

Enamel Matrix Derivative in Diffusion Chamber Implanted Subcutaneously in Rat Induces Formation of Fibrous Connective Tissue Containing Abundant Blood Vessels

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Abstract. *Background: Enamel matrix derivative (EMD) is widely used for regeneration therapy in dental clinical situations, but the mechanism of EMD bioactivity remains obscure. To clarify this mechanism, we focused on the formation of connective tissue and blood vessels. The aim of this study was to confirm whether EMD induces the formation of connective tissue and blood vessels by using the diffusion chamber (DC) technique. Materials and Methods: Individual DCs containing EMD (DC-EMD) or propylene glycol alginate (PGA) were implanted subcutaneously in rat dorsum. At 4 weeks after the implantation, histological analysis of DCs was performed using azan staining. Results: DC-EMD induced the formation of much larger amounts of connective tissue containing abundant blood vessels than did DC-PGA. Conclusion: The results indicated that EMD can induce the formation of both connective tissue and blood vessels. This bioactivity may contribute to the mechanism whereby EMD induces tissue regeneration.*

Enamel matrix derivative (EMD) appears to stimulate the healing of soft tissue as well as that of bone tissue, and has

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been widely applied in regeneration therapy for implant, periodontic, and endodontic treatments (1-3). EMD has been shown to stimulate osteoblast proliferation and differentiation (4, 5) and collagen production (6), implying that EMD may be a strong inducer of tissue mineralization. On the other hand, *in vitro* experiments have indicated that EMD has little effect on the bioactivities of osteoblasts or fibroblasts (7, 8). To resolve this contradiction, we hypothesized that EMD stimulates angiogenesis in tissues. In fact, some studies have reported that EMD strongly stimulates the accumulation of vascular endothelial growth factor (VEGF) in connective tissue (9, 10), thereby stimulating angiogenesis. This effect may represent a critical contribution to the wound-healing process in both soft and hard tissues. To test this hypothesis, we focused on the effects of EMD on angiogenesis, including the establishment of a unique experimental approach employing the diffusion chamber (DC) technique (11).

The DC technique was originally developed to examine osteoblast differentiation and the effects of various growth factors on osteoblast differentiation and proliferation (12). We placed EMDs into DCs and then implanted the resulting DC-EMDs subcutaneously in rat. This model was used to examine the effects of EMD on angiogenesis.

In the present study, we examined the effects of EMD on angiogenesis using the DC technique in combination with histochemical analysis.

Materials and Methods

DCs were assembled using a Millipore filter membrane in combination with plastic rings (pore size, 0.45 μm ; internal diameter, 9 mm; thickness, 2 mm; Millipore Corporation, Bedford, MA, USA) (Figure 1A). After sterilization using ethylene oxide gas,

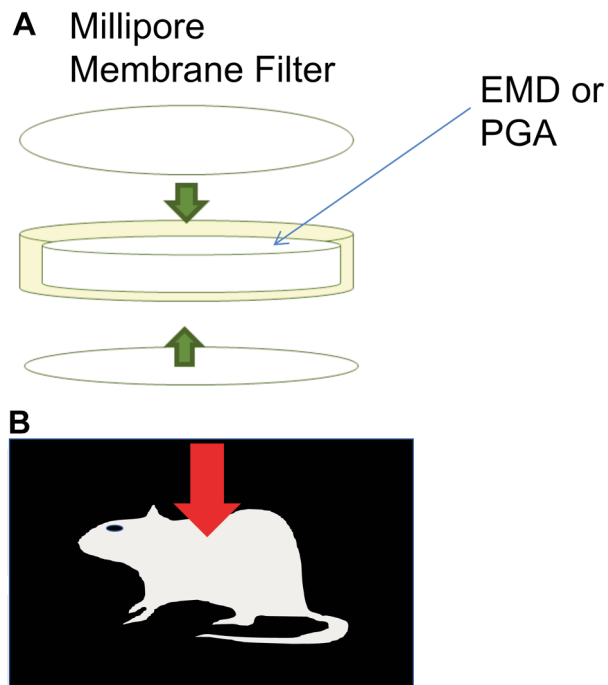


Figure 1. Schematic of the assembly of a diffusion chamber (DC), which is composed of two pieces of membrane filter and a plastic ring. The enamel matrix derivative (EMD) or propylene glycol alginate (PGA; control matrix) then was inserted into the DC (A). The resulting DC-EMD and DC-PGA then were implanted subcutaneously in rat dorsum (B).

EMD (Emdogain, Institute Straumann JP, Tokyo, Japan) was placed in the DC through a hole in the ring, which then was plugged; this construct was designated a DC-EMD. A control was generated using propylene glycol alginate (PGA; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), which is normally included in EMD as a carrier material. Specifically, a negative control DC (designated a DC-PGA) was constructed by placing PGA in a DC as above.

Animal experiments were performed according to a protocol approved by the Animal Affairs Committee of Meikai University (approval no. A1739). Six female Sprague-Dawley rats, 8 weeks of age, were used in the experiment. Each rat had one DC-EMD and one DC-PGA implanted subcutaneously on the right and left sides, respectively, of the dorsum (Figure 1B). At 4 weeks post-implantation, the DCs were recovered and fixed in 10% neutral buffered formalin. The DCs then were embedded in paraffin wax and subjected to serial sectioning. The resulting sections were stained with azan to visualize the structures of connective tissue and blood vessels. Staining was performed according to the technique described by Fukuda *et al.* (13). Specifically, the sections were immersed in 10% (w/v) potassium dichromate solution containing 10% (w/v) trichloroacetic acid for 15 min. After washing with distilled water (DW), the sections were stained with 0.1% (w/v) azocarmine G (Fujifilm Wako Pure Chemical Corporation) for 5 h. After washing with DW, the sections were immersed in 0.1% (v/v) aniline in 95% (v/v) ethanol for 5 s and then in 0.1% (v/v) acetic acid in 95% (v/v) ethanol for 1 min. The sections were washed with DW, and then

incubated in 5% (w/v) phosphotungstic acid solution for 1 h. After washing with DW, the sections were stained with aniline blue-orange G solution (Fujifilm Wako Pure Chemical Corporation) for 1 h. The stained sections then were observed under light microscopy, including counting of the number of blood vessels per field.

The results are expressed as mean±SD. Statistical analysis between the two groups was evaluated using the Wilcoxon *t*-test. A *p*-value less than 0.05 was considered significant.

Results

Figure 2 shows examples of DCs recovered from a rat at 4 weeks post-implantation. Each DC-EMD was surrounded by fibrous connective tissue. In contrast, each DC-PGA was surrounded by only a small amount of connective tissue.

Figure 3 shows histological structures for DCs recovered from rats at 4 weeks post-implantation. The sections of azan-stained DCs-EMD demonstrated the presence of connective tissue composed of blue-stained collagen bundles containing abundant blood vessels filled with red blood cells. On the other hand, the sections of azan-stained DCs-PGA revealed the presence of a thin layer of connective tissue covering the membrane. This tissue layer contained few blood vessels.

Figure 4 shows the results of counting the number of blood vessels in the connective tissue on each DC. The number of blood vessels in the connective tissue on the DCs-EMD was significantly greater compared to that on the DCs-PGA.

Discussion

EMD is a strong inducer of cell differentiation for various tissues. Multiple experiments have reported that EMD can stimulate wound healing in a number of different tissues, with these effects depending on the orchestrated bioactivities of many kinds of cells during wound healing. Although some articles have reported that EMD stimulates osteoblast differentiation (4, 5), other research has not detected biological effects in *in vitro* systems (7, 8, 14). We hypothesized that this apparent contradiction may reflect the intimate role of vascularization in tissue differentiation. If the bioactivities of EMD reflect the induction of angiogenesis, this putative contradiction would be partially resolved. Because the environmental changes caused by angiogenesis are unique in living organisms and are not seen under the culture conditions. To examine the role of EMD in the induction of vascularization, we employed the DC technique as part of our experimental design. Notably, EMD placed in a DC would be expected to provide slow diffusion of EMD and associated factors through the membrane, out of the chamber, and into the body, facilitating analysis of the bioactivities induced by EMD. In fact, DCs implanted subcutaneously in rat have been shown to provide various bioactivities induced by transforming growth factor-β, bone morphogenetic proteins, fibroblast growth factors, and

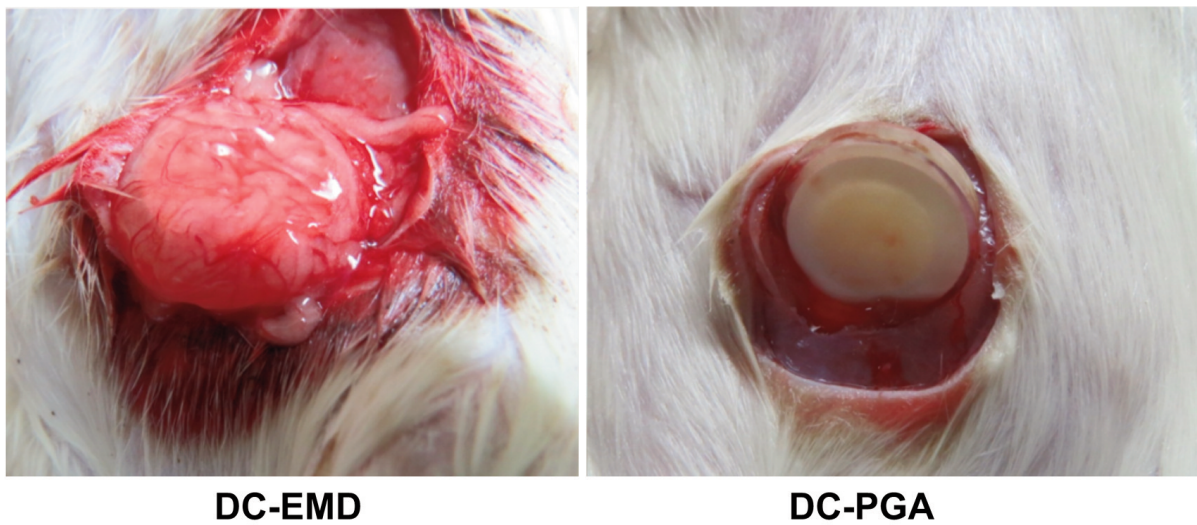


Figure 2. At 4 weeks after subcutaneous implantation, the diffusion chambers with enamel matrix derivative (DC-EMD) or propylene glycol alginate (DC-PGA) were recovered from rats.

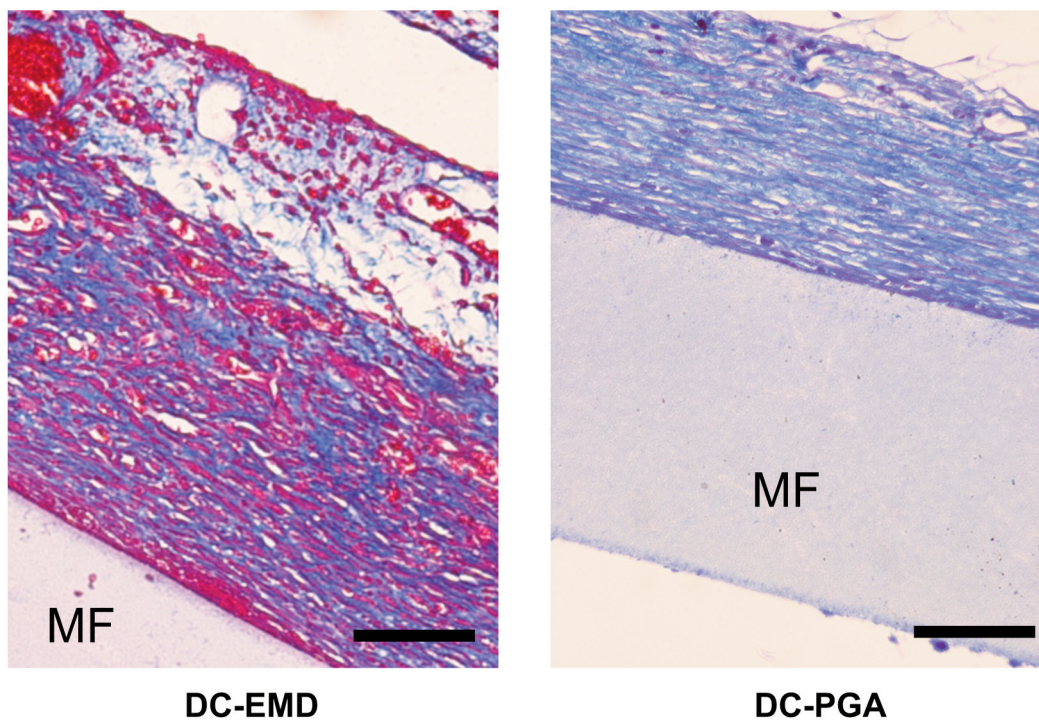


Figure 3. Azan staining was performed on sections of the diffusion chambers with enamel matrix derivative (DC-EMD) or propylene glycol alginate (DC-PGA, control matrix). Collagen bundles stained with blue and blood vessels filled with red blood cells can be clearly seen. Bar: 50 μ m. MF: Millipore filter membrane.

platelet-derived growth factors (15). On the other hand, DCs also can be employed to examine systemic factors. We have analyzed the mechanism of post-menopausal osteoporosis-induced estrogen deficiency using DCs (11). Experiments

using DCs containing osteoblast-like cells implanted subcutaneously in ovariectomized rats demonstrated that osteoblast differentiation is controlled by insulin-like growth factors acting as systemic factors under conditions of

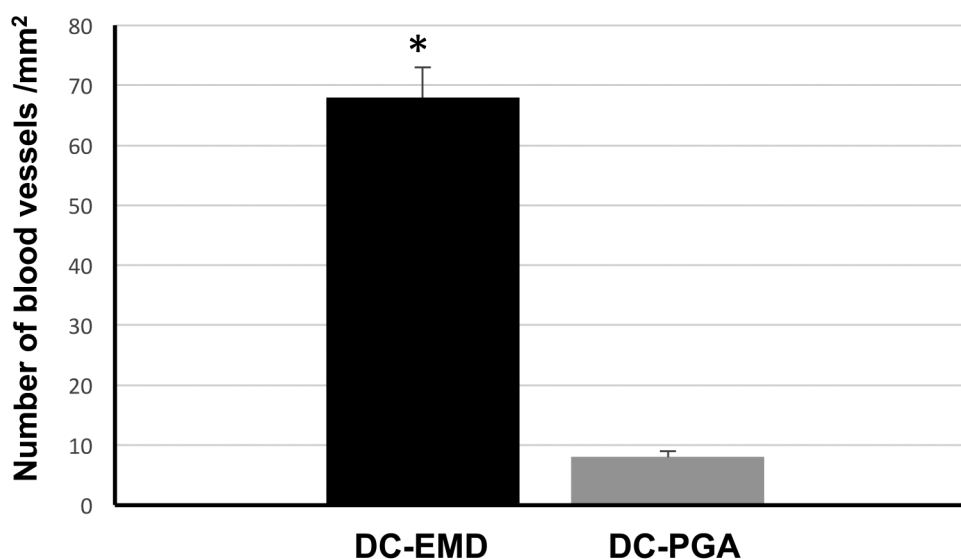


Figure 4. The number of blood vessels was counted in the connective tissue induced by each of diffusion chambers with enamel matrix derivative (DC-EMD) or propylene glycol alginate (DC-PGA, control matrix). Data are presented as mean \pm SD of six DCs. *Significantly different from DC-PGA at $p<0.05$ by Wilcoxon *t*-test).

estrogen deficiency. For these reasons, we used DCs to examine the bioactivities of EMD. The results of our experiment demonstrated that EMD can strongly induce the formation of connective tissue with abundant blood vessels. Previous work has shown that EMD induces VEGF expression in endothelial cells (9) and that the proliferation of those cells is stimulated by VEGF (9, 10, 16). Thus, EMD is capable of inducing vascularization in various tissues. Given that EMD is employed clinically in dentistry for tissue regeneration, it is notable that angiogenesis has a key role in the proliferation and differentiation of various tissues.

It has been reported that EMD induces VEGF expression in endothelial cells, a phenomenon intimately correlated with angiogenesis. On the other hand, the present work demonstrated that implanted DCs containing EMD apparently induce the formation of connective tissue containing abundant blood vessels. Together, these observations indicate that EMD induces the formation not only of blood vessels, but also fibrous connective tissue. The fibrous connective tissue induced by EMD presumably contains precursor cells that can differentiate into various kinds of tissues (17), and the resulting blood vessels play an active role in both cell proliferation and differentiation. We postulate that the strong induction of both fibrous connective tissue and blood vessel formation may be an important aspect of the biological effects of EMD.

In conclusion, we demonstrated that EMD in DCs induces the formation of fibrous connective tissue containing abundant blood vessels. These results suggest that EMD may find applications in regeneration therapy for multiple tissue types.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

S.Y. performed all experiments and wrote the article. Y.K. and K.M. supported the animal experiments. H.S., P.R.K., T.H., and H.K. edited and revised the article.

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