Effect of Emdogain® on human periodontal fibroblasts in an in vitro wound-healing model


Objective: The aim of this study was to evaluate the influence of Emdogain® (EMD) on cultured gingival fibroblasts, periodontal ligament fibroblasts and dermal fibroblasts, using an in vitro model of wound healing.

Background: Enamel matrix derivative has been demonstrated to promote periodontal regeneration. However, the precise mechanisms by which this agent acts are still unclear.

Methods: The effect of EMD on proliferation of the cells was studied using subconfluent cultures of gingival fibroblasts and periodontal ligament fibroblasts. The cells were made quiescent overnight and then stimulated with various concentrations of EMD (10, 50, 100 and 150 µg/ml) for 24 h. Negative and positive controls were cells cultured in media containing 0.2% and 10% fetal calf serum (FCS). The DNA synthesis was measured by the cellular uptake of [3H]thymidine. For in vitro wounding the cells were cultured, wounded and stimulated with 0.2% FCS, 10% FCS and EMD at a concentration of 20 µg/ml. The percentage of wound fill after treatment was measured after d 1, 4, 6, 12 and 16. The proliferation of cells was also calculated by the extent of incorporation of crystal violet.

Results: The results demonstrated that cells in vitro fill an empty space by a combination of proliferation and cell migration. The most rapid closure of a wound area occurred where both proliferation and migration can occur as was seen when wounded cultures were maintained in 10% FCS or at a concentration of 20 µg/ml EMD which promoted proliferation.

Conclusions: Therefore, EMD appears to exert an influence on cells that is compatible with improved wound healing.

A major goal of periodontal therapy is restoration of the damaged tissues to their original form and function and requires regeneration of the destroyed periodontal connective tissues through formation of new cementum, new bone and new attachment of new connective tissue fibers (1–5). The observation that enamel matrix proteins are involved in the formation of cementum (6) led to the proposal that these proteins may have a beneficial effect in inducing periodontal regeneration. This led to the isolation of enamel matrix proteins and their development for periodontal regeneration. In recent years many studies have confirmed that enamel matrix proteins are capable of aiding periodontal regeneration [see (7) for review]. However, the exact mechanisms involved in such induction are still unclear.

Wound healing is a complex process involving cell migration, cell attachment to various components of the extracellular matrix and cell proliferation. Many of these processes are controlled by cytokines and growth factors. While Emdogain® (EMD) is not strictly considered a growth factor...
it appears to possess a number of properties that are ‘growth factor-like’. The mode of action of EMD appears to be through a matrix cell interaction between the amelogenin aggregate of the EMD and the cells of periodontal lamina (8–14). Interestingly, enhanced soft tissue wound healing has been noted following the use of EMD (15).

A previous study from our laboratory has described an in vitro method to analyse cell biological events associated with wound healing using cells derived from the periodontium (16). To date, only a few studies have been carried out to study the effect of EMD on such events using periodontal cells (17, 18). Therefore this study, through the use of an in vitro model of wound healing, aimed to gain a better understanding about how EMD enhances wound repair, particularly soft tissue repair, by investigating its effect on the proliferation and migration of human periodontal cells.

Materials and methods

Fibroblast cultures

Following institutional ethics approval, fibroblasts were obtained from human gingiva and periodontal lamina as described previously (16). Tissue explants were cultured using Dulbecco’s Modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS) supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml) and fungizone (2.5 µg/ml). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The media were replaced every 2 or 3 d until confluence was reached. The gingiva and periodontal lamina fibroblast used for this study were between their 5th and 10th passage in culture. The dermal fibroblasts were a very kind gift from Dr Mark Hayes, Royal Children Hospital, Brisbane, Australia. These cell cultures had been obtained from explants taken with consent from patients undergoing facelift operations.

DNA synthesis

DNA synthesis was measured by the uptake of [³H]thymidine. Human periodontal ligament fibroblasts and human gingival fibroblasts were seeded in triplicate, into 24-well plates at an initial density of 20,000 cells per well, and allowed to attach and spread overnight in DMEM containing 10% FCS. The medium was then replaced with 500 µl/well of DMEM alone and incubated for a further 48 h. This medium was removed and replaced with either control or test media, DMEM + 0.2% FCS (negative control), DMEM + 10% FCS (positive control), and EMD at concentrations of 10, 50, 100 and 150 µg/ml. Lyophilized EMD was made to 1 mg/ml in 50 mM carbonate buffer pH 9.0, 1 h prior to use (19). After 20 h incubation in the test media, [³H]thymidine was added to each well to give a final concentration of 1 µCi/ml. The cells were incubated for a further 4 h, after which the media were removed and the cells washed three times with 500 µl PBS. Cells were lysed by the addition of 1% SDS and the radioactivity in the cell lysate was determined in a Beckman® LS-6000SC Liquid Scintillation Counter (20).

In vitro wounding

Dermal fibroblasts, gingival fibroblasts and periodontal ligament fibroblasts were cultured in 4-well chamber plates, and allowed to grow to confluence. The cell layer was then wounded using a sterilized silicon bung, which removed the cells within a circular area (8 mm diameter). After the wounding, the media were removed and the wells were washed twice with PBS. The cultures were exposed to media containing DMEM, supplements and either 0.2% FCS, 10% FCS or 20 µg/ml EMD. A low concentration of EMD was chosen to avoid excessive build up of insoluble components of EMD during the long-term cultures. Media were replaced every second day and incubation continued for 1, 4, 6, 12 and 16 d. One plate (4 wells) was used for each time and treatment. The cells were fixed in glutaraldehyde 2% and later stained with crystal violet (0.75% crystal violet, 0.25% NaCl) (Fig. 1a,b).

Measurement of wound area

Digital images of the wounded cell cultures were photographed (Olympus® Camedia C-3000) and the area of the wound determined using NIH Image Analysis System® and Software. The results were expressed as percentage of the original wound area.

Fig. 1. In vitro wound healing by gingival fibroblasts cells stained with crystal violet following treatment with 20 µg/ml EMD from d 0–16. (a) Edge of wound area immediately after wounding (original magnification ×2.5). (b) View of the gingival fibroblasts filling the wound area after 16 d of treatment with 20 g/ml EMD (original magnification ×2.5). (c–h) show wound areas following treatment with 20 µg/ml EMD at d 0, 4, 6, 12 and 16 (original magnification ×1). (c) Wound at d 0; (d) wound at d 1; (e) wound at d 4; (f) wound at d 6; (g) wound at d 12; (h) wound at d 16.
Cell proliferation

Once the photographs had been taken, the incorporated dye was released from the cells by the addition of 1 ml of 33% acetic acid. Measurements of the absorbance at 570 nm allowed the increase in cell number to be determined (21).

Statistical analyses

The values of each treatment group were presented as a mean with the standard deviation of quadruplicates. The data were compared using the One way ANOVA with Tukey–Kramer multiple comparisons post-test. Using InStat View 4.02 software (Abacus Concepts, Inc., Berkley, CA, USA).

Results

DNA synthesis

The maximum proliferative response of gingival fibroblasts and periodontal ligament fibroblasts was noted with EMD at concentrations of 50, 100 and 150 µg/ml with a highly significative increase over 0.2% FCS controls ($P < 0.001$). The effects of serum and different concentrations of EMD on gingival fibroblasts and periodontal ligament fibroblasts on $[^3]$H]thymidine incorporation into DNA are shown on Fig. 2a, b.

For the gingival fibroblasts, EMD resulted in a significant ($P < 0.001$) increase in DNA synthesis compared to the 0.2% and 10% FCS controls when added at a concentration of 50 µg/ml and greater. At concentrations less than 50 µg/ml there was no significant effect on DNA synthesis by the gingival fibroblasts.

A similar response was noted to the periodontal ligament fibroblasts. At concentrations of 50 µg/ml and greater there was a significant ($P < 0.001$) increase in thymidine uptake compared to the 0.2% FCS controls and at concentrations of 100 µg/ml of EMD. Responses at EMD concentrations of 10 and 50 µg/ml were not significant compared to the 0.2% and 10% FCS controls, respectively.

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Fig. 2. $[^3]$H]Thymidine incorporation for (a) gingival fibroblasts and (b) periodontal ligament fibroblasts. Quiescent subconfluent cultures were stimulated with various test media (0.2% FCS, 10% FCS, EMD 10, 50, 100 and 150 µg/ml) for 24 h. $[^3]$H]thymidine was added for the last 4 h of incubation. *Indicates a statistically significant difference between negative control and test media $P < 0.001$. 

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Wound healing

All cell lines showed a progressive reduction in the wound area when cultures were maintained in 10% FCS (Fig. 3). By d 16, the remnant area of the wound was 1.3% for the dermal fibroblasts, 1.6% for the periodontal ligament fibroblasts and 10.8% for the gingival fibroblasts. When wounded cultures were maintained in 20 μg/ml of EMD, the wound area also progressively reduced, although neither the rate nor the extent of the wound closure was as great as noted with the 10% FCS treatment. When wounded cultures were maintained in 0.2% FCS for 16 d, the wound area diminished, but this was not as great as that noted for the cells cultured in the presence of either 10% FCS or EMD.

Cell proliferation

All cell types showed an increase in cell number over the length of the experiment when maintained in 10% FCS. The level of this increase was greatest for the gingival fibroblasts (Fig. 4). In the presence of 20 μg/ml EMD, the wounded gingival fibroblast cultures showed a significant increase in cell number (threefold increase after 6 d) compared with the twofold increase in the presence of 0.2% FCS.

By contrast, the wounded periodontal ligament fibroblast cultures showed no significant increase in cell number when maintained in either 0.2% FCS or 20 μg/ml EMD. The wounded dermal fibroblasts did not increase in cell number when maintained in 0.2% FCS, and only increased slightly in cell number in the

Fig. 3. Percentage of wound area closure following different treatments during d 1, 4, 6, 12 and 16 for (a) gingival fibroblasts, (b) periodontal ligament fibroblasts and (c) dermal fibroblasts. *Indicates a statistically significant difference ($P < 0.01$) in wound closure compared to d 0.
presence of 20 μg/ml EMD (1.3-fold increase) (Fig. 4a–c).

Discussion

EMD has been shown to stimulate periodontal fibroblast proliferation in the short term at concentrations of 50 μg/ml, 100 μg/ml and 150 μg/ml, but not at 10 μg/ml. An EMD concentration of 20 μg/ml regularly replenished in a long-term culture was chosen for the in vitro wounding to minimize the effects of prolonged deposition of insoluble components of the EMD. At this concentration EMD was also determined to be at the lowest effective concentration with regards to its effect on fibroblast proliferation as judged by thymidine uptake. It could be expected that these conditions would promote proliferation if the mitotic component of EMD accumulated during the length of the assay.

In vitro wounding of cell cultures resulted in various degrees of wound closure depending on the type of cell studied and the media in which the cultures were maintained. EMD at 20 μg/ml was almost as effective at promoting wound closure as 10% FCS for all cells types. Indeed, some level of

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Fig. 4. Changes in cell number during in vitro wound healing. (a) gingival fibroblasts, (b) periodontal ligament fibroblasts and (c) dermal fibroblasts. The fold increase in cell number was determined relative to the number of cells present at d 1. *Indicates a statistically significant difference ($P < 0.01$) in cell numbers compared to those recorded at d 0.
wound closure was seen in all cell types when cultures were maintained in 0.2% FCS, but this wound closure was not maintained over the full 16 d of this experiment, possibly reflecting the loss of viability of the fibroblasts cultured for long-term in reduced serum conditions. A previous study using a different wound-healing model has described a similar effect of EMD on wound closure responses of periodontal ligament fibroblasts and gingival fibroblasts (18, 22). The main difference in the models is the relative area of wound in the cultures. The model used in our laboratory (16) created a relatively large area of wound, which permits longer-term observations to be made.

Continuous replenishment of EMD at low concentration was conducive to the proliferation of gingival fibroblasts, but not to the periodontal ligament fibroblasts or the dermal fibroblasts. This resulted in a more rapid and complete wound closure for gingival fibroblasts than for the periodontal ligament fibroblasts or dermal fibroblasts. This is in contrast to other studies, which have shown a marked influence on periodontal ligament fibroblast proliferation after 7–10 d (16). Whether this reflects the significant variability and heterogeneity of primary periodontal ligament fibroblast cultures (23) remains to be established.

In conclusion the results from this study indicate that, in vitro, the most rapid closure of a wound area occurs when both proliferation and migration can occur as was seen when wounded cultures were maintained in 10% FCS or at a concentration of 20 μg/ml EMD. Such findings may help, in part, to explain the clinical results noted following the use of Endogain. It has been reported that in addition to promoting periodontal regeneration, Endogain also seems to promote rapid soft tissue repair. The results from this study indicate that gingival fibroblasts respond vigorously to exposure to EMD and this could account for the excellent gingival responses noted following its use. Although the periodontal ligament fibroblasts in our study did not respond as much as the gingival cells this is not inconsistent with our understanding of regeneration whereby matrix synthesis and proliferation are often mutually exclusive. Thus, as has been reported elsewhere, EMD may well enhance matrix synthesis by periodontal ligament cells thereby permitting them to undertake the processes necessary for reconstruction of the periodontal tissues without the complicating factor of going through unnecessary replicative processes.

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**References**