/mL, but in cultures treated with
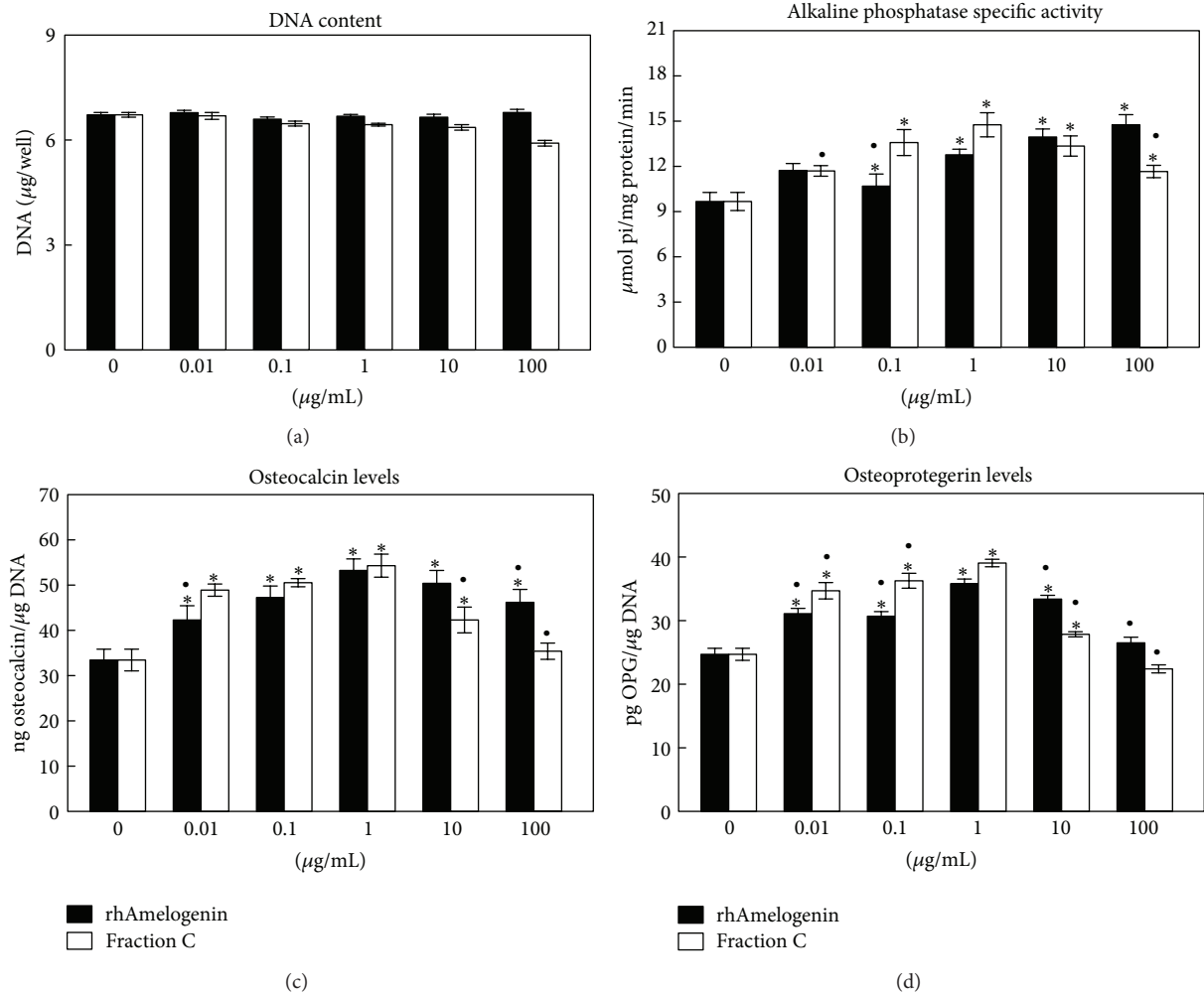
(a)

(b)

(c)

(d)

FIGURE 2: Effect of rhAmelogenin and Fraction C on DNA content and osteoblast phenotype of MG63 cells. MG63 cells were grown to confluence and then treated with $0\,\mu g/mL$ or $0.01\,\mu g/mL$–$100\,\mu g/mL$ rhAmelogenin or Fraction C for 24 hours. DNA content (a) and alkaline phosphatase-specific activity (b) were measured in the cell lysate, and osteocalcin (c) and osteoprotegerin (d) measured in the conditioned media. $^{*}P < 0.05$, versus $0\,\mu g/mL$; $^{\bullet}P < 0.05$, versus $1\,\mu g/mL$.

the highest concentration of Fraction C OPG levels were comparable to those seen in control cultures.

PGE$_2$ was increased by all proteins (Figure 3(a)). Effects of rhAmelogenin were independent of dose. In contrast, Fraction C was stimulatory only at the higher concentrations of 10 and $100\,\mu g/mL$.

TGF-β1 was differentially regulated by each fraction (Figure 3(b)). rhAmelogenin increased active TGF-β1 at doses of 1 and $10\,\mu g/mL$. However, at $100\,\mu g/mL$ it was no longer stimulatory. In contrast, Fraction C was stimulatory at all doses tested and to a comparable extent. All protein fractions increased latent TGF-β1 (Figure 3(c)), an effect independent of dose.

VEGF-A and FGF-2 were also differentially regulated (Figures 4(a) and 4(b)). VEGF-A was increased to the greatest extent by $100\,\mu g/mL$ rhAmelogenin and Fraction C. rhAmelogenin induced highest FGF-2 production in cultures treated with $1\,\mu g/mL$, while Fraction C induced FGF-2 production in a dose-dependent manner.

3.2. Osteogenic Response of Human Osteoblasts. HOB cells were regulated in a similar manner. There was no effect on cell number by any of the proteins (Figure 5(a)). Both protein fractions increased alkaline phosphatase-specific activity (Figure 5(b)), osteocalcin levels (Figure 5(c)), and OPG levels (Figure 5(d)). Whereas there were no differences in response between 1 and $10\,\mu g/mL$ for these parameters, VEGF-A (Figure 5(e)) and PGE$_2$ (Figure 5(f)) were differentially regulated. VEGF-A was increased by Fraction C only and only at $1\,\mu g/mL$. PGE$_2$ was decreased by rhAmelogenin and Fraction C, but only at $10\,\mu g/mL$. Moreover, the inhibitory effect of Fraction C was more robust.

Long-term effects of rhAmelogenin and Fraction C on osteoblast mineralization were examined after 14 days. rhAmelogenin promoted Alizarin red staining in HOB cultures in the absence of osteogenic media supplements (Figure 6). However, application of Fraction C also yielded higher mineralization than control cultures and produced
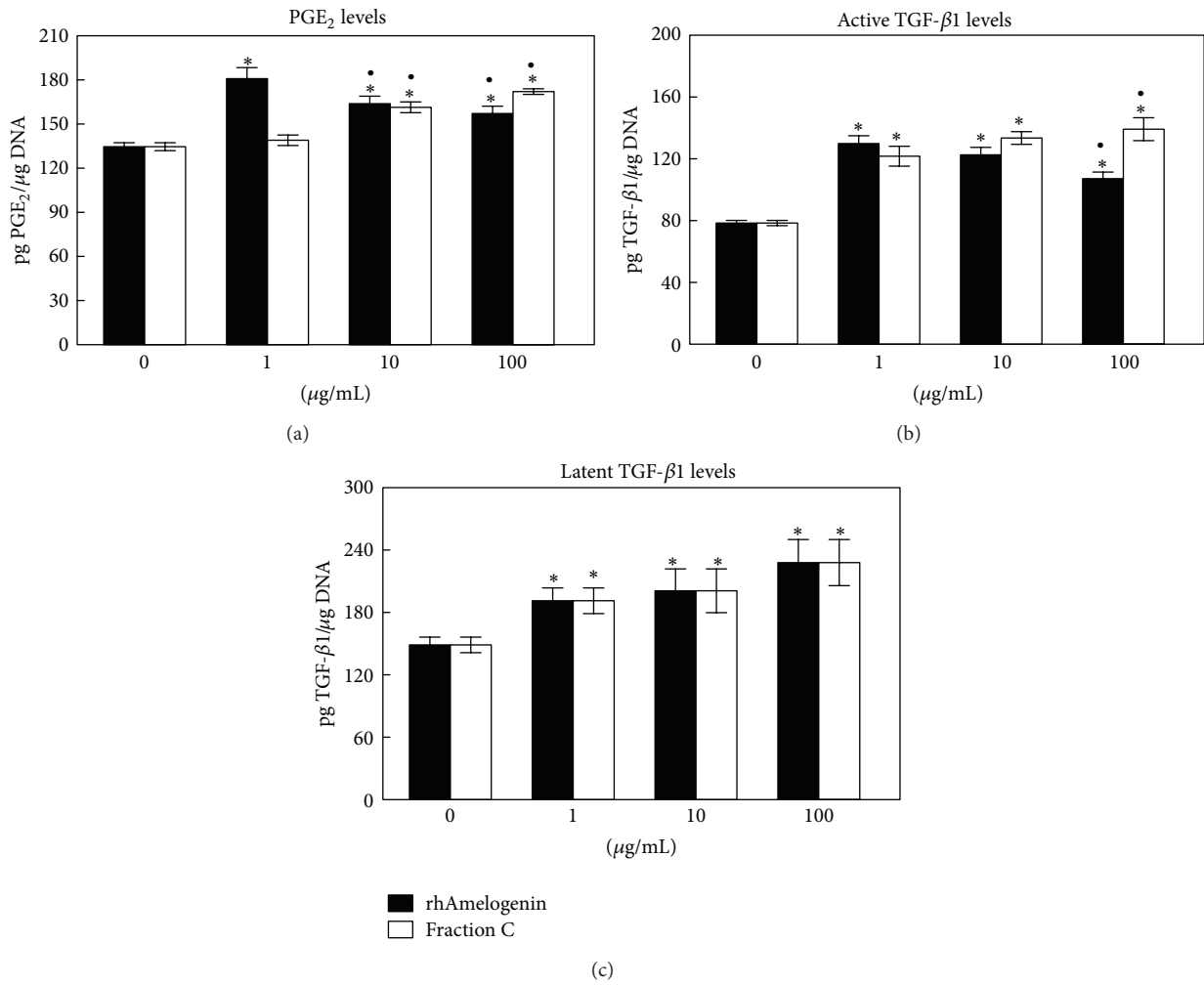
(a)

(b)

(c)

FIGURE 3: Effect of rhAmelogenin and Fraction C on growth factor production of MG63 cells. MG63 cells were grown to confluence and then treated with 0 μg/mL or 0.01 μg/mL–100 μg/mL rhAmelogenin or Fraction C for 24 hours. PGE$_2$ (a), active TGF-β1 (b), and latent TGF-β1 (c) were measured in the conditioned media. $^*P < 0.05$, versus 0 μg/mL; $^\bullet P < 0.05$, versus 1 μg/mL.
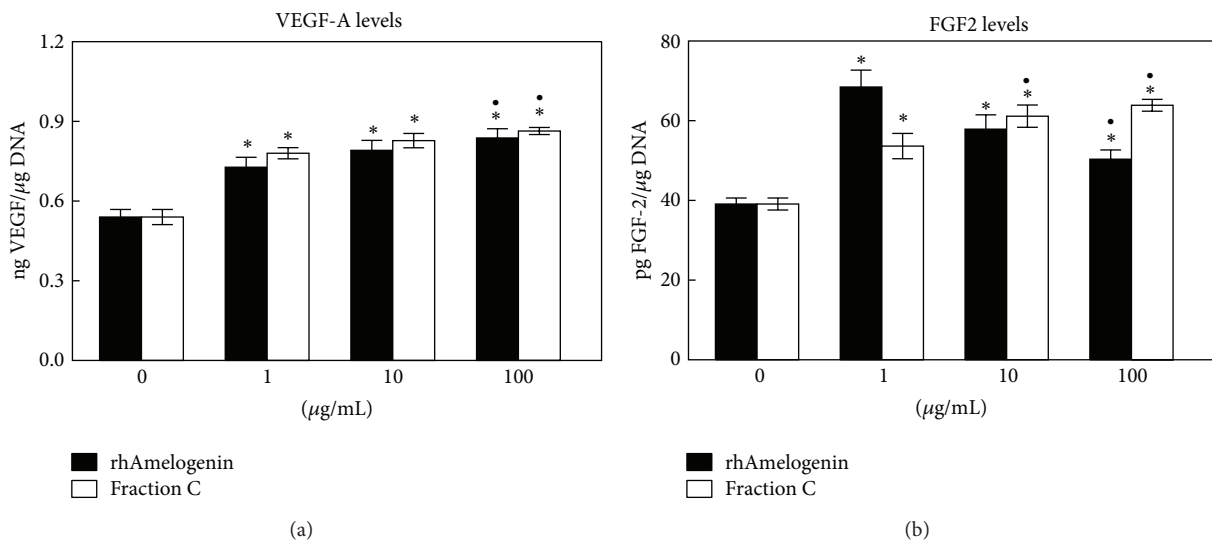

(a)

(b)

FIGURE 4: Effect of rhAmelogenin and Fraction C on angiogenic factor production of MG63 cells. MG63 cells were grown to confluence and then treated with 0 μg/mL or 1, 10, or 100 μg/mL rhAmelogenin or Fraction C for 24 hours. VEGF-A (a) and FGF-2 (b) levels were measured in the conditioned media. $^*P < 0.05$, versus 0 μg/mL; $^\bullet P < 0.05$, versus 1 μg/mL.

(a)

(b)

(c)

(d)

rhAmelogenin
Fraction C
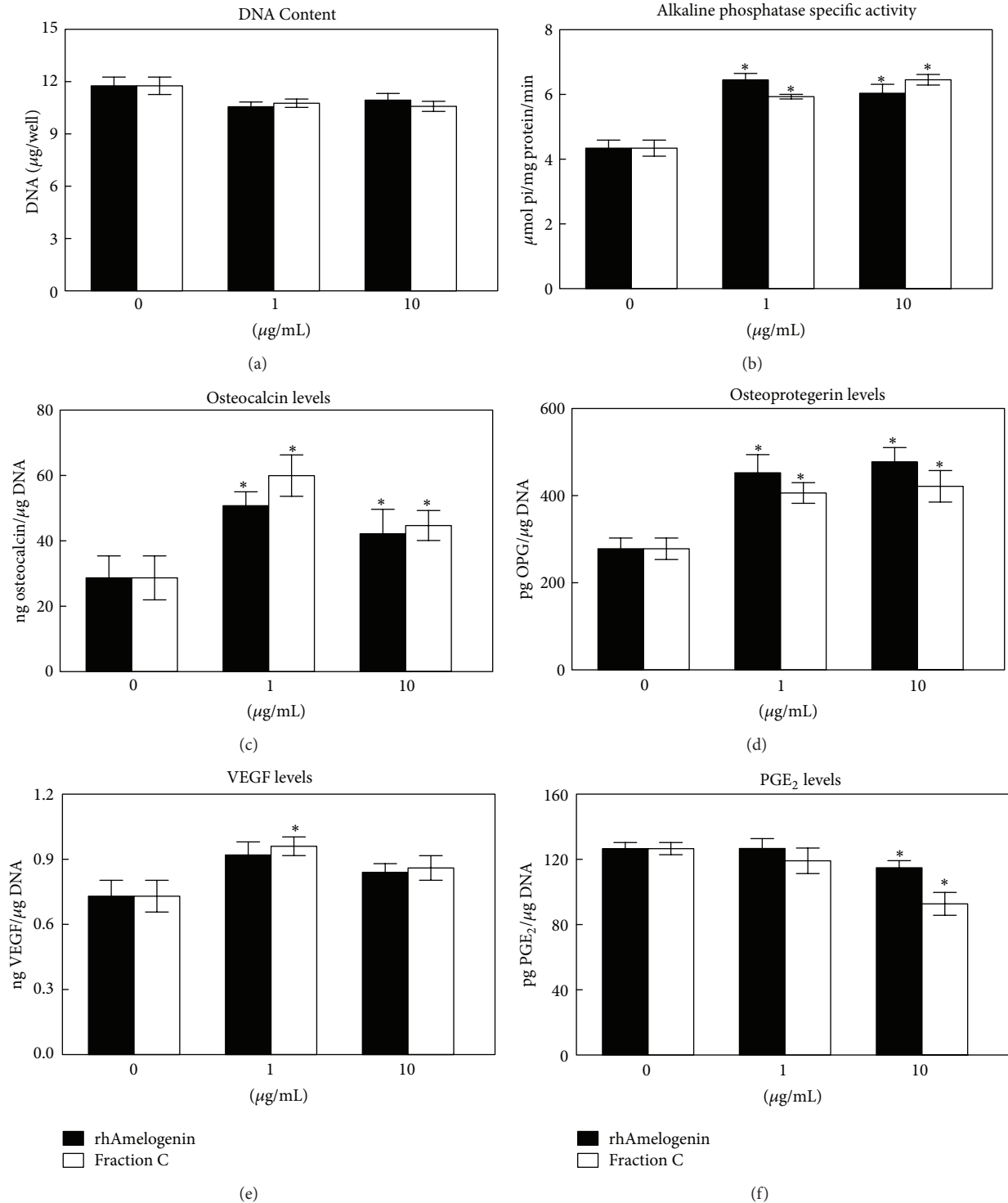
(e)

rhAmelogenin
Fraction C

(f)

FIGURE 5: Effect of rhAmelogenin and Fraction C on DNA content and osteoblast phenotype of human osteoblasts. Human osteoblasts were grown to confluence and then treated with 0, 1, or 10 μg/mL rhAmelogenin or Fraction C for 24 hours. DNA content (a) and alkaline phosphatase-specific activity (b) were measured in the cell lysate, and osteocalcin (c), osteoprotegerin (d), VEGF-A (e), and PGE$_2$ (f) measured in the conditioned media. $^*P < 0.05$, versus 0 μg/mL; $^\bullet P < 0.05$, versus 1 μg/mL.
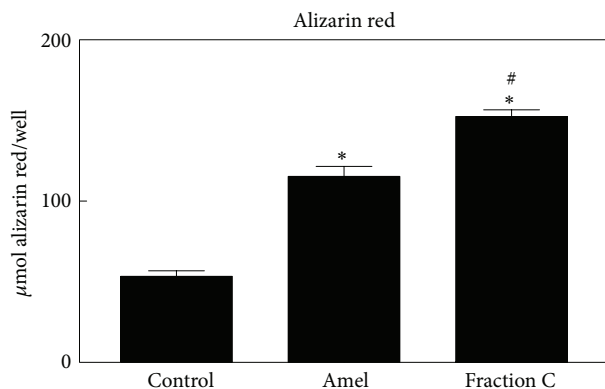
FIGURE 6: Effect of rhAmelogenin and Fraction C on human osteoblast mineralization. Human osteoblasts were cultured in basal media supplemented with $1\,\mu g$/mL rhAmelogenin (Amel) or Fraction C for 14 days and Alizarin red staining examined. $^{*}P < 0.05$, versus control; $^{\#}P < 0.05$, versus Amel.

significantly more Alizarin red staining than rhAmelogenin treatment (Figure 6).

3.3. Effect on MG63 Cell Apoptosis.

The possible protective effects of rhAmelogenin and Fraction C on osteoblast apoptosis were determined in cells pretreated with the apoptogen chelerythrine. Treatment with chelerythrine decreased MTT absorbance (Figure 7(a)). However, this decrease was less robust in cells pretreated with rhAmelogenin or Fraction C. DNA fragmentation was higher in chelerythrine-treated cells than in control cells (Figure 7(b)). Pretreatment with rhAmelogenin reduced this effect, while cells pretreated with Fraction C were not different from control. While chelerythrine increased caspase-3 activity, pretreatment with rhAmelogenin or Fraction C decreased the effect by 33% (Figure 7(c)). Chelerythrine induced a 100% increase in control cells, but pretreatment with rhAmelogenin or Fraction C blocked this effect (Figure 7(d)).

4. Discussion

The results of this study demonstrate that individual components of decellularized matrices can contribute to overall tissue regeneration by acting on cells involved in the formation of new tissue in a differential manner. As noted previously, EMD is processed from the unmineralized or partially mineralized enamel matrix of porcine tooth germs, resulting in a bioscaffold for wound healing and tissue regeneration in periodontal, orthopaedic, and dermatologic applications [2, 3, 45]. The major protein in EMD, amelogenin, is present at the epithelial/mesenchymal transition zone in the tooth germ [6], making it a candidate for modulating osteoblastic differentiation and osteogenesis. Our data support this hypothesis, showing that rhAmelogenin and Fraction C enhance markers of osteoblastic maturation and stimulate osteoblasts to produce mineral-like tissue *in vitro*. In addition, our results suggest that Fraction C has an antiapoptotic effect on osteoblasts.

It is not clear whether the full-length amelogenin has only a structural function or if it also activates signaling pathways that induce cell activities. It has been suggested that amelogenin may play both roles, working as a structural extracellular protein and working as an active peptide that induces cell differentiation [46] by then creating an optimal environment for osteoblast differentiation. A variety of amelogenin peptides are produced *in vivo* during tooth morphogenesis by alternative splicing and proteolytic degradation [47, 48], but the biological function of many of these peptides is not known. In contrast to the four peaks found in the original fractionation [29], here we found that Fraction C contained just two peaks, one of 43 amino acids and the other one corresponding to 45 amino acids in length, which correspond to the previously reported TRAP species [28]. The LRAP portion of amelogenin has been shown to induce osteogenesis *in vivo* [49] and osteoblast maturation *in vitro* [50, 51], but the effect of Fraction C, containing the TRAP portion, was unclear. In the current study, we provide evidence that primary human osteoblasts and osteoblast-like cells are sensitive to the Fraction C and that this active peptide increases osteoblast maturation.

Our results indicate that amelogenin and Fraction C have no effect on cell number in either MG63 cells or human osteoblasts, which is in agreement with the previously published literature [52–56]. Our results suggest that osteoblast maturation was promoted at the expense of cell proliferation. Several studies showed an increase in osteocalcin levels and alkaline phosphatase activity after recombinant human amelogenin treatment in many cell types, supporting our observations [53, 54].

Our study supports the hypothesis that the N-terminal sequence of amelogenin, also found in Fraction C as TRAP, enhances the stimulatory effects of amelogenin on osteoblastic differentiation. rhAmelogenin and Fraction C had similar effects on alkaline phosphatase-specific activity in HOB cultures and on production of osteocalcin at both treatment doses. MG63 cells were less sensitive to rhAmelogenin than to Fraction C, exhibiting biphasic increases in alkaline phosphatase-specific activity and osteocalcin levels. The higher osteogenic response to Fraction C suggests that immature osteoblast-like cells are more sensitive to shorter amelogenin isoforms and proteolytic peptides than full-length amelogenin.

Osteoblasts participate in bone formation not only by producing and mineralizing osteoid, but also by creating a suitable osteogenic microenvironment that controls osteoblastic differentiation of progenitor cells, differentiation and maturation of osteoclasts, and angiogenesis [57, 58]. The autocrine and paracrine factors of osteoprotegerin, TGF-β1, and PGE$_2$ are associated with this osteogenic environment [59–61]. OPG functions as a decoy receptor of the nuclear factor-kappa B ligand (RANKL), a member of the tumor necrosis factor superfamily that induces osteoclast activation. OPG blocks RANKL from binding to its specific receptor, protecting bone from osteoclast resorption [62]. TGF-β1 stimulates osteoblastic differentiation [63] and inhibits osteoclast activity [64], but at high concentrations it can also increase scar formation [65, 66]. Thus, the ratio of
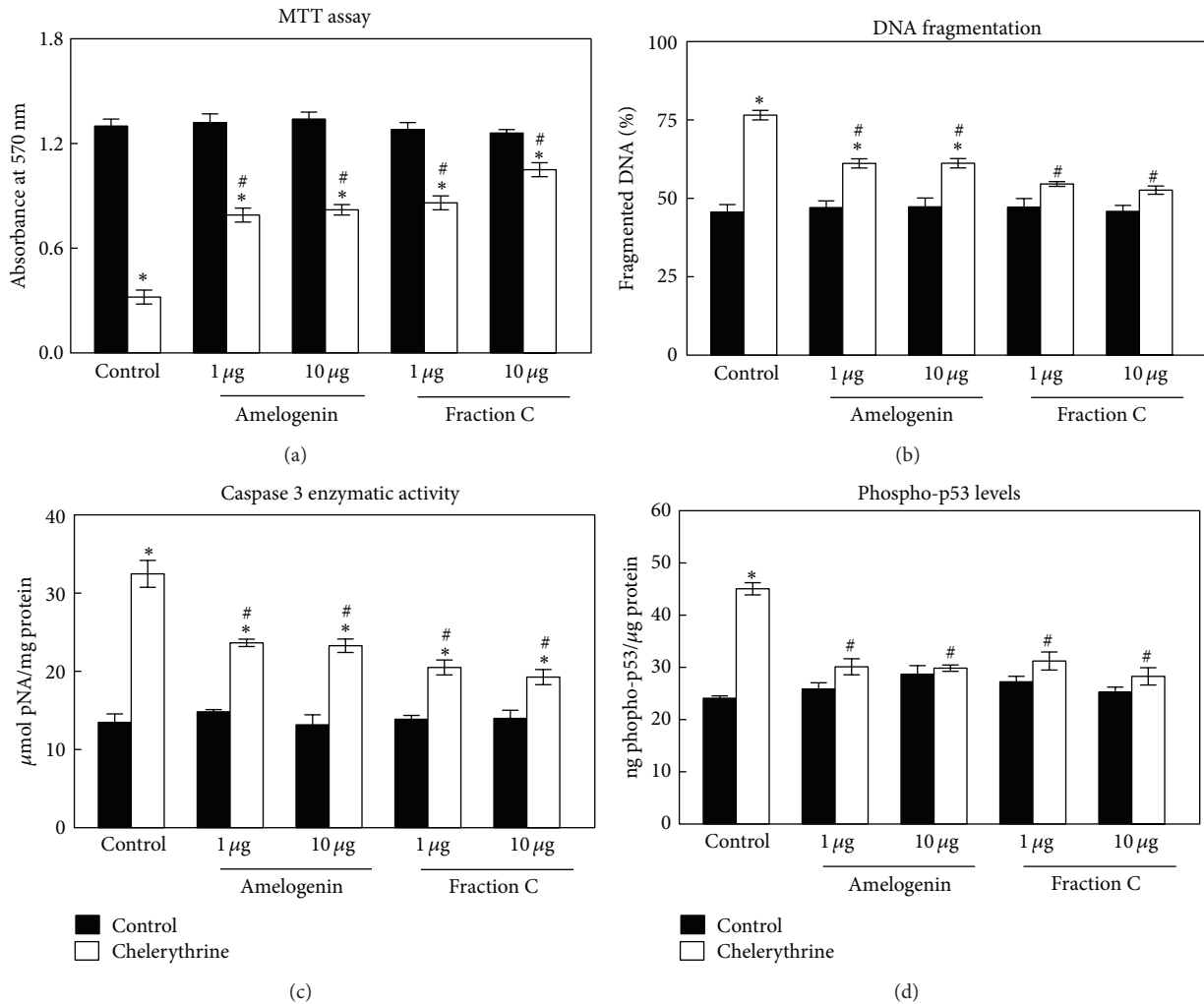
FIGURE 7: Protective effect of rhAmelogenin and Fraction C on osteoblast apoptosis. MG63 cells were grown to confluence. Cultures were pretreated for 30 minutes with 10 μM chelerythrine to induce apoptosis and then treated with rhAmelogenin or Fraction C. MTT (a), DNA fragmentation (b), caspase-3 activity (c), and phospho-p53 (d) were measured. $^*P < 0.05$, versus 0 μg/mL; $^\bullet P < 0.05$, versus 1 μg/mL.

active growth factor to latent growth factor may be an important variable. Finally, PGE_2 is required for osteoblastic differentiation [67]. Our results indicate that rhAmelogenin and Fraction C increase the levels of all three of these factors, suggesting that these molecules contribute to osteogenesis *in vivo* by their effects on local factor production by osteoblasts. Moreover, osteoblasts at different states of maturation may be more responsive to individual amelogenin isoforms and subsequent peptide formation by proteolytic degradation. This is supported by the observation that OPG levels significantly increased when MG63 cells were treated with lower doses of Fraction C in comparison to rhAmelogenin.

Our results show that the levels of both active and latent TGF-β1 in the conditioned medium were increased by rhAmelogenin and Fraction C, confirming previous studies in which treatment with rhAmelogenin increased TGF-β1 mRNA or protein levels [30, 68]. The increase in TGF-β1 may cause an indirect effect on cell proliferation, differentiation, or extracellular matrix and growth factor production

[69, 70]. EMD has been reported to have BMP- and TGF-β1-like activities [54, 71], and studies have found that EMD stimulates BMP and TGF-β signal transduction [72]. These hypotheses are also supported by studies in which antibodies to TGF-β1 inhibited the effect of EMD on epithelial cells [71]. Our results suggest the possibility that the growth factor effects being observed are due to production of these factors induced by protein constituents of EMD and their subsequent autocrine/paracrine actions. Further support of this hypothesis is the fact that the rhAmelogenin in our study was produced in *Escherichia coli* and yet had similar effects as Fraction C. Moreover, Fraction C was purified using reverse-phase, high-pressure liquid chromatography, which had a molecular weight of 5 kDa, and an amino acid composition distinct from any epitope of TGF-β1.

Angiogenesis, sprouting of new blood vessels, is a crucial step in bone formation and regeneration. Among the factors involved in the new vessel formation, VEGF-A and FGF-2 are two of the factors necessary to initiate angiogenesis

and recruit endothelial cells [73]. VEGF-A and FGF-2 are present in and regulate the formation of enamel and dentin [74, 75]. However, the effects of amelogenin on angiogenesis or in the regulation of these growth factors are unclear. Our results showed an increase in VEGF-A and FGF-2 levels in the conditioned media of MG63 cells after 24-hour treatment with rhAmelogenin or Fraction C. However, in normal human osteoblasts only the lowest dose of Fraction C increased VEGF-A. The differences in the secretion of angiogenic factors may be attributed to the maturation state of the MG63 cells.

Osteoblasts undergo apoptosis as a normal process in terminal differentiation. Carinci et al. showed that EMD regulates expression of genes involved in cell cycle regulation, proliferation, and apoptosis using DNA microarray technology [38]. Interestingly, the authors found an upregulation in genes that induce apoptosis such as MADD and TNF-α but also genes that inhibit apoptosis and increase cell survival as MCL1. Here we found that both rhAmelogenin and Fraction C were able to inhibit chelerythrine-induced apoptosis. It is possible that the net bone formation seen because of clinical EMD application is due to a delay in osteoblast apoptosis, allowing continued matrix mineralization by osteoblasts.

Our results demonstrate that both MG63 osteoblast-like cells and primary human osteoblasts are sensitive to Fraction C. In the more homogeneous MG63 cells, the effect of the peptides is dependent on dose. In committed human osteoblasts, osteogenic markers and local factors increase after treatment regardless of dose, suggesting differential roles of EMD ECM components on periodontal regeneration. The results of this study indicate that Fraction C induces an angiogenic and osteogenic environment that may be responsible for the effects of EMD on periodontal regeneration and ulcer healing. Taken together, the results suggest that Fraction C could be a suitable candidate peptide for tissue engineering applications as part of scaffold or as release peptide due to the stronger osteogenic effect and apoptosis inhibition on osteoblastic cells.

Conflict of Interests

Corinna Mauth and Anja C. Gemperli were employees of Institut Straumann AG at the time this study was performed. The other authors have no conflicts to disclose.

Acknowledgments

References

[1] C. D. R. Kalpidis and M. P. Ruben, "Treatment of intrabony periodontal defects with enamel matrix derivative: a literature review," *Journal of Periodontology*, vol. 73, no. 11, pp. 1360–1376, 2002.

[2] E. Venezia, M. Goldstein, B. D. Boyan, and Z. Schwartz, "The use of enamel matrix derivative in the treatment of periodontal defects: a literature review and meta-analysis," *Critical Reviews in Oral Biology and Medicine*, vol. 15, no. 6, pp. 382–402, 2004.

[3] M. Esposito, M. G. Grusovin, N. Papanikolaou, P. Coulthard, and H. V. Worthington, "Enamel matrix derivative (Emdogain(R)) for periodontal tissue regeneration in intrabony defects," *Cochrane Database of Systematic Reviews*, no. 4, Article ID CD003875, 2009.

[4] S. Gestrelius, S. P. Lyngstadaas, and L. Hammarström, "Emdogain—periodontal regeneration based on biomimicry," *Clinical oral investigations*, vol. 4, no. 2, pp. 120–125, 2000.

[5] P. D. A. Owens, "A light and electron microscopic study of the early stages of root surface formation in molar teeth in the rat," *Archives of Oral Biology*, vol. 24, no. 12, pp. 901–907, 1979.

[6] D. D. Bosshardt and A. Nanci, "Hertwig's epithelial root sheath, enamel matrix proteins, and initiation of cementogenesis in porcine teeth," *Journal of Clinical Periodontology*, vol. 31, no. 3, pp. 184–192, 2004.

[7] H. C. Slavkin, P. Bringas Jr., C. Bessem et al., "Hertwig's epithelial root sheath differentiation and initial cementum and bone formation during long-term organ culture of mouse mandibular first molars using serumless, chemically-defined medium," *Journal of Periodontal Research*, vol. 24, no. 1, pp. 28–40, 1989.

[8] A. Sculean, P. Windisch, D. Szendröi-Kiss et al., "Clinical and histologic evaluation of an enamel matrix derivative combined with a biphasic calcium phosphate for the treatment of human intrabony periodontal defects," *Journal of Periodontology*, vol. 79, no. 10, pp. 1991–1999, 2008.

[9] U. Mirastschijski, D. Konrad, E. Lundberg, S. P. Lyngstadaas, L. N. Jorgensen, and M. S. Ågren, "Effects of a topical enamel matrix derivative on skin wound healing," *Wound Repair and Regeneration*, vol. 12, no. 1, pp. 100–108, 2004.

[10] J. D. Termine, A. B. Belcourt, and P. J. Christner, "Properties of dissociatively extracted fetal tooth matrix proteins. I. Principal molecular species in developing bovine enamel," *Journal of Biological Chemistry*, vol. 255, no. 20, pp. 9760–9768, 1980.

[11] P. Chadwick and C. Acton, "The use of amelogenin protein in the treatment of hard-to-heal wounds," *British Journal of Nursing*, vol. 18, no. 6, pp. S22–S24, 2009.

[12] M. Romanelli, E. Kaha, H. Stege et al., "Effect of amelogenin extracellular matrix protein and compression on hard-to-heal venous leg ulcers: follow-up data," *Journal of Wound Care*, vol. 17, no. 1, pp. 17–23, 2008.

[13] S. Delgado, M. Girondot, and J.-Y. Sire, "Molecular evolution of amelogenin in mammals," *Journal of Molecular Evolution*, vol. 60, no. 1, pp. 12–30, 2005.

[14] M. Zeichner-David, "Is there more to enamel matrix proteins than biomineralization?" *Matrix Biology*, vol. 20, no. 5-6, pp. 307–316, 2001.

[15] P. H. Krebsbach, S. K. Lee, Y. Matsuki, C. A. Kozak, K. M. Yamada, and Y. Yamada, "Full-length sequence, localization, and chromosomal mapping of ameloblastin: a novel tooth-specific gene," *Journal of Biological Chemistry*, vol. 271, no. 8, pp. 4431–4435, 1996.

[16] C.-C. Hu, M. Fukae, T. Uchida et al., "Cloning and characterization of porcine enamelin mRNAs," *Journal of Dental Research*, vol. 76, no. 11, pp. 1720–1729, 1997.

[17] A. G. Fincham, J. Moradian-Oldak, and J. P. Simmer, "The structural biology of the developing dental enamel matrix," *Journal of Structural Biology*, vol. 126, no. 3, pp. 270–299, 1999.

[18] H. B. Wen, A. G. Fincham, and J. Moradian-Oldak, "Progressive accretion of amelogenin molecules during nanospheres assembly revealed by atomic force microscopy," *Matrix Biology*, vol. 20, no. 5-6, pp. 387–395, 2001.

[19] J. Moradian-Oldak, J. Tan, and A. G. Fincham, "Interaction of amelogenin with hydroxyapatite crystals: an adherence effect through amelogenin molecular self-association," *Biopolymers*, vol. 46, no. 4, pp. 225–238, 1998.

[20] H. B. Wen, J. Moradian-Oldak, W. Leung, P. Bringas Jr., and A. G. Fincham, "Microstructures of an amelogenin gel matrix," *Journal of Structural Biology*, vol. 126, no. 1, pp. 42–51, 1999.

[21] S. J. Brookes, J. Kirkham, R. C. Shore, S. R. Wood, I. Slaby, and C. Robinson, "Amelin extracellular processing and aggregation during rat incisor amelogenesis," *Archives of Oral Biology*, vol. 46, no. 3, pp. 201–208, 2001.

[22] A. Veis, "Amelogenin gene splice products: potential signaling molecules," *Cellular and Molecular Life Sciences*, vol. 60, no. 1, pp. 38–55, 2003.

[23] A. Veis, K. Tompkins, K. Alvares et al., "Specific amelogenin gene splice products have signaling effects on cells in culture and in implants in vivo," *Journal of Biological Chemistry*, vol. 275, no. 52, pp. 41263–41272, 2000.

[24] K. Tompkins, K. Alvares, A. George, and A. Veis, "Two related low molecular mass polypeptide isoforms of amelogenin have distinct activities in mouse tooth germ differentiation in vitro," *Journal of Bone and Mineral Research*, vol. 20, no. 2, pp. 341–349, 2005.

[25] R. E. Grayson, Y. Yamakoshi, E. J. Wood, and M. S. Ågren, "The effect of the amelogenin fraction of enamel matrix proteins on fibroblast-mediated collagen matrix reorganization," *Biomaterials*, vol. 27, no. 15, pp. 2926–2933, 2006.

[26] S. Lacerda-Pinheiro, N. Jegat, D. Septier et al., "Early in vivo and in vitro effects of amelogenin gene splice products on pulp cells," *European Journal of Oral Sciences*, vol. 114, supplement 1, pp. 232–238, 2006.

[27] M. Zeichner-David, L.-S. Chen, Z. Hsu, J. Reyna, J. Caton, and P. Bringas, "Amelogenin and ameloblastin show growth-factor like activity in periodontal ligament cells," *European Journal of Oral Sciences*, vol. 114, supplement 1, pp. 244–253, 2006.

[28] A. G. Fincham and J. Moradian-Oldak, "Amelogenin post-translational modifications: carboxy-terminal processing and the phosphorylation of bovine and porcine "TRAP" and "LRAP" amelogenins," *Biochemical and Biophysical Research Communications*, vol. 197, no. 1, pp. 248–255, 1993.

[29] A. Mumulidu, B. Hildebrand, B. Fabi et al., "Purification and analysis of a 5 kDa component of enamel matrix derivative," *Journal of Chromatography B*, vol. 857, no. 2, pp. 210–218, 2007.

[30] Z. Schwartz, D. L. Carnes Jr., R. Pulliam et al., "Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 219 cells, inhibits proliferation and stimulates differentiation of osteoblast-like MG63 cells, and increases proliferation and differentiation of normal human osteoblast NHOst cells," *Journal of Periodontology*, vol. 71, no. 8, pp. 1287–1296, 2000.

[31] J. C. Rincon, Y. Xiao, W. G. Young, and P. M. Bartold, "Enhanced proliferation, attachment and osteopontin expression by porcine periodontal cells exposed to Emdogain," *Archives of Oral Biology*, vol. 50, no. 12, pp. 1047–1054, 2005.

[32] S. Hägewald, N. Pischon, P. Jawor, J.-P. Bernimoulin, and B. Zimmermann, "Effects of enamel matrix derivative on proliferation and differentiation of primary osteoblasts," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*, vol. 98, no. 2, pp. 243–249, 2004.

[33] J. van den Dolder, A. P. G. Vloon, and J. A. Jansen, "The effect of Emdogain on the growth and differentiation of rat bone marrow cells," *Journal of Periodontal Research*, vol. 41, no. 5, pp. 471–476, 2006.

[34] D. R. Davenport, J. M. Mailhot, J. C. Wataha, M. A. Billman, M. M. Sharawy, and M. K. Shrout, "Effects of enamel matrix protein application on the viability, proliferation, and attachment of human periodontal ligament fibroblasts to diseased root surfaces in vitro," *Journal of Clinical Periodontology*, vol. 30, no. 2, pp. 125–131, 2003.

[35] R. J. Miron, D. D. Bosshardt, E. Hedbom et al., "Adsorption of enamel matrix proteins to a bovine-derived bone grafting material and its regulation of cell adhesion, proliferation, and differentiation," *Journal of Periodontology*, vol. 83, no. 7, pp. 936–947, 2011.

[36] H. M. Grandin, A. C. Gemperli, and M. Dard, "Enamel matrix derivative: a review of cellular effects in vitro and a model of molecular arrangement and functioning," *Tissue Engineering B*, vol. 18, no. 3, pp. 181–202, 2012.

[37] K. Bertl, N. An, C. Bruckmann et al., "Effects of enamel matrix derivative on proliferation/viability, migration, and expression of angiogenic factor and adhesion molecules in endothelial cells in vitro," *Journal of Periodontology*, vol. 80, no. 10, pp. 1622–1630, 2009.

[38] F. Carinci, A. Piattelli, L. Guida et al., "Effects of Emdogain on osteoblast gene expression," *Oral Diseases*, vol. 12, no. 3, pp. 329–342, 2006.

[39] S. Gestrelius, C. Andersson, D. Lidström, L. Hammarström, and M. Somerman, "In vitro studies on periodontal ligament cells and enamel matrix derivative," *Journal of Clinical Periodontology*, vol. 24, no. 9, pp. 685–692, 1997.

[40] Z. Schwartz, R. Dennis, L. Bonewald, L. Swain, R. Gomez, and B. D. Boyan, "Differential regulation of prostaglandin E2 synthesis and phospholipase A2 activity by 1,25-(OH)2D3 in three osteoblast-like cell lines (MC-3T3- E1, ROS 17/2.8, and MG-63)," *Bone*, vol. 13, no. 1, pp. 51–58, 1992.

[41] J. Y. Martin, D. D. Dean, D. L. Cochran, J. Simpson, B. D. Boyan, and Z. Schwartz, "Proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63) cultured on previously used titanium surfaces," *Clinical Oral Implants Research*, vol. 7, no. 1, pp. 27–37, 1996.

[42] C. A. Gregory, W. G. Gunn, A. Peister, and D. J. Prockop, "An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction," *Analytical Biochemistry*, vol. 329, no. 1, pp. 77–84, 2004.

[43] S. Yamamoto, K. Seta, C. Morisco, S. F. Vatner, and J. Sadoshima, "Chelerythrine rapidly induces apoptosis through generation of reactive oxygen species in cardiac myocytes," *Journal of Molecular and Cellular Cardiology*, vol. 33, no. 10, pp. 1829–1848, 2001.

[44] M. Zhong, L. J. Wike, J. T. Ryaby, D. H. Carney, B. D. Boyan, and Z. Schwartz, "Thrombin peptide TP508 prevents nitric oxide mediated apoptosis in chondrocytes in the endochondral developmental pathway," *Biochimica et Biophysica Acta*, vol. 1783, no. 1, pp. 12–22, 2008.

[45] J. F. Guest, E. Nagy, E. Sladkevicius, P. Vowden, and P. Price, "Modelling the relative cost-effectiveness of amelogenin in non-healing venous leg ulcers," *Journal of Wound Care*, vol. 18, no. 5, pp. 216–224, 2009.

[46] D. D. Dean, C. H. Lohmann, V. L. Sylvia et al., "Effect of porcine fetal enamel matrix derivative on chondrocyte proliferation, differentiation, and local factor production is dependent on cell maturation state," *Cells Tissues Organs*, vol. 171, no. 2-3, pp. 117–127, 2002.

[47] J. P. Simmer and J. C.-C. Hu, "Expression, structure, and function of enamel proteinases," *Connective Tissue Research*, vol. 43, no. 2-3, pp. 441–449, 2002.

[48] C. Robinson, S. J. Brookes, R. C. Shore, and J. Kirkham, "The developing enamel matrix: nature and function," *European Journal of Oral Sciences*, vol. 106, supplement 1, pp. 282–291, 1998.

[49] C. W. Gibson, Y. Li, B. Daly et al., "The leucine-rich amelogenin peptide alters the amelogenin null enamel phenotype," *Cells Tissues Organs*, vol. 189, no. 1–4, pp. 169–174, 2009.

[50] R. Warotayanont, D. Zhu, M. L. Snead, and Y. Zhou, "Leucine-rich amelogenin peptide induces osteogenesis in mouse embryonic stem cells," *Biochemical and Biophysical Research Communications*, vol. 367, no. 1, pp. 1–6, 2008.

[51] R. Warotayanont, B. Frenkel, M. L. Snead, and Y. Zhou, "Leucine-rich amelogenin peptide induces osteogenesis by activation of the Wnt pathway," *Biochemical and Biophysical Research Communications*, vol. 387, no. 3, pp. 558–563, 2009.

[52] F. Boabaid, C. W. Gibson, M. A. Kuehl et al., "Leucine-rich amelogenin peptide: a candidate signaling molecule during cementogenesis," *Journal of Periodontology*, vol. 75, no. 8, pp. 1126–1136, 2004.

[53] H. L. Viswanathan, J. E. Berry, B. L. Foster et al., "Amelogenin: a potential regulator of cementum-associated genes," *Journal of Periodontology*, vol. 74, no. 10, pp. 1423–1431, 2003.

[54] T. Iwata, Y. Morotome, T. Tanabe, M. Fukae, I. Ishikawa, and S. Oida, "Noggin blocks osteoinductive activity of porcine enamel extracts," *Journal of Dental Research*, vol. 81, no. 6, pp. 387–391, 2002.

[55] E. C. Swanson, H. K. Fong, B. L. Foster et al., "Amelogenins regulate expression of genes associated with cementoblasts in vitro," *European Journal of Oral Sciences*, vol. 114, supplement 1, pp. 239–243, 2006.

[56] A. Gurpinar, M. A. Onur, Z. C. Cehreli, and F. Tasman, "Effect of enamel matrix derivative on mouse fibroblasts and marrow stromal osteoblasts," *Journal of Biomaterials Applications*, vol. 18, no. 1, pp. 25–33, 2003.

[57] A. Javed, H. Chen, and F. Y. Ghori, "Genetic and transcriptional control of bone formation," *Oral and Maxillofacial Surgery Clinics of North America*, vol. 22, no. 3, pp. 283–293, 2010.

[58] L. J. Raggatt and N. C. Partridge, "Cellular and molecular mechanisms of bone remodeling," *Journal of Biological Chemistry*, vol. 285, no. 33, pp. 25103–25108, 2010.

[59] L. F. Bonewald and S. L. Dallas, "Role of active and latent transforming growth Factor,B in bone formation," *Journal of Cellular Biochemistry*, vol. 55, no. 3, pp. 350–357, 1994.

[60] X. Li, C. C. Pilbeam, L. Pan, R. M. Breyer, and L. G. Raisz, "Effects of prostaglandin E2 on gene expression in primary osteoblastic cells from prostaglandin receptor knockout mice," *Bone*, vol. 30, no. 4, pp. 567–573, 2002.

[61] Y. Kobayashi, N. Udagawa, and N. Takahashi, "Action of RANKL and OPG for osteoclastogenesis," *Critical Reviews in Eukaryotic Gene Expression*, vol. 19, no. 1, pp. 61–72, 2009.

[62] B. F. Boyce and L. Xing, "Functions of RANKL/RANK/OPG in bone modeling and remodeling," *Archives of Biochemistry and Biophysics*, vol. 473, no. 2, pp. 139–146, 2008.

[63] R. A. Kanaan and L. A. Kanaan, "Transforming growth factor β1, bone connection," *Medical Science Monitor*, vol. 12, no. 8, pp. RA164–RA169, 2006.

[64] K. Janssens, P. Ten Dijke, S. Janssens, and W. van Hul, "Transforming growth factor-β1 to the bone," *Endocrine Reviews*, vol. 26, no. 6, pp. 743–774, 2005.

[65] N. Chegini, "The role of growth factors in peritoneal healing: transforming growth factor beta (TGF-beta)," *The European Journal of Surgery*, no. 577, pp. 17–23, 1997.

[66] K. R. Cutroneo, "TGF-β-induced fibrosis and SMAD signaling: oligo decoys as natural therapeutics for inhibition of tissue fibrosis and scarring," *Wound Repair and Regeneration*, vol. 15, supplement 1, pp. S54–S60, 2007.

[67] H. Kawaguchi, C. C. Pilbeam, J. R. Harrison, and L. G. Raisz, "The role of prostaglandins in the regulation of bone metabolism," *Clinical Orthopaedics and Related Research*, no. 313, pp. 36–46, 1995.

[68] S. Yoneda, D. Itoh, S. Kuroda et al., "The effects of enamel matrix derivative (EMD) on osteoblastic cells in culture and bone regeneration in a rat skull defect," *Journal of Periodontal Research*, vol. 38, no. 3, pp. 333–342, 2003.

[69] N. H. M. Heng, P. D. N'Guessan, B.-M. Kleber, J.-P. Bernimoulin, and N. Pischon, "Enamel matrix derivative induces connective tissue growth factor expression in human osteoblastic cells," *Journal of Periodontology*, vol. 78, no. 12, pp. 2369–2379, 2007.

[70] E. Shimizu, R. Saito, Y. Nakayama et al., "Amelogenin stimulates bone sialoprotein (BSP) expression through fibroblast growth factor 2 response element and transforming growth factor-β1 activation element in the promoter of the BSP gene," *Journal of Periodontology*, vol. 76, no. 9, pp. 1482–1489, 2005.

[71] T. Kawase, K. Okuda, H. Yoshie, and D. M. Burns, "Anti-TGF-β antibody blocks enamel matrix derivative-induced upregulation of p21WAF1/cip1 and prevents its inhibition of human oral epithelial cell proliferation," *Journal of Periodontal Research*, vol. 37, no. 4, pp. 255–262, 2002.

[72] S. Suzuki, T. Nagano, Y. Yamakoshi et al., "Enamel matrix derivative gel stimulates signal transduction of BMP and TGF-β," *Journal of Dental Research*, vol. 84, no. 6, pp. 510–514, 2005.

[73] M. M. L. Deckers, M. Karperien, C. van der Bent, T. Yamashita, S. E. Papapoulos, and C. W. G. M. Löwik, "Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation," *Endocrinology*, vol. 141, no. 5, pp. 1667–1674, 2000.

[74] M. Aida, T. Irié, T. Aida, and T. Tachikawa, "Expression of protein kinases C βI, βII, and VEGF during the differentiation of enamel epithelium in tooth development," *Journal of Dental Research*, vol. 84, no. 3, pp. 234–239, 2005.

[75] T. Tsuboi, S. Mizutani, M. Nakano, K. Hirukawa, and A. Togari, "FGF-2 regulates enamel and dentine formation in mouse tooth germ," *Calcified Tissue International*, vol. 73, no. 5, pp. 496–501, 2003.