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In vitro clonogenic capacity of periodontal ligament fibroblasts cultured with Emdogain

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Abstract – The aim of the present study was to evaluate the efficiency of Emdogain (EMD) in preserving the size of the periodontal ligament progenitor pool (clonogenic capacity) and in promoting their proliferation. Periodontal ligament fibroblasts (PDLF) were obtained from explants of young permanent healthy tooth. After initial outgrowth (10 days to 2 weeks following explantation), the culture medium of experimental flasks was replaced with medium supplemented with 100 μg ml^{-1} EMD, whereas the other served as controls and were fed with regular medium. Following 5 weeks, the cells were washed (3×), harvested (trypsin + EDTA), and evaluated for their viability. Viable cells from each group were inoculated into six 96-well plates at a concentration of one viable cell per two wells and were allowed to grow for 5 weeks. The percentage of cells with clonogenic capacity was determined as the number of colonies formed/number of cells seeded × 100 in the experimental and control groups. Three degrees of dish area coverage were utilized: up to 25%, between 25% and 75% and higher than 75%. This experiment was repeated four times from four different donors. A total of 2328 cells were evaluated, half of which, were cultured with EMD. The mean percentage of cells (from all donors) who exhibited any clonogenic capacity in the presence of EMD was comparable with that of cells cultured in the absence of EMD: 26.6 ± 14.3% when compared with 34.6 ± 20.6% respectively ($P = 0.186$). Similarly, the percentage of clones that proliferated to cover up to 25% of the well area was comparable in the two groups 7.5 ± 8.6 for EMD-treated clones and 7.1 ± 7.8 for untreated clones ($P = 0.674$). The percentage of clones that proliferated to cover 25% up to 75% of the well area was greater EMD-treated clones as compared with the untreated cells: 8.1 ± 6.7% vs 3.8 ± 3%. However this difference was not statistically significant ($P = 0.277$). In contrast, the percentage of clones that covered more than 75% of the well area was significantly lower in the EMD-treated clones when compared with the untreated clones (10.9 ± 11.1 vs 23.8 ± 14.7; $P = 0.022$) . In conclusion, EMD decreased the percentage of PDLF with capabilities of arising colonies with 75–100% confluency probably by increasing their differentiation.

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Current evidence suggests that periodontal ligament fibroblasts (PDLF) play a major role in the periodontal wound healing process following periodontal disease and dental trauma (1, 2). The hypothesis is that PDLF have the ability to migrate, proliferate and differentiate into the three main groups of cells that regenerate the periodontium: fibroblasts and mineralized tissue forming progenitors (1, 3, 4). The

mineralized tissue forming progenitors are believed to give rise to the cementoblastic and osteoblastic lineage (1, 3–7). The key for successful treatment of avulsed tooth is the preservation of the fibroblastic progenitor pool in order to facilitate immediate repopulation of the denuded root surface by fibroblasts and consequently prevent the osteoblasts from attaching the cementum (8, 9). Previous approaches to stimulate the growth and/or differentiation of PDLF by using growth factors have shown that, although it is possible to enhance tissue formation, the lack of cellular specificity could generate potential problems when applying this treatment in clinical periodontal regeneration (10–15).

A new approach for promoting periodontal regeneration has utilized enamel matrix derived proteins (16–18). During enamel tooth development, the enamel-forming cells or ameloblasts secrete a protein matrix that becomes organized into sheets of crystallites and ultimately forms layers of enamel (19). Amelogenins, the major proteins of these intercellular layers, are well conserved through evolution and are thought to play a significant role in cementogenesis (20, 21). The porcine enamel matrix (Emdogain, EMD), which consists predominantly of amelogenins, but also contains other growth factors, was shown to promote *in vitro* periodontal ligament cell proliferation, collagen production and to enhance mineralization (22). *In vivo*, when EMD was applied in experimental periodontal defects, *de novo* formation of cementum, periodontal ligament-like tissue and bone was observed (23, 24).

As EMD has been proposed to support the regeneration of periodontal tissues we hypothesized that these proteins might also be efficient in preserving the size of the periodontal ligament progenitor pool and in promoting the proliferation of its progenitors. The aim of the present study was to test this hypothesis by evaluating the clonogenic capacity of PDLF that were explanted and cultured in the presence of EMD.

Materials and methods

Periodontal ligament cells

Cultures of human periodontal liagment (PDL) cells were obtained by explantation and maintained in our laboratory as previously described (25). PDLF were obtained from explants of fully erupted premolar teeth extracted from four children aged 12–17 years for orthodontics reasons. Following extraction, the tooth was transferred to a test tube containing culture medium with 10 times concentrated AB. Under sterile conditions, the PDL tissue was mechanically removed by scraping the middle third of the root surface with a sharp blade. These tissue explants were placed in 25 cm^2 tissue culture flasks (Falcon, Oxnard, CA, USA) precoated with foetal calf serum (FCS). The cells were incubated at 37°C in humidified air with 7% CO_2 for 6 weeks. The medium was replaced every 3–4 days for 5 weeks. Trypsin/EDTA incubation was used to harvest the cells.

General procedure

Explants of periodontal ligament from a young permanent healthy tooth were attached to four FCS-coated flasks and cultured in culture medium composed of α-minimum essential medium (MEM) supplemented with 15% FCS, and an antibiotic solution (ABX1) consisting of Penicillin (100 units ml^{-1}), Gentamicin (50 μg ml^{-1}) and Fungizone (0.3 μg ml^{-1}). After initial outgrowth (10 days to 2 weeks following explantation), the culture medium of two experimental flasks was replaced with medium supplemented with 100 μg ml^{-1} EMD, whereas the other two served as controls and were fed with regular medium. The culture medium was changed every 4 days for 5 weeks. At the day of the experiment, the medium was suctioned; the cells were washed (X3) with phosphate buffered saline containing Ca^{++} Mg^{++} and harvested with 1 ml 0.025% trypsin + 0.02% EDTA. The detached cells were harvested to a test tube, centrifuged (250 ***g***, 5 min, 15°C) and the pellet was re-suspended in 2 ml of fresh culture medium. The cells from experimental and control flasks were then evaluated for their viability and tested for their clonogenic capacities as described below. This experiment was repeated four times from four different donors. The Ethics Committee of Tel Aviv University approved the study. Written consent was obtained from all parents of the participating patients before extraction, which was performed free of charge.

Emdogain

A new batch of a commercial Emdogain® gel (Biora AB, Malmö, Sweden), a formulation of porcine Enamel Matrix Proteins (which is co-packed with PerioGlas to improve handling of the material) was used for each of the three experiments. In the fourth experiment, 10 mg of lyophilized EMD (porcine enamel matrix derivate, as lyophilized protein, received from the Biora AB) was dissolved in 5 ml of α-MEM for 24 h at 4°C, and stored at 4°C until use. EMD or Emdogain® was added to the prepared culture medium to reach a concentration of 100 μg ml^{-1} EMD.

Viability

The cells were counted in hemocytometer and the viability was determined by trypan blue exclusion test (26). Cells from each flask were counted four times and the average of the counts recorded. The viable cells were analyzed for clonogenic capacities as described below.

Clonogenic capacity

To evaluate the clonogenic capacity of the cells, cells from each group were inoculated into six 96-well plate at a concentration of one viable cell/two wells and were allowed to grow for 5 weeks as described above (in each experiment 12, 96-well-plate were used). The culture medium of experimental cells was supplemented with 100 μg ml^{-1} EMD, whereas the controls were fed with regular medium. The percentage of cells with clonogenic capacity was determined as the number of colonies formed/number of cells seeded × 100 in the experimental and control groups. To determine the capacity of EMD to support the proliferation of the progenitors, the percentage of well area covered by the cloned cultured was evaluated by a blind assessor. Three degrees of dish area coverage were utilized: up to 25%, between 25 and 75% and higher than 75%.

The experiments were repeated four times, in each of which the cells were from explants of different tooth.

Alkaline phosphatase expression

Cloned PDLF were plated in 24-well plates and cultured for 48 h in α-MEM supplemented with 12% FCS and antibiotics. Thereafter, the cultured cells were washed with saline, stained with Naphtol AS-MX and Fast Violet B salt (Sigma, St Louis, MO, USA) for 30 min and fixed in 70% ethanol for 10 min (5). The percentage of alkaline phosphatase positive cells among cells were determined for each clone.

Mineralized tissue formation

Cloned PDLF were plated in 24-well plate (20 000 cells per well) and cultured in medium supplemented with 50 μg ml^{-1} vit C (Merck, West Point, PA, USA) 10^{-8} M dexamethasone and 10 mM β-glycerophosphate; Sigma). The medium replaced every third day. After 28 days the cultures were fixed in a solution of water:methanol:formalin (1:1:1.5 v/v) for 10 min, stained in a saturated solution of alizarin red S (BDH chemicals Ltd, Poole, UK) pH < 4, for 30 min, washed in water and dried. Utilizing image analysis, the amount of mineralized-like tissue formed in culture by each culture was estimated by determining the percentage of dish area occupied by red-stained material in each of the wells (6)

Statistical Analysis

Paired *T*-test was used to evaluate the differences between clonogenic capacities of EMD treated vs untreated cultures.

Results

A total of 2328 cells were evaluated, half of which were cultured with Emdogain. The results of the clonogenic capacity at different stages of confluency are summarized in Table 1. Out of the evaluated cells, 576, 576, 576, and 600 cells were evaluated from each of the four different donors aged 12–17 years. A total of 864 cells were cultured in the presence of commercial Emdogain while 300 in the presence of lyophilized EMD. The mean percentage of cells (from all donors) that exhibited any clonogenic capacity in the presence of EMD was comparable with that of cells cultured in the absence of EMD: 26.6 ± 14.3% as compared with 34.6 ± 20.6% ($P = 0.186$). Similarly, the percentage of clones that proliferated to cover up to 25% of the well area was comparable in the two groups 7.5 ± 8.6 for EMD-treated clones and 7.1 ± 7.8 for untreated clones ($P = 0.674$). The percentage of clones that proliferated to cover 25% up to 75% of

Table 1. Data presented as number of cells proliferated to the level of confluency/cells seeded

	75–100% confluent		25–75% confluent		25% confluent		Total	
	Without EMD	With EMD	Without EMD	With EMD	Without EMD	With EMD	Without EMD	With EMD
Explant 1	36/288	19/288	4/288	12/288	5/288	12/288	45/288	43/288
Explant II	120/288	76/288	15/288	3/288	6/288	2/288	141/288	81/288
Explant III	86/288	30/288	21/288	45/288	53/288	58/288	160/288	133/288
Explant IV	33/300	1/300	4/300	35/300	18/300	15/300	55/300	51/300
Total	275/1164	126/1164	44/1164	95/1164	82/1164	87/1164	401/1164	308/1164

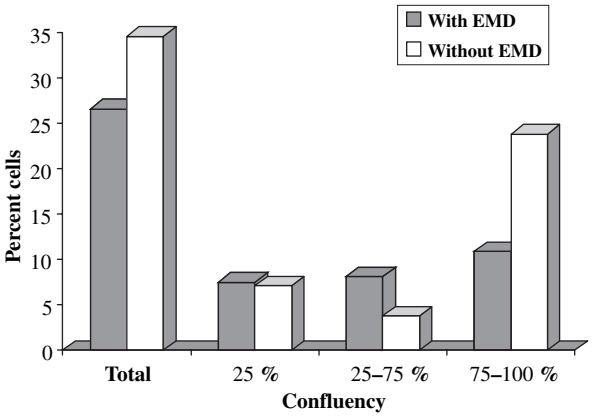

Fig. 1. Comparison of the percent of periodontal ligament cells proliferating into the various levels of confluency. Summarized data of 2328 seeded cells.

the well area was greater EMD-treated clones when compared with the untreated cells: 8.1 ± 6.7 vs 3.8 ± 3. However this difference was not statistically significant ($P = 0.277$). In contrast, the percentage of clones that covered more than 75% of the well area was significantly lower in the EMD-treated clones as compared with the untreated clones (10.9 ± 11.1 vs 23.8 ± 14.7; $P = 0.022$) (Fig. 1).

The percentage of PDLF clones that expressed alkaline phospatase was higher in clones grown with EMD (62.5% vs 12.5%, sum of 40 clones in two experiments). Similarly, the percentage of PDLF-clones that expressed mineralized tissue formation was higher in clones grown with EMD (58.8% vs 35.7%, sum of 31 clones in two experiments).

Discussion

The present study showed that enamel matrix derivates decreased the percentage of PDLF with capabilities of arising colonies with 75–100% confluency. The reasons for this decrease could be attributed to decreased adhesion, or to increased differentiation of PDLF that were grown in the presence of EMD. The possibility of decreased adhesion ability could be ruled out because the total clonogenic capacity of PDLF grown with or without the presence of EMD was statistically comparable ($P = 0.19$). This finding is in accordance with Gestrelius et al. (22) who showed that EMD had no significant effect on attachment and spreading of periodontal cells. Thus, it could be speculated, that EMD decreased the percentage of PDLF with capabilities of arising colonies with 75–100% confluency by increasing the differentiation of the cultured PDLF. This assumption is reinforced by the findings that the percentage of PDLF clones expressed alkaline phospatase and mineralized tissue formation was higher in clones grown with EMD and by the finding that the percent of PDLF arising colonies with 25–75% confluency was higher by 113% in cells cultured in the presence of EMD, thus more progenitor cells underwent differentiation process, which limited their ability to proliferate into confluency. These findings are in accordance with other *in vitro* studies, which showed that EMD increased the differentiation of cells into mineralized tissue forming cells (22, 27–30). The differentiation of the PDLF into mineralized tissue forming cells is also confirmed by several clinical studies, which showed that EMD improved periodontal healing following periodontal surgery as a result of periodontitis (16–18). In contrast to periodontal healing following periodontitis, which necessitates increase in differentiation of PDLF into mineralized tissue forming cells, periodontal healing following dental trauma necessitates the opposite differentiation pathway. Therefore optimal healing will take place in conditions that will preserve the progenitors cells or increase the differentiation into fibroblasts forming cells.

A few clinical studies used EMD to prevent the development of replacement resorption following avulsion, or to limit the progression of already developed replacement resorption. Most of these clinical studies were published following short follow-up periods, and most of them admitted that EMD may delay but not really prevent the development of replacement resorption which is one of the most serious complication following dental trauma (31–33).

Moreover, a recent study by Schjott and Andreasen (33) evaluated the ability of Emdogain to prevent progressive root resorption following replantation in 16 teeth, or to revert the ankylosis stage to a normal PDL situation in 11 teeth. They concluded that Emdogain was not able to prevent or cure ankylosis.

The results of the present study indicated that EMD decreased the percentage of PDLF with capabilities of arising colonies with 75–100% confluency, and thus might decrease the ability of the PDLF to repopulate the denuded root surface following the re-implantation of avulsed teeth. Thus the data of the present study and previous published data that EMD increased the differentiation of cells into mineralized forming cells suggest that EMD by itself is not efficient in regenerating the traumatized periodontal tissues of avulsed teeth. Further studies should address the question whether the utilization of EMD in combination with mitogenic factors might be an effective treatment modality in this respect.

Conclusion

EMD decreased the percentage of PDLF with capabilities of arising colonies with 75–100%

confluency probably by increasing their differentiation.

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