Gene array of primary human osteoblasts exposed to enamel matrix derivative in combination with a natural bone mineral

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Abstract

Objectives The application of an enamel matrix derivative (EMD) for regenerative periodontal surgery has been shown to promote formation of new cementum, periodontal ligament, and alveolar bone. In intrabony defects with a complicated anatomy, the combination of EMD with various bone grafting materials has resulted in additional clinical improvements, but the initial cellular response of osteoblasts coming in contact with these particles have not yet been fully elucidated. The objective of the present study was to evaluate the in vitro effects of EMD combined with a natural bone mineral (NBM) on a wide variety of genes, cytokines, and transcription factors and extracellular matrix proteins on primary human osteoblasts.

Material and methods Primary human osteoblasts were seeded on NBM particles pre-coated with versus without EMD and analyzed for gene differences using a human osteogenesis gene super-array (Applied Biosystems). Osteoblast-related genes include those transcribed during bone mineralization, ossification, bone metabolism, cell growth and differentiation, as well as gene products representing extracellular matrix molecules, transcription factors, and cell adhesion molecules.

Results EMD promoted gene expression of various osteoblast differentiation markers including a number of collagen types and isoforms, SMAD intracellular proteins, osteopontin, cadherin, alkaline phosphatase, and bone sialoprotein. EMD also upregulated a variety of growth factors including bone morphogenetic proteins, vascular endothelial growth factors, insulin-like growth factor, transforming growth factor, and their associated receptor proteins.

Conclusion The results from the present study demonstrate that EMD is capable of activating a wide variety of genes, growth factors, and cytokines when pre-coated onto NBM particles.

Clinical relevance The described in vitro effects of EMD on human primary osteoblasts provide further biologic support for the clinical application of a combination of EMD with NBM particles in periodontal and oral regenerative surgery.

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Introduction

Enamel matrix derivative (EMD) has been shown to promote periodontal regeneration by inducing formation of cementum, periodontal ligament (PDL), and alveolar bone which is clinically evidenced by probing depth reduction, attachment gain, and radiographic defect fill [1–3]. The major components of EMD are amelogenins, a family of hydrophobic proteins derived from different splice variants

and controlled by post-secretory processing from a single gene that account for more than 95 % of the total protein content [4]. These proteins self-assemble into supramolecular aggregates that form an insoluble extracellular matrix that function to control the ultrastructural organization of the developing enamel crystallites [4]. Other proteins found in the enamel matrix include enamelin, ameloblastin (also called amelin or sheathlin), amelotin, apin, and various proteinases [5, 6]. The rationale for the clinical use of EMD is the observation that enamel matrix proteins are deposited onto the surface of developing tooth roots prior to cementum formation [7].

Although histological studies in animals and humans have provided evidence for periodontal regeneration and substantial clinical improvements following the use of EMD, concerns have been expressed regarding the viscous nature of EMD, which may not be sufficient to prevent flap collapse in periodontal defects with a complicated anatomy [8, 9]. In order to overcome this potential limitation and improve the clinical outcomes, various combinations of EMD and different types of grafting materials have been used [1–3, 10–16]. The combination of EMD with a natural bone mineral of bovine origin (NBM) has provided additional periodontal regeneration and substantial clinical improvements when compared to either NBM alone or EMD alone [1, 3, 10–14]. Recently, we have demonstrated that the combination of EMD with NBM particles enhances osteoblast and PDL cell proliferation and differentiation in vitro [17]. The aim of the present study was to investigate more deeply the initial behavior of primary human osteoblasts exposed to this combination by assessing a wide variety of osteoblast cytokines, growth factors, differentiation markers, and extracellular matrix molecules using a commercially available super-array.

Materials and methods

Surface coating with EMD

EMD was prepared according to Institut Straumann AG standard operating protocols as previously discussed [18]. Thirty milligrams of EMD was dissolved in 3 ml of sterile 0.1 % acetic acid at 4 °C. For experiments, stock EMD was diluted 100× in 0.1 M carbonate buffer at 4 °C to a working concentration of 100 μg/ml in order to maintain physiological pH. One milliliter of EMD solution was poured onto 100 mg of NBM particles (BioOss, Geistlich Pharma AG, Wolhusen, Switzerland) in 24-well culture dishes and incubated overnight at 4 °C. Following incubation, dishes were rinsed twice with 1 ml phosphate buffered saline twice.

Human primary osteoblast isolation and differentiation

Human bone chips from a single donor were cultured according to an explant model [19] under a protocol approved by the Ethics Committee, Katon Bern, Switzerland as previously described [20]. Primary human osteoblasts were detached from the tissue culture plastic using trypsin solution (Invitrogen, Basel, Switzerland). Cells used for experimental seeding were from passages 4–6. Osteoblasts were seeded at a density of 50,000 cells in 24-well culture plates (Falcon) for experimental seeding.

Super-array of osteogenic potential

The initial expression of osteoblast-related genes was examined after culture of cells for 24 h. Total RNA was isolated using TRIZOL reagent and RNAeasy Mini kit (QIAGEN, Basel, Switzerland). A TaqMan® Human Osteogenesis 96-well Plate Super-array (4414096, Applied Biosystems, Rotkreuz, Switzerland) was employed for the analysis. Osteoblast-related genes include those transcribed during bone mineralization, ossification, bone metabolism, cell growth, and differentiation. The gene products represent extracellular matrix molecules, transcription factors, and cell adhesion molecules among others. Real-time RT-PCR was performed according to manufacturer's protocol using 20 μl final reaction volume of TaqMan®'s One step Master Mix kit (Applied Biosystems) as previously described [21]. RNA quantification was performed using a Nanodrop 2000c (Thermo Scientific, Waltham, MA, USA) and 100 ng of total RNA was used per sample well. Gene fold increase represent data from NBM particles pre-coated with EMD versus NBM particles that were left uncoated.

Statistical analysis

Gene array analysis was performed for both control ($n=4$) and experimental groups ($n=4$). Means and standard deviations (SE) were calculated, and the statistical significance of differences among each group were examined by student t test between both groups (*, p values<0.05).

Results

Osteoblast regulation of transcription factors and differentiation parameters

Analysis of gene array data revealed an increase of osteoblast differentiation genes across a wide variety of osteoblast differentiation markers and secreted proteins (Table 1). Specifically, many collagen types and isoforms were upregulated including collagen type I alpha 2 (3.4772 fold,

Table 1 Gene fold increase in osteoblast differentiation markers, transcription factors, and extracellular matrix proteins

GenBank	Name	Fold change	SE	p value
SPARC-Hs00277762_m1	Secreted protein, acidic, cysteine-rich (osteonectin)	5.1523	0.4429	0.0026
SPP1-Hs00959010_m1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	4.1211	0.9317	0.0441
SMAD2-Hs00183425_m1	SMAD family member 2	3.6036	0.3579	0.0054
COL1A2-Hs00164099_m1	Collagen, type I, alpha 2	3.4772	0.3908	0.0079
SMAD3-Hs00232222_m1	SMAD family member 3	3.4075	0.3000	0.0040
COL5A1-Hs00609088_m1	Collagen, type V, alpha 1	3.1376	1.0815	0.1425
COL3A1-Hs00164103_m1	Collagen, type III, alpha 1 (Ehlers–Danlos syndrome type IV, autosomal dominant)	3.0964	0.5120	0.0263
CDH11-Hs00156438_m1	Cadherin 11, type 2, OB-cadherin (osteoblast)	3.0693	0.6402	0.0456
IBSP-Hs00173720_m1	Integrin-binding sialoprotein (bone sialoprotein, bone sialoprotein II)	2.5758	0.3907	0.0274
COL1A1-Hs00164004_m1	Collagen, type I, alpha 1	2.5394	0.4282	0.0369
COL12A1-Hs00189184_m1	Collagen, type XII, alpha 1	2.5253	0.3229	0.0180
ALPL-Hs00758162_m1	Alkaline phosphatase, liver/bone/kidney	2.4090	0.1523	0.0027
SMAD5-Hs00195437_m1	SMAD family member 5	2.0202	0.2773	0.0348
SMAD4-Hs00232068_m1	SMAD family member 4	1.9375	0.3029	0.0525
MINPP1-Hs00245149_m1	Multiple inositol polyphosphate histidine phosphatase, 1	1.7856	0.0613	0.0010
COL14A1-Hs00385388_m1	Collagen, type XIV, alpha 1	1.7729	0.2257	0.0417
TWIST1-Hs00361186_m1	Twist homolog 1 (acrocephalosyndactyly 3; Saethre–Chotzen syndrome) (*Drosophila*)	1.3874	0.3476	0.3463
ARSE-Hs00163677_m1	Arylsulfatase E (chondrodysplasia punctata 1)	1.0910	0.0624	0.2415
COL18A1-Hs00181017_m1	Collagen, type XVIII, alpha 1	0.6487	0.1004	0.0396

$p < 0.0079$), collagen type V alpha 1 (3.1376, $p < 0.1425$), collagen type 1, alpha 1 (2.5394, $p < 0.0369$), collagen type X11 alpha 1 (2.5253, $p < 0.0180$), and collagen type XIV, alpha 1 (1.7729, $p < 0.0417$). Intracellular proteins responsible for transducing extracellular signals (SMAD family) were also upregulated by EMD when compared to control uncoated samples (upregulation varied between 1.9375 and 3.6036 fold). EMD also upregulated a number of osteoblast differentiation markers including osteonectin (5.1523 fold, $p < 0.0026$), osteopontin (4.1211, $p < 0.0441$), cadherin 11 (3.0693, $p < 0.0456$), bone sialoprotein (2.5758, $p < 0.0274$), and alkaline phosphatase (2.4090, $p < 0.0027$).

Osteoblast regulation of osteoblast growth factors

An array of growth factors essential for osteoblast differentiation were also quantified using real-time RT-PCR (Table 2). In general, EMD had a positive impact on the release of bone morphogenetic proteins (BMPs) including BMP1 (6.6867 fold increase, $p < 0.0033$), BMP2 (4.2685, $p < 0.0028$), BMP6 (3.7190, $p < 0.0028$), and BMP4 (3.0751 < 0.0226). EMD also upregulated vascular endothelial growth factor A and B (VEGF-A, VEGF-B, 4.8375, $p < 0.0231$ and 2.3501, $p < 0.0104$, respectively), fibroblast growth factor 1 (FGF1, 3.4801, $p < 0.0132$), insulin-like growth factor 2 (IGF2,

Table 2 Gene fold increase in osteoblast growth factors and cytokines

GenBank	Name	Fold change	SE	p value
BMP1-Hs00241807_m1	Bone morphogenetic protein 1	6.6867	0.6575	0.0033
VEGFA-Hs00900054_m1	Vascular endothelial growth factor A	4.8375	0.8922	0.0231
BMP2-Hs00154192_m1	Bone morphogenetic protein 2	4.2685	0.3577	0.0028
BMP6-Hs00233470_m1	Bone morphogenetic protein 6	3.7190	0.2980	0.0028
FGF1-Hs00265254_m1	Fibroblast growth factor 1 (acidic)	3.4801	0.4692	0.0132
BMP4-Hs00370078_m1	Bone morphogenetic protein 4	3.0751	0.4782	0.0226
VEGFB-Hs00173634_m1	Vascular endothelial growth factor B	2.3501	0.2348	0.0104
IGF2-Hs00171254_m1	Insulin-like growth factor 2 (somatomedin A)	2.1332	0.2599	0.0223
TGFB1-Hs99999918_m1	Transforming growth factor, beta 1	1.8082	0.0965	0.0036
TGFB3-Hs00234245_m1	Transforming growth factor, beta 3	1.5939	0.1298	0.0196

2.1332, $p < 0.0223$) as well as transforming growth factor, beta 1 and 3 (TGFβ1, TGFβ3, 1.8082, $p < 0.0036$ and 1.5939, $p < 0.0196$ fold, respectively). Interestingly, EMD showed an even more pronounced effect on the receptors associated with each growth factor (Table 3). EMD increased epidermal growth factor receptor 17.0332 fold, TGFβ receptor 14.9025 fold, IGF1 receptor 6.2826 fold, BMP receptor 5.7594 fold, and FGF receptor 1 2.6171 fold (Table 3).

Discussion

The results from the present study demonstrate that EMD has the ability to enhance cytokine and growth factor gene expression as well as increase osteoblast differentiation markers and transcription factors when combined onto NBM particles. Previous in vitro research has documented the role of EMD in both osteoblasts and PDL cells in various cell culture systems [22]. EMD has a significant influence on cell adhesion, cell proliferation, and cell differentiation of many cell types by mediating cell attachment, spreading, proliferation, and survival as well as expression of transcription factors, growth factors, cytokines, extracellular matrix constituents, and other molecules involved in the regulation of bone remodeling [22].

The rational for choosing osteoblasts as a primary cell source as opposed to PDL cells was to simulate in vivo situations. Although EMD stimulates periodontal regeneration, the cells that come in contact with bone grafting particles pre-coated with EMD are typically osteoblasts as PDL cells themselves generally attach and proliferate along the root cementum. Despite primary human osteoblasts being harvested from a single donor, these conditions represent a more realistic clinical similarity when compared to other single donor cell lines derived from various species [23, 24]. Previously, many investigators have demonstrated that EMD influence osteoblast differentiation when seeded on standard tissue culture plastic using a wide variety of cell lines from various species (MG63, SaOS, MC3T3, HSC-2, mice/rat calvarial osteoblasts) [22].

In the present study, we observed that EMD enhanced many growth factors and cytokines including cadherin gene expression (Table 1). Interestingly, we have previously shown that EMD upregulates the expression of vital osteoblast cell–cell communication and adhesion molecules N-cadherin and connexin43 (intercellular gap junction channel proteins) at early time points, which enhances the differentiation and mineralization activity of osteoblasts [20]. EMD also had a pronounced effect on SMAD intercellular proteins (Table 1). The family of SMAD signal proteins is utilized by many cell types including osteoblasts to transduce extracellular signals from TGFβ from the cell membrane to the nucleus [25]. Previously, it was observed that EMD induced rapid translocation of SMAD2 into the nucleus causing an increase in cell proliferation [26, 27]. Interestingly, results from our super-array revealed upregulation of multiple SMAD proteins, with over threefold increases in SMAD2 and SMAD3 (Table 1) demonstrating a very plausible role of TGFβ for EMD-treated osteoblasts. Furthermore, TGFβ1 and TGFB receptor were increased 1.8082 and 14.9025 fold, respectively, on EMD-coated NBM particles. These results are consistent with other authors who have demonstrated that EMD increased the secretion of TGFβ1 and PDGF through intracellular cAMP [28, 29].

EMD also stimulated a variety of growth factors contributing to osteoblast maturation (Table 2). These findings are consistent with other authors who have analyzed the effects of EMD on cells grown on cell culture plastic [30–32]. Recently, it was demonstrated that the effect of EMD on cell proliferation was mediated through binding to amelogenins while the differentiation of progenitor cells was caused mainly by the release of BMPs [30]. Furthermore, it was shown that the receptors for BMPs played an important role in differentiation of PDL cells in response to mechanical stimulation and interleukin 1β [31]. In a previous in vitro gene expression assay on periodontal ligament cells treated with EMD on cell culture plastic, EMD upregulated growth factors PDGF, BMPs, TGFβ, and VEGF [32]. In this study, EMD was not only capable of

Table 3 Gene fold increase in osteoblast receptors associated with growth factors and cytokines

GenBank	Name	Fold change	SE	p value
EGFR-Hs00193306_m1	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	17.0332	4.8205	0.0449
TGFBR1-Hs00610319_m1	Transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53 kDa)	14.9025	3.7939	0.0351
IGF1R-Hs00609566_m1	Insulin-like growth factor 1 receptor	6.2826	1.6289	0.0477
BMPR1A-Hs00831730_s1	Bone morphogenetic protein receptor, type IA	5.7594	1.0529	0.0202
FGFR1-Hs00241111_m1	Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	2.6171	0.9069	0.1748
TGFBR2-Hs00559661_m1	Transforming growth factor, beta receptor II (70/80 kDa)	2.3731	0.5244	0.0791

increasing expression of osteoblast growth factors but also their respective membrane surface receptors (Table 3). The role of each of these receptors on EMD-induced proliferation and differentiation requires further investigation.

Taken together, the present study has demonstrated that the addition of EMD to NBM particles improves the initial cell response of primary human osteoblast in vitro. The results provide further evidence that EMD has an influence on secreted extracellular matrix proteins, osteoblast transcription factors, and differentiation markers as well as growth factors and their associated receptors thus supporting the clinical use of a combination of EMD with bone grafting particles.

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