

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.

<http://hdl.handle.net/2066/49673>

Please be advised that this information was generated on 2021-09-21 and may be subject to change.

The effect of Emdogain® on the growth and differentiation of rat bone marrow cells

J. van den Dolder, A. P. G. Vloon,
J. A. Jansen

Department of Periodontology and Biomaterials,
Radboud University Nijmegen Medical Center,
Nijmegen, the Netherlands

van den Dolder J, Vloon APG, Jansen JA. The effect of Emdogain® on the growth and differentiation of rat bone marrow cells. J Periodont Res 2006; 41: 471–476.

© 2006 The Authors. Journal compilation © 2006 Blackwell Munksgaard

Background and Objective: The major extracellular matrix (ECM) proteins in developing enamel can induce and maintain the formation and mineralization of other skeletal hard tissue, such as bone. Therefore, dental matrix proteins are ideal therapeutic agents when direct formation of functional bone is required for a successful clinical outcome. Emdogain® (EMD) consists of enamel matrix proteins which are known to stimulate bone formation. However, only a few studies in the literature have reported the effect of EMD on osteoblast-like cells *in vitro*.

Material and Methods: In this study, rat bone marrow cells, obtained from the femora of Wistar rats, were precultured for 7 d in osteogenic medium. Then, the cells were harvested and seeded in 24-well plates at a concentration of 20,000 cells/well. The wells were either precoated with 100 µg/ml EMD, or left uncoated. The seeded cells were cultured in osteogenic medium for 32 d and analysed for cell attachment (by using the Live and Dead assay), cell growth (by determining DNA content) and cell differentiation (by measuring alkaline phosphatase activity and calcium content, and by using scanning electron microscopy and the reverse transcription–polymerase chain reaction).

Results: The results showed that at the 4-h time point of the experiment, more cells were attached to EMD-negative wells, but this effect was no longer apparent at 24 h. DNA analysis revealed that both groups showed a similar linear trend of cell growth. No differences in alkaline phosphatase activity or calcium content were observed, and no differences in gene expression (osteocalcin, alkaline phosphatase and collagen type I) were found between the groups.

Conclusion: Based on our results, we conclude that EMD had no significant effect on the cell growth and differentiation of rat bone marrow cells.

Dr J. van den Dolder, Department of Periodontology and Biomaterials, Radboud University Nijmegen Medical Center, PO Box 9101, 6500 HB Nijmegen, the Netherlands
Tel: +31 243614086
Fax: +31 243614657
e-mail: j.vandendolder@dent.umcn.nl

Key words: bone marrow cells; cell growth; Emdogain; matrix mineralization

Accepted for publication February 14, 2006

A strategy to enhance the osteogenic capability of a bone graft substitute is the use of a scaffold material loaded with bone-inductive growth factors prior to implantation. These growth factors are released at the implant site and act upon existing cells or recruit other cells to form new bone tissue.

However, there is another group of proteins that can support and enhance bone formation, namely extracellular (enamel) matrix proteins. Extracellular matrix (ECM) is produced by osteoblasts and consists of several classes of molecules that regulate modeling and remodeling of bone.

ECM contains structural proteins that serve multiple roles in bone formation, ranging from cell attachment (e.g. fibronectin, collagen type I, osteopontin and bone sialoprotein) to nucleators for mineralization (e.g. osteopontin and bone sialoprotein) (1,2). Teeth differ from other skeletal

tissues in that they are more highly mineralized and more resistant to mechanical, chemical and biological breakdown. Dental matrix proteins produced by ameloblasts are also inducers for the mineralization of bony tissues. From literature reports it is known that the major ECM proteins in developing enamel can induce and maintain the formation and mineralization of other skeletal hard tissue, such as bone. Therefore, dental matrix proteins are ideal therapeutic agents where the direct formation of functional bone is required for a successful clinical outcome (3–6).

A purified enamel matrix protein product, Emdogain® (EMD), has been introduced commercially. EMD is prepared from developing porcine teeth and consists mainly of amelogenin and some undetectable growth factors (7). Amelogenins are hydrophobic proteins that are known to self-assemble into supramolecular aggregates which form an insoluble extracellular matrix (8) with high affinity for hydroxyapatite and collagens (9). Animal experiments and clinical studies of periodontal treatment have already demonstrated that EMD stimulates the regeneration of periodontal tissue, including acellular cementum, periodontal ligament (PDL) cells and alveolar bone (10–14). The effect of EMD on PDL cells *in vitro* has been examined in different studies, which found that EMD stimulates cellular proliferation, alkaline phosphatase (ALP) activity, mineralized nodule formation and transforming growth factor- β 1 (TGF- β 1) production (9,15,16). However, only a few studies have reported the effect of EMD on osteoblast-like cells or bone marrow cells. Yoneda *et al.* (17) used two mouse osteoblastic cell lines and showed that the effect of EMD was cell-type dependent. They found no effect for EMD with the ST2 cell line, but with the KUSA/A1 cell line they observed enhanced cell proliferation, ALP activity and mineralized nodule formation. Other studies also observed different responses of their osteoblastic cell lines with EMD (18,19). Keila *et al.* (20) performed a study with rat bone marrow cells and observed an increase in the osteogenic capacity (ALP activity

and mineralized nodule formation) of the cells in the presence of EMD.

Therefore, the aim of the present study was to evaluate the effect of EMD on the proliferation and differentiation of rat bone marrow cells.

Material and methods

Cell isolation

Rat bone marrow (RBM) cells were isolated and cultured using the method described by Maniopoulos (21). Femora of male Wistar rats were washed in α -minimal essential medium (MEM; Gibco BRL, Life Technologies B.V. Breda, the Netherlands) containing 0.5 mg/ml gentamycin and 3 μ g/ml fungizone (Sigma Chemical Co., St Louis, MO, USA). Epiphyses were cut off and diaphyses flushed out with 15 ml of α -MEM supplemented with 10% foetal calf serum (FCS) (Gibco BRL, Life Technologies), 50 μ g/ml ascorbic acid, 50 μ g/ml gentamycin, 10 mM sodium β -glycerophosphate and 10^{-8} M dexamethasone (Sigma Chemical Co.). The cells were incubated in a humidified atmosphere of 95% air, 5% CO₂, at 37°C for 7 d. The medium was changed every 2–3 d.

Cell seeding

After 7 d of primary culture, cells were seeded as a cell suspension (20,000 cells/well) in 24-well plates or on Thermanox® discs (Nalge Nunc Int., Naperville, IL, USA). However, before seeding, half of the 24-well plates or Thermanox® discs were first coated with 100 μ g/ml EMD (Straumann AG, Waldenburg, Switzerland). Cells were cultured in the presence of osteogenic medium (α -MEM, supplemented with 10% FCS) containing 50 μ g/ml ascorbic acid, 50 μ g/ml gentamycin, 10 mM sodium β -glycerophosphate and 10^{-8} M dexamethasone (Sigma Chemical Co.) and incubated in a humidified atmosphere of 95% air, 5% CO₂, at 37°C. The medium was changed every 2–3 d.

Cell attachment and spreading

After an incubation period of 1, 4, 8 or 24 h, cells were evaluated on their attachment and spreading. The Live

and Dead viability assay (Molecular Probes, Leiden, the Netherlands) was performed on the cells, and consisted of washing with phosphate-buffered saline (PBS), covering with substance A (1.0 μ l) and B (3.5 μ l), incubation for 30–45 min at 37°C, washing with PBS, and finally analysis under the fluorescence microscope. Green cells were viable cells, where red cells were not.

DNA analysis

After 1, 2, 4, 8 or 12 d of incubation, samples were used for DNA analysis. Medium was removed and the cell layers were washed twice with PBS. One millilitre of MilliQ was added to each sample. The samples were frozen and thawed repeatedly.

A DNA standard curve was made with Lambda DNA. One-hundred microlitres of sample or standard was added to 100 μ l of Picogreen working solution (Molecular Probes) and the samples were incubated for 10 min at room temperature in the dark. After incubation, DNA was measured using a fluorescence microplate reader with excitation filter 365 nm and emission filter 450 nm. The same samples were also used for the ALP activity assay.

ALP activity

ALP activity was measured on days 2, 4, 8 and 12 of incubation. For the assay (Sigma Chemical Co.), 96-well plates were used. Eighty microlitres of sample and 20 μ l of buffer solution (5 mM MgCl₂, 0.5 M 2-amino-2-methyl-1-propanol) were added to the wells. Subsequently, 100 μ l of substrate solution (5 mM paranitrophenylphosphate) was added to the wells and the plate was incubated for 1 h at 37°C. The reaction was stopped by adding 100 μ l of stop solution (0.3 M NaOH). For the standard curve, serial dilutions of 4-nitrophenol were added to final concentrations of 0–25 nM. The plate was read in an enzyme-linked immunosorbent assay (ELISA) reader at 405 nm.

Calcium content

The calcium content in the samples was measured by the ortho-cresolphthalein

complexone (OCPC) method (Sigma Chemical Co.). The calcium content was examined on days 8, 16, 24 and 32 of incubation. Cell layers were washed twice in PBS. Five-hundred microlitres of 0.5 N acetic acid was added to the wells and the samples were incubated overnight. Samples were frozen at -20°C until use.

OCPC solution was prepared as follows: 80 mg of OCPC was added to 75 ml of demineralized H_2O together with 0.5 ml of 1 M KOH and 0.5 ml of 0.5 N acetic acid. To prepare sample solution, 5 ml of OCPC solution was added to 5 ml of 14.8 M ethanolamine/boric acid buffer (pH 11), 2 ml of 8-hydroxyquinoline (5 g in 100 ml of 95% ethanol) and 88 ml of demineralized water. Three-hundred microlitres of sample solution was added to 10 μl of sample. To generate a standard curve, serial dilutions of CaCl_2 were made (1–200 $\mu\text{g}/\text{ml}$). The plate was incubated at room temperature for 10 min then read at 575 nm.

Scanning electron microscopy (SEM)

After days 8 and 24 of incubation, Thermanox® discs seeded with cells were washed twice with PBS. Fixation was carried out for 10 min in 2% glutaraldehyde, then the substrates were washed twice with 0.1 M sodium-cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol and dried by tetramethylsilane (Sigma Chemical Co.). The specimens were sputter-coated with gold, and examined and photographed using a Jeol 6310 scanning electron microscope (JEOL (Europe) B.V., Nieuw, Vennep, the Netherlands) at an acceleration voltage of 10 kV.

SEM was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Centre for Molecular Life Sciences (NCMLS), the Netherlands.

RNA isolation

RNA was isolated from the cells of six wells of each group (with or without EMD) with the aid of an RNA-isolation kit (RNeasy kit; Qiagen, Venlo, the Netherlands). The RNA obtained from six wells was pooled to retrieve

sufficient RNA for analysis. Cells were pelleted at 300 g in a tube and the supernatant was removed. The cells were then disrupted by adding 350 μl of RLT lysis buffer. The samples were stored at -70°C until use.

The volume of the sample was doubled by adding 70% ethanol and then mixed by pipetting. Seven-hundred microlitres of the samples were added to an RNeasy mini column that was placed in a collection tube. This was centrifuged for 15 s at 3 g and the flow-through was discarded. Seven-hundred microlitres of wash buffer RW1 was added to the RNeasy columns. The columns were then centrifuged for 15 s at 3 g and the flow-through was discarded. Then, the column was transferred to a new 2-ml collection tube, and 500 μl of RPE buffer diluted five-fold was added to the columns. These columns were again centrifuged for 15 s at 3 g and the flow-through was discarded. The columns were washed again with 500 μl of diluted RPE buffer, centrifuged for 2 min at 3 g and the flow-through was discarded. Finally, the columns were transferred to a new 1.5-ml tube and 40 μl of RNase-free water was added directly onto the RNeasy membrane in the columns. The columns were centrifuged for 1 min at 3 g for RNA elution. The RNA obtained was quantified by adding 1 μl of sample to 49 μl of RNase-free water and the absorbance was measured in a spectrophotometer (Bio-Rad Laboratories, Veenendaal, the Netherlands) at 260 nm.

Reverse transcription–polymerase chain reaction (RT–PCR)

After isolation of the RNA, the RT reaction was performed. One microgram of total RNA, 1 μl of 100 ng of random primers, 1 μl of dNTP mix (10 mM each) and 10 μl of distilled water were added to a nuclease-free microcentrifuge tube. The mixture was heated to 65°C for 5 min and quickly chilled on ice. The contents of the tube were collected by brief centrifugation. One microlitre of 5 \times First-Strand Buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl_2) and 2 μl of 0.1 M

dithiothreitol (DTT) were added. This was incubated at 25°C for 10 min, and the contents of the tube were mixed gently and incubated at 42°C for 2 min. Finally, 1 μl (200 U) of Superscript II (Invitrogen, Breda, the Netherlands) was added and incubated at 42°C for 50 min. The reaction was inactivated by heating the mixture at 70°C for 15 min. The cDNA thus obtained was used as a template in the PCR.

Semiquantitative PCR

Five microlitres of 10 \times PCR Buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1.5 μl of 50 mM MgCl_2 , 1 μl of 10 mM dNTP Mix, 1 μl of Amplification Primer 1 (10 μM), 1 μl of Amplification Primer 2 (10 μM), 0.2–0.5 μl of *Taq* DNA polymerase (2–5 U/ml) (Invitrogen), 1 μg of cDNA, and autoclaved, distilled water, were added to a PCR reaction tube to achieve a final volume of 50 μl . The following genes were analysed: osteocalcin as a late-differentiation marker, collagen type I as a marker for the extracellular matrix, and ALP as an early differentiation marker. All data were normalized to the housekeeping gene, β -actin.

Statistical analysis

This study was performed by using two separate runs of experiments. In each run, samples were present in triplicate. In the results section, the data of both runs are described, but only the first run is presented in the Figures. Statistical analysis was performed for each run by using an unpaired *t*-test (GraphPad Software, Inc., San Diego, CA, USA).

Results

In this study, a heterogeneous cell population was obtained from bone marrow. As a result of this heterogeneity, differences can exist between various experimental runs, and, in this study, discrepancies between the different runs were indeed observed.

The results of the Live and Dead assay revealed that after 4 h, more cells were attached to the noncoated surfa-

ces than to the EMD-coated surfaces. However, this difference was not observed at 24 h. During the first hours of incubation, cells were not spread and had a round shape, but after 24 h, the cells were observed to spread more.

DNA analysis confirmed the results of the Live and Dead assay, namely that equal numbers of cells were attached to EMD-coated and noncoated wells on day 1. Furthermore, DNA analysis revealed no differences in cell proliferation between the noncoated and EMD-coated groups. Both experimental runs showed a similar linear cell growth with the same amount of DNA (Fig. 1).

The ALP activity was higher in the second run and had a peak on day 12. The first run had a peak on day 8. No differences in ALP activity were observed between the noncoated and EMD-coated groups (Fig. 2).

The first run of the calcium measurements showed that the EMD-coa-

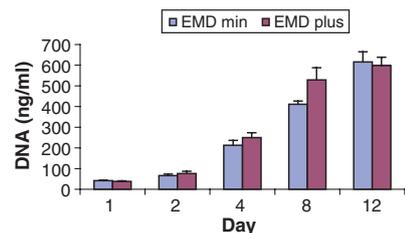


Fig. 1. The cell growth of rat bone marrow cells cultured on Emdogain® (EMD)-coated (EMD plus) and EMD-noncoated (EMD min) (first run) wells. Bars represent the standard deviation (SD).

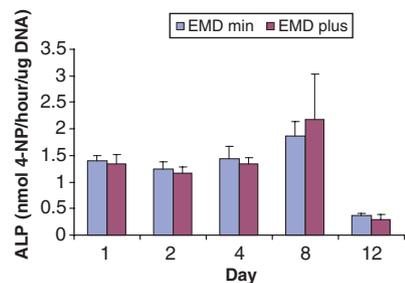


Fig. 2. The alkaline phosphatase (ALP) activity of rat bone marrow cells cultured on Emdogain® (EMD)-coated (EMD plus) and EMD-noncoated (EMD min) (first run) wells. Bars represent the standard deviation (SD).

ted group had a significantly enhanced calcium content compared with the uncoated cells on day 32 ($p < 0.01$). Unfortunately, this difference was not observed in the second run (Fig. 3).

SEM examination of both groups indicated that cells did proliferate. On day 8, multilayers of cells were already evident. However, no differences between noncoated and EMD-coated specimens were observed. After 24 d of incubation, a layer of calcified globular accretions, associated with collagen bundles, was deposited on the specimens. No clear differences in appearance between the noncoated and

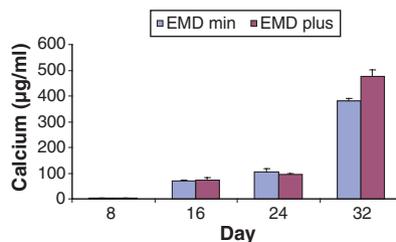


Fig. 3. The calcium content of of rat bone marrow cells cultured on Emdogain® (EMD)-coated (EMD plus) and EMD-noncoated (EMD min) (first run) wells. Bars represent the standard deviation (SD).

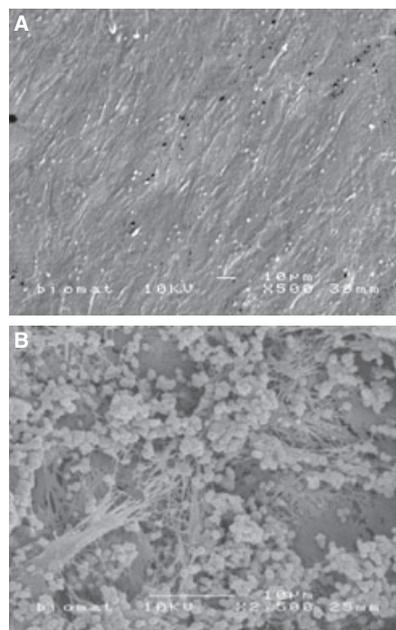


Fig. 4. Scanning electron micrographs of day 8 (A) and day 24 (B).

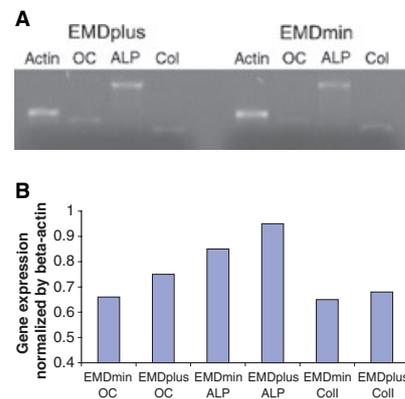


Fig. 5. Results of semiquantitative polymerase chain reaction (PCR). (A) Gene expression bands of α -actin (Actin), osteocalcin (OC), alkaline phosphatase (ALP) and collagen type I (Col). (B) Semiquantitative values of gene expression normalized by the household gene, α -actin. EMDplus, Emdogain® coated and EMD min, Emdogain® noncoated (first run) wells.

EMD-coated specimens were visible (Fig. 4).

Furthermore, a semiquantitative PCR analysis was performed on both groups. Unfortunately, no clear differences in the gene expression of ALP, collagen type I and osteocalcin were observed. However, a trend was seen in favor of EMD. In addition, all the genes were expressed and normalized to the household gene, β -actin (Fig. 5).

Discussion

In the present study, the effect of EMD on the proliferation and differentiation of rat bone marrow cells was examined. Unfortunately, no consistent effect of EMD on cell growth or differentiation was observed in this heterologous primary cell culture. Calcium measurements showed a significant stimulatory effect on matrix mineralization of EMD in the first run, but not in the second run. Also, the results of the PCR showed only a limited trend for increased expression of the osteogenic genes ALP and osteocalcin. However, the PCR method used was semiquantitative and, as a consequence, strong conclusions cannot be made.

From previous studies, we know that the effect of EMD on proliferation

and differentiation varies among different cell types, and the effect of EMD on different osteoblastic cell lines has been reported. Yoneda *et al.* (17) reported that EMD stimulated KUSA/A1 cell proliferation, although it did not affect ST2 cell proliferation. EMD stimulated the ALP activity and mineralized nodule formation of KUSA/A1 cells, but did not affect the osteoblastic differentiation of ST2 cells. Keila *et al.* (20) reported that bone marrow stromal cells were stimulated by EMD in their osteoblastic differentiation by enhanced ALP activity and mineralized nodule formation. The growth of murine calvarial osteoblasts was stimulated by the addition of EMD (19). Schwartz *et al.* (18) found that EMD stimulated the proliferation, but not the differentiation, of preosteoblastic 2T9 cells, and inhibited the proliferation and stimulated differentiation of osteoblast-like MG63 cells. They also found that the proliferation and differentiation of normal human osteoblast NHOst cells increased. However, in agreement with our observations, Gurpinar *et al.* (22) revealed that EMD had no impact on the cell growth of rat marrow stromal osteoblasts.

In addition to cell type, most studies differ in cell source. Some researchers used calvarial cells (19) to examine the osteogenic capacity, while others used bone marrow cells (20). Moreover, different types of rats were used for cell retrieval [i.e. Wistar (the present study) and Sprague-Dawley (20)]. All these discrepancies in primary conditions can have severe implications for the final results.

Besides differences in cell types, variation in experimental designs may also be responsible for inconsistencies in results between different studies. For example, in the present study we coated the 24-well plates with EMD, while other studies used diluted EMD in their medium (20). Furthermore, various concentrations of EMD were used in different studies. Some studies reported that 100 µg/ml EMD is the optimal concentration for stimulating the osteogenic potential of PDL cells, while others (20) reported that 25 µg/ml EMD stimulated the osteogenic

potential of bone marrow stromal cells. When 100 µg/ml EMD was used, no difference between control and 100 µg/ml EMD in ALP activity and matrix mineralization was observed. In contrast, Jiang *et al.* (19) observed that EMD gave an enhanced response on the growth of primary osteoblasts digested from mouse calvaria with a higher concentration of EMD (100 µg/ml) compared with a lower concentration (25 µg/ml).

In this study, the 24-well plates were first coated with 100 µg/ml EMD before cells were added to the wells. Freeze-dried EMD was diluted in 0.1% acetic acid. We observed that the hydrophobic enamel matrix proteins aggregated at the bottom. This has been mentioned by previous researchers and also observed when EMD was diluted in culture media at neutral pH (9,16,17). Jiang *et al.* (19) and Yoneda *et al.* (17) diluted EMD in culture media and found that EMD had an enhancing effect on osteoblastic cells. Therefore, we believe that diluting EMD in culture medium can give a more predictable result than using EMD as a coating.

The EMD product has been extracted from porcine tooth germs. Therefore, osteoinductive factors may be present in the extract. However, Gestrelus *et al.* (7) used different immunoassays to examine whether certain factors were present. Granulocyte-macrophage colony-stimulating factor (GM-CSF), calbindin D, epithelial growth factor (EGF), fibronectin, basic fibroblast growth factor (bFGF), α -interferon, interleukin-1 α , -2, -3 and -6, insulin growth factor-1 (IGF-1) and -2, neurotrophic growth factor (NGF), platelet-derived growth factor (PDGF), tumour necrosis factor (TNF) and TGF- α were examined and none was detected. Suzuki *et al.* (22) detected bone sialoprotein (BSP) in EMD. BSP is known to play an important role in the mineralization of hard tissues as well as in tooth development (23). Furthermore, it is known that PDL cells secrete, under the influence of EMD, several growth factors, such as TGF- α 1, interleukin-6 and PDGF-AB (16,24). Some studies of TGF- α 1 showed enhanced cell proliferation

with bone marrow stromal cells, but no enhancement in osteogenic differentiation (25–27), while others found stimulation of ALP activity with TGF- α 1 (28,29).

Several *in vivo* experiments have demonstrated that EMD stimulates alveolar bone regeneration (6,13,14) and regeneration of a femoral bone defect (5), but ectopic studies revealed no osteoinductivity (17). Based on our results, and on the results of other research groups, it is still unclear how EMD exerts its function. Some claim that EMD has mainly an angiogenic effect, which probably contributes to the acceleration of wound healing and bone regeneration (30). Others claim that EMD maintains the viability of adherent stromal cells and promotes their osteogenic potential (20). However, such an effect was not confirmed by our current RBM cell culture study.

Therefore, we conclude that EMD, applied as a coating on a tissue culture polystyrene substrate surface, has no evident effect on the proliferation and differentiation of rat bone marrow cells.

Acknowledgements

This research was financially supported by a VENI grant (NGT6052) of the Dutch Technology Foundation (STW), the applied science division of the NWO and the technology program of the Ministry of Economic Affairs.

References

1. Davies JE. In vitro modeling of the bone/implant interface. *Anat Rec* 1996;**245**:426–445.
2. Reddi AH. Morphogenesis and tissue engineering of bone and cartilage: inductive signals, stem cells and biomimetic biomaterials. *Tissue Eng* 2000;**6**:351–359.
3. Veis A, Tompkins K, Alvares K *et al.* Specific amelogenin gene splice products have signaling effects on cells in culture and in implants *in vivo*. *J Biol Chem* 2000;**275**:41263–41272.
4. Boyan BD, Weesner TC, Lohmann CH *et al.* Porcine fetal enamel matrix derivative enhances bone formation induced by demineralized freeze dried bone allograft *in vivo*. *J Periodontol* 2000;**71**:1278–1286.
5. Kawana F, Sawae Y, Sahara T *et al.* Porcine enamel matrix derivative enhances

- trabecular bone regeneration during wound healing of injured rat femur. *Anat Rec* 2001;**264**:438–446.
6. Tonetti MS, Lang NP, Cortellini P *et al*. Enamel matrix proteins in the regenerative therapy of deep intrabony defects. *J Clin Periodontol* 2002;**29**:317–325.
 7. Gestrelus S, Andersson C, Lidstrom D, Hammarstrom L, Somerman M. In vitro studies on periodontal ligament cells and enamel matrix derivative. *Clin Periodontol* 1997;**24**:685–692.
 8. Fincham AG, Moradian-Oldak J, Simmer JP *et al*. Self-assembly of a recombinant amelogenin protein generates supramolecular structures. *J Struct Biol* 1994;**112**:103–109.
 9. Gestrelus S, Andersson C, Johansson AC *et al*. Formulation of enamel matrix derivative for surface coating. Kinetics and cell colonization. *J Clin Periodontol* 1997;**24**:678–684.
 10. Heden G, Wennstrom J, Lindhe J. Periodontal tissue alterations following Emdogain® treatment of periodontal sites with angular bone defects. *J Clin Periodontol* 1999;**26**:855–860.
 11. Heijl L, Heden G, Svardstrom G, Ostgren A. Enamel matrix derivative (Emdogain®) in the treatment of intrabony periodontal defects. *J Clin Periodontol* 1997;**24**:705–714.
 12. Rasperini G, Ricci G, Silvestri M. Surgical technique for treatment of intrabony defects with enamel matrix derivative (Emdogain®): 3 case reports. *Int J Periodontics Restor Dent* 1999;**19**:578–587.
 13. Sculean A, Windisch P, Keglevich T, Gera I. Clinical and histologic evaluation of an enamel matrix protein derivative combined with abioactive glass for the treatment of intrabony periodontal defects in humans. *Int J Periodontics Restor Dent* 2005;**25**:139–147.
 14. Sculean A, Donos N, Windisch P *et al*. Healing of human intrabony defects following treatment with enamel matrix proteins or guided tissue regeneration. *J Periodont Res* 1999;**34**:310–322.
 15. Hoang AM, Oates TW, Cochran DL. In vitro wound healing responses to enamel matrix derivative. *J Periodontol* 2000;**71**:1270–1277.
 16. van der Pauw MT, van den Bos T, Everts V, Beertsen W. Enamel matrix-derived protein stimulates attachment of periodontal ligament fibroblasts and enhances alkaline phosphatase activity and transforming growth factor beta1 release of periodontal ligament and gingival fibroblasts. *J Periodontol* 2000;**71**:31–43.
 17. Yoneda S, Itoh D, Kuroda S *et al*. The effects of enamel matrix derivative (EMD) on osteoblastic cells in culture and bone regeneration in a rat skull defect. *J Periodont Res* 2003;**38**:333–342.
 18. Schwartz Z, Carnes DL, Pulliam R *et al*. Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 2T9 cells, inhibits proliferation and stimulates differentiation of osteoblast-like MG63 cells, and increases proliferation and differentiation of normal human osteoblast NHOst cells. *J Periodontol* 2000;**71**:1287–1296.
 19. Jiang J, Safavi KE, Spangberg LSW, Zhu Q. Enamel matrix derivative prolongs primary osteoblast growth. *J Endodont* 2001;**27**:110–112.
 20. Keila S, Nemcovsky CE, Moses O, Artzi Z, Weinreb M. In vitro effects of enamel matrix proteins on rat bone marrow cells and gingival fibroblasts. *J Dent Res* 2004;**83**:134–138.
 21. Maniatopoulos C, Sodek J, Melcher AH. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* 1988;**254**:317.
 22. Gurpinar A, Onur MA, Cehreli ZC, Tasman F. Effect of enamel matrix derivative on mouse fibroblasts and marrow stromal osteoblasts. *J Biomat Appl* 2003;**18**:25–33.
 23. Suzuki N, Ohyama M, Maeno M, Ito K, Otsuka K. Attachment of human periodontal ligament cells to enamel matrix-derived protein is mediated via interaction between BSP-like molecules and integrin alpha (v) beta3. *J Periodontol* 2001;**72**:1520–1526.
 24. MacNeal RL, Berry J, Strayhorn C, Somerman MJ. Expression of bone sialoprotein mRNA by cells lining the mouse tooth root during cementogenesis. *Arch Oral Biol* 1996;**41**:827–835.
 25. Lyngstadaas SP, Lundberg E, Ekdahl H, Andersson C, Gestrelus S. Autocrine growth factors in human periodontal ligament cells cultured on enamel matrix derivative. *J Clin Periodontol* 2001;**28**:181–188.
 26. Siebers MC, Walboomers XF, Leeuwenburgh SCG, Wolke JCG, Jansen JA. TGF-β1-release from a porous electrostatic spray deposition (ESD) derived calcium phosphate coating. *Tissue Eng* 2006; in press.
 27. Blom EJ, Klein-Nulend J, Klein CPAT, Kurashina K, van Waas MAJ, Burger EH. Transforming growth factor-α1 incorporated during setting in calcium phosphate cement stimulates bone cell differentiation in vitro. *J Biomed Mater Res* 2000;**50**:67–74.
 28. Harris SE, Bonewald LF, Harris MA, *et al*. Effects of transforming growth factor beta on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase, and type I collagen mRNA in long-term cultures of fetal rat calvarial osteoblasts. *J Bone Miner Res* 1994;**9**:855–863.
 29. Lu L, Yaszemski MJ, Mikos AG. TGF-α1 release from biodegradable polymer microparticles: its effect on marrow stromal osteoblast function. *J Bone Joint Surg* 2001;**83**:S82–S92.
 30. Ingram RT, Bonde SK, Riggs BL, Fitzpatrick LA. Effects of transforming growth factor beta (TGFβ) and 1,25 dihydroxyvitamin D3 on the function, cytochemistry and morphology of normal human osteoblast-like cells. *Differentiation* 1994;**55**:153–163.