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ORIGINAL ARTICLE

Gene array of PDL cells exposed to Osteogain in combination with a bone grafting material

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Abstract
Objectives The aim of the present study was to investigate the effects of Osteogain, a new formulation of enamel matrix derivative (EMD) in combination with a grafting material on a wide variety of genes for cytokines, transcription factors and extracellular matrix proteins involved in osteoblast differentiation.
Materials and methods Primary human periodontal ligament (PDL) cells were seeded on natural bone mineral (NBM) particles coated with Osteogain for 24 h and analyzed for regulated gene expression using a human osteogenesis gene superarray kit. 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 Osteogain significantly upregulated the expression of over 20 of the 100 genes examined including bone morphogenetic protein 2 (BMP2), TGFβ1, fibroblast growth factor (FGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) as well as some of their associated receptors. Osteogain also promoted gene expression of a number of osteoblast differentiation markers including collagen1α2 and alkaline phosphatase as well as cell adhesion molecules including fibronectin and a variety of integrin binding proteins. Interestingly, Osteogain promoted calcitonin receptor 55-fold and also promoted annexin A5 gene expression over 12-fold.
Conclusion The present study demonstrates that Osteogain is capable of either upregulating or downregulating the expression of a wide variety of genes including those for growth factors and cytokines when combined with a bone grafting material.
Clinical relevance The results from the present study demonstrate the large and potent effect of addition of Osteogain in combination to a bone grafting material over a wide variety of genes supporting osteogenesis.

Keywords Enamel matrix derivative · Enamel matrix proteins · Emdogain · Periodontal regeneration · Bone graft

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Introduction

Enamel matrix derivative (EMD) has been shown to promote periodontal regeneration by inducing new root cementum, periodontal ligament and alveolar bone formation as well as to speed up tissue wound healing [1–4]. The major components of EMD are amelogenins, a family of hydrophobic proteins that account for more than 95 % of the total protein content [5]. 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 [5]. Other proteins found in the enamel matrix include enamelin, ameloblastin (also called amelin or sheathlin), amelotin, apin and various proteinases [6, 7]. The rationale for the clinical use of EMD is the observation that enamel matrix proteins (EMPs) are deposited onto the surface of developing tooth roots prior to cementum formation [8].

Although histological studies in animals and humans have provided evidence for periodontal regeneration and substantial clinical improvements following the use of EMD (commercially available under the trademark name Emdogain; Straumann AG, Basel, Switzerland), concerns have been expressed regarding the viscous nature of Emdogain gel, which may not be sufficient to prevent flap collapse in periodontal defects with a complicated anatomy [9, 10]. In order to overcome this potential limitation and improve the clinical outcomes, various combinations of Emdogain and different types of grafting materials have been used [1]. The combination of Emdogain with a natural bone mineral (NBM) of bovine origin has provided increased periodontal regeneration power and substantial clinical improvements when compared to either NBM alone or Emdogain alone [1]. Recently, a new carrier system for enamel matrix derivative was designed to improve protein adsorption of enamel matrix proteins to grafting materials [11–13]. In a pioneering study, it was found that more proteins are adsorbed on the material surface when EMD was delivered in liquid formulation when compared to the gel formulation found in Emdogain [11]. Advantages also included better surface coating of the protein and penetration of EMPs within the bone grafting material surface [11]. Following preliminary experiments, a new carrier system was developed in liquid formulation for EMD (Osteogain; Straumann AG). The aim of the present study was therefore to investigate the effects of Osteogain in combination with NBM on the initial periodontal ligament (PDL) cell behaviour when compared to NBM alone by assessing the gene expression of a wide variety of osteoblast cytokines, growth factors, differentiation markers and extracellular matrix molecules using a commercially available super-array kit.

Methods

Osteogain and bone grafting material

For all in vitro experiments, Osteogain (0.3-ml vials, working concentration 30 mg/ml) was kindly provided by Straumann AG, Malmo, Sweden. In order to reach the in vitro working concentration of 100 μg/ml, Osteogain was diluted in cell culture media containing 10 % FBS. For all in vitro cell experiments, cells were exposed to culture media containing Osteogain at time point 0 until the end of the experiment. A NBM bone grafting material (Bio-Oss, Geistlich, Switzerland) was utilized as the material of choice for the present study due to our laboratories previously handling its use as well as its ability to adsorb enamel matrix proteins within the material graft [11].

PDL cell isolation and differentiation

Primary human PDL cells were isolated from the middle third of extracted premolars for orthodontic reasons from patients demonstrating no signs of periodontal disease as previously described [14, 15]. Informed consent was obtained from all the participants. Primary PDL cells were detached from the tissue culture plastic using trypsin solution. Cells used for experimental seeding were from passages 4–6. Primary PDL cells were seeded at a density of 500,000 cells on top of 500 mg NBM material per well in 6-well dishes for super-array analysis. Assays were performed in quadruplicates with four independent experiments performed from four different human donors.

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 RNeasy 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 the manufacturer's protocol using the 20 μl final reaction volume of TaqMan® One-Step Master Mix Kit (Applied Biosystems) as previously described [16]. RNA quantification was performed using a NanoDrop 2000c (Thermo Scientific, Waltham, MA, USA), and 100 ng of the total RNA was used per sample well. A gene fold increase represents data from cells grown in the presence of NBM particles precoated or not with Osteogain.

Statistical analysis

Gene array analysis was performed for both control ($n = 4$) and Osteogain ($n = 4$) groups. Means and standard deviations (SDs) were calculated, and the statistically significant differences were examined by Student's t test between both groups ($^*p$ values <0.05 was considered significant).

Results

The full set of genes investigated in the present study is listed in Table 1 in alphabetical order. As depicted, a number of genes involved in osteogenesis were upregulated with the vast majority between 1- and 10-fold when cells were cultured in the presence of Osteogain (Table 1). For the purpose of this experiment, only genes having been upregulated over 3-fold or those that demonstrated a significance of greater than a p value of 0.05 are discussed. Alpha-2-HS-glycoprotein was upregulated 5.09-fold with a p value of 0.002. Alkaline phosphatase and annexin 5 were upregulated [3.19 ($p = 0.022$) and 12.08 ($p = 0.001$), respectively]. From the number of tested bone morphogenetic proteins (BMPs), bone morphogenetic protein 2 (BMP2) showed the highest upregulation of 6.96 ($p = 0.039$) with its associated receptor upregulated 3.09-fold when compared to control cells seeded on NBM alone. Interestingly, the addition of Osteogain upregulated calcitonin receptor 55.95-fold ($p = 0.008$). Both epidermal growth factor (EGF) and fibroblast growth factor (FGF) demonstrated increased expression with their associated receptors being upregulated also (Table 1). Fibronectin, a cell attachment protein, was upregulated 3.4-fold ($p = 0.018$). A number of integrin binding proteins were upregulated between 2.6- and 9.04-fold (Table 1). Platelet-derived growth factor (PDGF) was upregulated 1.74-fold; however, this demonstrated statistically significant differences from control ($p = 0.019$). SMAD1 and SMAD2, members of intracellular pathways for BMPs, were significantly upregulated 1.89- and 2.62-fold ($p < 0.05$). Lastly, both Twist homolog 1 and vitamin D were both upregulated 2.66- and 5.58-fold, respectively, with both demonstrating significant differences from controls ($p = 0.025$ and $p = 0.047$, respectively).

Discussion

The aim of the present study was to investigate the role of Osteogain when combined with bone grafting materials in comparison to NBM alone on a wide array of genes related to osteogenesis. As such, the use of a gene array with a predesigned set of roughly 100 genes was utilized in order to determine possible mechanisms/roles of enamel matrix proteins on osteoblast differentiation. Interestingly, of the 100 genes investigated, 21 genes demonstrated either a 3-fold upregulation or a $p < 0.05$ when compared to control samples (Table 1). Many of these genes were upregulated between 2- and 10-fold.

One of the surprising results from this study was that calcitonin receptor was upregulated over 55-fold following the addition of Osteogain to a bone grafting material. Calcitonin has several roles in the body. More specifically, it is responsible for lowering blood Ca^{2+} primarily by inhibiting osteoclast activity and stimulating osteoblast activity [17]. Furthermore, what is impressive about these results is that we used different primary PDL cells from four different human donors. Thus, this significant upregulation is independent of slight human variations and it seems plausible that Osteogain may have some effect and/or some regulation through calcitonin/calcitonin receptors. Furthermore, a second gene for which was most significantly upregulated was annexin A5 with a 12.08-fold increase when treated with Osteogain. Annexin A5 is a calcium-dependent phospholipid binding protein which has been suggested to play a role as a mechanosensitive ion channel [18]. Disruption of its expression impairs mechanically induced calcium signalling in osteoblasts, and it has the ability to interact with both extracellular matrix proteins and cytoskeletal elements [18]. Thus, the pairing of upregulated genes calcitonin receptor and annexin A5 suggests a possible role of calcium regulation via enamel matrix proteins. Further investigations in this area are necessary.

The use of Osteogain also elicited a significant upregulation of many growth factors and their associated receptors (Table 1). Of the group of BMPs, BMP2 was the most upregulated with a significant increase of 6.96-fold when treated with Osteogain + NBM in comparison to NBM alone (Table 1). Furthermore, both bone morphogenetic protein receptor 2 and intracellular protein SMAD2 were upregulated 3.07- and 2.69-fold, respectively. Thus, the present study reveals that enamel matrix proteins have a significant influence on the BMP/cell signalling events that take place following cell culture with Osteogain. This is in accordance with previous publications that have demonstrated this phenomenon [19–21]. The family of SMAD signal proteins are found in many cell types and are responsible for transducing extracellular signals from TGFβ and BMPs from the cell membrane to the nucleus [22]. Kawase et al. showed in two consecutive studies that EMD was able to induce rapid translocation of SMAD2 to the nucleus and this was responsible for increased cell proliferation [23, 24]. In the present study, we also observed a significant increase of SMAD2 by 2.69-fold in as short as 24 h following exposure to Osteogain (Table 1).

Apart from BMP signalling, it was also found that gene expressions of TGFβ1, PDGF, EGF and FGF were all upregulated in response to Osteogain (Table 1). Furthermore, the associated receptors of both EGF and FGF were also upregulated. This is in accordance with our previously published gene array which has demonstrated the large effects of EMD on many growth factors, cytokines and their associated receptors [20]. Thus, this study further confirms the effects of Osteogain as opposed to gel-like substance Emdogain and demonstrates its ability to elicit a large effect on gene expression from primary human PDL cells.

Interestingly, Osteogain was also able to have effects on cell adhesion molecules including fibronectin and various integrin binding proteins (Table 1). Fibronectin is a known

Table 1 Osteogenic gene array of primary PDL cells exposed to Osteogain in combination with NBM bone graft in comparison to NBM alone

Symbol	Name	AVG ΔC_t (Ct(GOI) − Ave Ct (HKG))		Fold change	*t* test
		Test sample	Control sample	Test sample/ control sample	*p* value
ACVR1	Activin A receptor, type I	−7.80	−7.47	1.25	0.915332
AHSG	Alpha-2-HS-glycoprotein	−11.85	−9.51	*5.09*	*0.001627*
ALPL	Alkaline phosphatase, liver/bone/kidney	−9.80	−8.13	*3.19*	*0.021668*
ANXA5	Annexin A5	−9.36	−5.76	*12.08*	*0.000579*
BGLAP	Bone gamma-carboxyglutamate (gla) protein	−2.41	−2.82	0.75	0.620107
BGN	Biglycan	−6.86	−7.30	0.74	0.596608
BMP1	Bone morphogenetic protein 1	−3.08	−4.07	0.51	0.247237
BMP2	Bone morphogenetic protein 2	−7.12	−4.32	*6.96*	*0.038526*
BMP3	Bone morphogenetic protein 3	−3.56	−3.48	1.06	0.596056
BMP4	Bone morphogenetic protein 4	−4.00	−3.61	1.31	0.228776
BMP5	Bone morphogenetic protein 5	−9.34	−9.17	1.13	0.673938
BMP6	Bone morphogenetic protein 6	−10.05	−9.89	1.11	0.734613
BMP7	Bone morphogenetic protein 7	−3.29	−2.29	1.99	0.202071
BMPR1A	Bone morphogenetic protein receptor, type IA	−3.60	−3.87	0.83	0.942792
BMPR1B	Bone morphogenetic protein receptor, type IB	−12.16	−10.98	2.27	0.208567
BMPR2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	−4.80	−3.18	*3.07*	0.145458
CALCR	Calcitonin receptor	−9.28	−3.47	*55.95*	*0.007783*
CD36	CD36 molecule (thrombospondin receptor)	−3.90	−4.72	0.56	0.439813
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	−6.06	−6.89	0.56	0.778036
CHRD	Chordin	−11.23	−11.17	1.04	0.343664
COL10A1	Collagen, type X, alpha 1	−12.43	−11.34	2.13	0.460268
COL14A1	Collagen, type XIV, alpha 1	−7.23	−5.96	2.42	0.463013
COL15A1	Collagen, type XV, alpha 1	−7.84	−6.37	2.76	0.337634
COL1A1	Collagen, type I, alpha 1	−5.50	−5.52	0.99	0.977061
COL1A2	Collagen, type I, alpha 2	−12.75	−11.14	*3.06*	0.822122
COL2A1	Collagen, type II, alpha 1	−6.53	−7.49	0.51	0.072133
COL3A1	Collagen, type III, alpha 1	−3.59	−3.73	0.91	0.793750
COL5A1	Collagen, type V, alpha 1	−2.21	−3.36	0.45	0.243179
COMP	Cartilage oligomeric matrix protein	−13.01	−13.39	0.77	0.273670
CSF1	Colony-stimulating factor 1 (macrophage)	−9.87	−8.68	2.27	0.325348
CSF2	Colony-stimulating factor 2 (granulocyte-macrophage)	−1.68	−1.71	0.98	0.950459
CSF3	Colony-stimulating factor 3 (granulocyte)	−5.76	−6.45	0.62	0.630748
CTSK	Cathepsin K	−5.41	−5.97	0.68	0.649570
DLX5	Distal-less homeobox 5	−9.36	−8.41	1.93	0.425777
EGF	Epidermal growth factor	−13.41	−11.60	*3.50*	*0.035436*
EGFR	Epidermal growth factor receptor	−11.16	−8.51	*6.28*	*0.015134*
FGF1	Fibroblast growth factor 1 (acidic)	−7.57	−5.38	*4.58*	*0.040644*
FGF2	Fibroblast growth factor 2 (basic)	−16.41	−15.07	2.54	*0.002678*
FGFR1	Fibroblast growth factor receptor 1	−2.73	−2.74	0.99	0.928878
FGFR2	Fibroblast growth factor receptor 2	−2.98	−2.08	1.87	*0.031555*
FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	−6.56	−7.33	0.59	0.338385
FN1	Fibronectin 1	−8.03	−6.26	*3.40*	*0.018460*
GDF10	Growth differentiation factor 10	−6.67	−6.01	1.58	0.489789
GLI1	GLI family zinc finger 1	−6.86	−6.86	1.00	0.730111
ICAM1	Intercellular adhesion molecule 1	−7.58	−7.61	0.98	0.596638

Table 1 (continued)

Symbol	Name	AVG ΔC_t (Ct(GOI) − Ave Ct (HKG))		Fold change	*t* test
		Test sample	Control sample	Test sample/ control sample	*p* value
IGF1	Insulin-like growth factor 1 (somatomedin C)	−10.81	−10.31	1.42	0.185206
IGF1R	Insulin-like growth factor 1 receptor	−6.53	−6.48	1.03	0.968599
IGF2	Insulin-like growth factor 2 (somatomedin A)	−3.10	−3.69	0.66	0.553169
IHH	Indian hedgehog	1.59	1.23	0.78	0.995198
ITGA1	Integrin, alpha 1	−13.98	−12.60	2.60	*0.009439*
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	−9.18	−6.01	*9.04*	*0.000284*
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	−13.77	−12.70	2.10	*0.003393*
ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	−1.79	−2.06	0.83	0.544331
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	0.65	1.16	1.42	0.448769
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	−4.23	−4.63	0.76	0.749247
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	−4.92	−4.88	1.02	0.820871
MMP8	Matrix metallopeptidase 8 (neutrophil collagenase)	−5.12	−5.51	0.77	0.619721
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	−6.82	−6.59	1.17	0.504086
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	−7.03	−6.10	1.90	0.243775
NOG	Noggin	−4.77	−4.42	1.28	0.632229
PDGFA	Platelet-derived growth factor alpha polypeptide	−8.05	−7.26	1.74	*0.018625*
PHEX	Phosphate-regulating endopeptidase homolog, X linked	−9.62	−9.33	1.22	0.223625
RUNX2	Runt-related transcription factor 2	−5.28	−5.53	0.84	0.571962
SERPINH1	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1 (collagen binding protein 1)	−9.13	−8.93	1.15	0.418260
SMAD1	SMAD family member 1	−9.10	−8.18	1.89	*0.019281*
SMAD2	SMAD family member 2	−7.81	−6.42	2.62	*0.009082*
SMAD3	SMAD family member 3	−5.55	−4.31	2.36	0.514189
SMAD4	SMAD family member 4	−4.79	−3.93	1.81	0.250318
SMAD5	SMAD family member 5	−4.42	−4.56	0.91	0.732234
SOX9	SRY (sex-determining region Y)-box 9	−5.49	−5.53	0.97	0.382991
SP7	Sp7 transcription factor	−11.41	−10.75	1.58	0.338706
SPP1	Secreted phosphoprotein 1	−11.44	−10.56	1.85	0.593599
TGFB1	Transforming growth factor, beta 1	−10.76	−9.47	2.45	0.214655
TGFB2	Transforming growth factor, beta 2	−1.49	−2.79	0.41	*0.039454*
TGFB3	Transforming growth factor, beta 3	−2.73	−2.86	0.92	0.884488
TGFBR1	Transforming growth factor, beta receptor 1	−6.57	−7.15	0.67	0.076319
TGFBR2	Transforming growth factor, beta receptor II (70/80 kDa)	−5.44	−7.79	*0.20*	0.171507
TNF	Tumour necrosis factor	−9.49	−8.05	2.71	0.278587
TNFSF11	Tumour necrosis factor (ligand) superfamily, member 11	−9.43	−7.98	2.72	0.235600
TWIST1	Twist homolog 1 (*Drosophila*)	−12.02	−10.61	2.66	*0.024769*
VCAM1	Vascular cell adhesion molecule 1	−10.06	−9.62	1.36	0.635539
VDR	Vitamin D (1,25-dihydroxyvitamin D_3) receptor	−9.13	−6.65	*5.58*	*0.046791*
VEGFA	Vascular endothelial growth factor A	−6.57	−5.97	1.51	0.452665
VEGFB	Vascular endothelial growth factor B	−7.84	−7.62	1.17	0.419889

Italicized values represent significant difference between NBM+Osteogain group versus NBM alone ($p < 0.05$)

extracellular matrix protein secreted by various cell types able to increase cell attachment [25, 26]. Furthermore, the effects of integrin binding have a long history of well-documented effects on cell attachment [27, 28]. Previous investigation on the attachment of human periodontal ligament cells to enamel matrix-derived protein demonstrated that it is mediated via an interaction between BSP-like molecules and integrin αvβ3 [29]. Furthermore, other investigators demonstrated that both PDL and gingival fibroblasts showed increased expression of the integrin subunits α_2, α_5 and β_1 and integrin $\alpha_v\beta_3$ following cell seeding with enamel matrix derivative.

It must be noted that in the present study, the control of utilizing Osteogain alone was not evaluated purely to simulate a clinical setting. Osteogain was developed as a new liquid carrier system for EMD in order to maximize better adsorption of enamel matrix proteins onto a bone grafting material surface. Clinically, the combination approach is often utilized due to the principal reason that Emdogain gel alone is unable to prevent a flap collapse for large intrabony defects, thus necessitating the combination with a bone grafting material. Clinically, it is also possible to use a bone grafting material alone for the treatment of large intrabony defects; thus, it was chosen as a control in the present study. However, the use of Osteogain alone would never be utilized clinically as the liquid formulation of the material would have handling issues. For these reasons, Osteogain alone was not used as a secondary control. Future investigation following the results from the present study will determine the effects of Osteogain on cell behaviour at later time points on specific gene transcription of osteoblast differentiation markers as well as at the protein level.

Conclusion

The present study demonstrates the potent effects of Osteogain when combined with a bone grafting material on gene expression of many cytokines, growth factors, adhesion molecules and intracellular signalling proteins. It was found that Osteogain was able to significantly upregulate BMP2, TGFβ1, FGF, EGF and PDGF as well as some of their associated receptors. Osteogain also promoted a number of osteoblast differentiation markers including collagen1α2 and alkaline phosphatase as well as cell adhesion molecules including fibronectin and a variety of integrin binding proteins. Further research analyzing protein quantification and later time points are now necessary to further investigate the role of Osteogain on new bone formation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. For this type of study, formal consent was not required.

Informed consent Informed consent was obtained for cells utilized in this study.

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