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Initial attachment of osteoblasts to various guided bone regeneration membranes: an *in vitro* study

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Objective and background: Guided bone regeneration (GBR) has proved to be a suitable and somehow predictable technique for promoting bone regeneration. A variety of synthetic and naturally derived GBR barriers have been used in clinics to facilitate bone regeneration. These barriers may differ in composition and structure and these may affect the outcomes of GBR. Therefore, the present study was undertaken to evaluate the *in vitro* ability of osteoblasts (MC3T3-E1) to attach to various GBR membranes.

Materials and methods: Six GBR/GTR (guided tissue regeneration) membranes [BioMend® (BM), Resolut® (RL), Guidor® (GD), EpiGuide® (EG), Gore-Tex® (GT) and Millipore filter® (MP)] were tested. For controls, cells were directly plated on culture dishes (CD). Each test membrane was secured to the bottom of a culture dish with a double-sided adhesive tape. All samples were triplicate. At 1.5 and 24 h after plating of 2 ml (5×10^4 cells/ml) of MC3T3-E1 (passage 7) cells, the specimens were rinsed with phosphate-buffered saline to wash out any unattached cells and then fixed with a 10% buffered formalin solution for 1 d. After washing with distilled water, the cells were stained with hematoxylin. The number of attached cells was counted under a light microscope equipped with an ocular-micrometer in a unit area of 0.25 mm^2 (five areas on each membrane). In addition, cell morphology attached to the membranes was evaluated under scanning electron microscope.

Results: Data were presented as mean ± standard error and analyzed for statistical difference using a generalized Wilcoxon's test. Cell attachment at 1.5 h was as follows: MP (27.5 ± 2.1) > RL (17.0 ± 1.4) ≈ BM (14.5 ± 1.4) ≈ EG (11.4 ± 1.0) > GD (5.2 ± 0.8) ≈ GT (3.1 ± 0.6); and at 24 h was: MP (67.6 ± 3.6) > RL (35.8 ± 1.8) > BM (15.4 ± 0.9) ≈ EG (13.3 ± 1.3) > GD (5.9 ± 0.7) ≈ GT (5.6 ± 1.3). At 24 h, the scanning electron microscope finding revealed that cells attached on MP, RL, BM and EG were flatter in shape, like cells on CD, than cells on GD and GT, where cells were rather round.

Conclusions: Results from this study suggested that MP, BM, RL and EG enhanced the early osteoblast attachment. However, the true benefit of this observation in clinic remains to be determined.

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Guided bone regeneration (GBR) has slowly become an acceptable method in clinical dentistry to facilitate augmentation of alveolar ridge defects, to promote implant wound healing, and to repair/regenerate implant defects. A variety of synthetic and naturally derived GBR barriers have been developed, tried and have showed promising results (1). These barriers may differ in composition and structure, but they all function as mechanical barriers to prevent epithelial and connective tissue cell migration from gingiva in order to facilitate regenerative potential cells (2, 3).

Ideally, barriers should facilitate cell attachment and promote migration of the progenitor cells. In order for osteoblastic progenitor cells to proceed with the wound healing cascade, proliferation, differentiation and tissue maturation, cells need to adhere to a substrata first (4–6). This attachment process involves a four-step sequence that includes adsorption of glycoproteins to the substrate surface, cell contact, attachment and spreading (5, 6). Cell replication begins only after these events have occurred (7).

Salonen and Persson reported, in an *in vitro* model, that low protein binding capacity of the expanded polytetrafluoroethylene (Gore-Tex®; GT, W.L. Gore and Associates Inc., Flagstaff, AZ, USA) material and the rough-textured surface of the GT inhibit epithelial cell migration (7). Payne *et al.* further demonstrated that calcium sulfate appeared to facilitate human gingival fibroblasts attachment and spreading while cells on GT and polylactic acid barrier (Guidor®; GD, John O. Butler Co., Chicago, IL, USA) exhibited a morphology not conducive to migration and had very limited number of cell attachment (8). A similar finding was also illustrated by Simain-Sato *et al.*, who reported that fibroblasts cultured on Resolut® (RL, W.L. Gore and Associates Inc., Flagstaff, AZ, USA) showed rounded oval cells and cell fragments (9). In addition, Gabriel *et al.* indicated that only a small amount (< 4.6 cells/mm^2) of human gingival fibroblasts attached to GT, GD and non-expanded high density PTFE membrane, and that there were no significant differences between membranes (10). Furthermore, Machtei *et al.* reported that the presence of connective tissue cells on the inner surface of the retrieved membrane is one of the factors that promotes periodontal bone regeneration (11). However, little is known about how different composition and structures of membranes may influence osteoblastic attachment. Thus osteoblast affinity and morphology to the commonly used GBR barriers should be studied. Therefore, the purpose of this investigation was to examine the ability of osteoblast (MC3T3-E1), *in vitro*, to attach on six various commercially available GBR barrier materials.

Materials and methods

Membranes examined

Six commercially available GBR/GTR (guided tissue regeneration) membranes were examined: Millipore filter® (cellulose ester mixture) (MP, Millipore Corp., Bedford, MA, USA), Gore-Tex® (expanded polytetrafluoroethylene) (GT, W.L. Gore and Associates Inc., Flagstaff, AZ, USA), BioMend® (collagen) (BM, Sultzer Calcitek Inc., Carlsbad, CA, USA), Guidor® (polylactic acid ester) (GD, John O. Butler Co., Chicago, IL, USA), Resolut (polylactic and polyglycolic polymers) (RL, W.L. Gore and Associates Inc., Flagstaff, AZ, USA) and EpiGuide® (polylactic acid) (THM Biochemical, Inc., Duluth, MN, USA).

Cells

MC3T3-E1 cells, a mouse osteoprogenitor cell line, were maintained in alpha-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum and antibiotics. Cells of passage 7 were used for the attachment assay.

Attachment asay

A double-sided adhesive tape (5 × 5 mm) was used to fix a 7 × 7 mm portion of each test membrane to the bottom of a culture dish. Each test membrane was soaked in the α-MEM solution with 10% fetal bovine serum for 30 to create the environment that MC3T3-E1 cells have been maintained. The medium was then removed and 2 ml (5×10^4 cells/ml) of cells were plated on the membranes. As controls, cells were plated on culture dishes (CD). All samples were made in triplicate. At 1.5 and 24 h after plating, the specimens were rinsed with phosphate-buffered saline to wash out any unattached cells and then fixed with a 10% buffered formalin solution for 1 d at room temperature. After washing with distilled water, the cells were stained with hematoxylin for 10 min. The number of attached cells included in a unit area of 0.25 mm^2 (five areas on each membrane) was counted under a light microscope equipped with an ocular-micrometer.

Scanning electron microscope evaluation

Upon completion of a 24-h cultivation period, each barrier membrane was also prepared for examination under a scanning electron microscope to evaluate cell morphology and attachment to the membranes. Cells grown on the different barriers were fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer, pH 7.4, 4°C, for 1 h and postfixed for 30 min with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4, 4°C). After dehydration in graded ethanols, specimens were transferred into t-butyl alcohol (2-methyl-2-propanol) and freeze dried. Specimens were then sputter-coated with 20 nm of gold and subsequently examined in a JEOL JSM-6300 scanning electron microscope. Photographs were taken at 15 kV using 300 to 600 magnification.

Statistical analysis

The results were statistically analyzed using Wilcoxon's test for non-paired examination. The significance level for rejection was chosen at $P < 0.05$.

Results

During the experimental period, there was no evidence indicating any influence

Table 1. Mean cellular attachment (cells/0.25 mm^2) at 1.5 and 24 h

Barriers	Mean cell attached 1.5 h	Mean cell attached 24 h
CD	38.8 ± 1.7[b]	102.5 ± 4.8[a,b]
MP	27.5 ± 2.1[b]	67.6 ± 3.6[a,b]
RL	17.0 ± 1.4	35.8 ± 1.8[a,b]
BM	14.5 ± 1.4	15.4 ± 0.9
EG	11.4 ± 1.0	13.3 ± 1.3
GD	5.2 ± 0.8[c]	5.9 ± 0.7[c]
GT	3.1 ± 0.6[c]	5.6 ± 1.3[c]

[a]Significant increase ($P < 0.05$) of cells attachment from 1.5 to 24 h.
[b]Significantly great ($P < 0.05$) when compared to all membranes.
[c]Significantly small ($P < 0.05$) when compared to CD, MP, RL, BM and EG.
CD: Culture dish; MP: Millipore filter®; RL: Resolut®; BM: BioMend®; EG: EpiGuide®; GD: Guidor ®; GT: Gore-Tex®.

from membrane toxic residues or the glue from the double-sided adhesive tape. Osteoblastic cell growth on a culture dish adjacent to the membranes and tape was not interfered by the membranes or the glue.

Data were presented as mean ± standard error. Table 1 summarized the mean cellular attachment to the different barrier membranes. CD showed a significant higher amount of cells attached than all the tested materials at 1.5 h and 24 h after the cell seeding. The cell number on CD at 24 h after seeding was around three times that at 1.5 h. Of the six barriers tested, MP had the best osteoblast attachment at both earlier (1.5 h) and later (24 h) time periods and is statistically significant ($P < 0.05$) than all other tested barriers.

RL showed a similar amount of cells attached as that noted in the BM; however, cell number on RL was significantly increased to twice as much at 24 h ($P < 0.05$). At both time periods, BM and EG were equally attached and were significantly better than GD and GT. GD and GT showed the least number of attached cells among the membranes, both at 1.5 h and 24 h.

Figure 1 shows the light microscope view of osteoblasts attached to various barriers at 1.5 and 24 h. Figure 2 illustrates the cell morphology attached to various barriers from scanning electron microscope at 24 h. Cell morphology at 24 h showed that osteoblasts attached to CD were flattened, with numerous cytoplasmic extension and lamellopodia. Similar cell attached morphology was also noted on the RL, BM and EG membranes. On the contrary, cells attached to GT and GD appeared to be round in shape and no observable differences were noted.

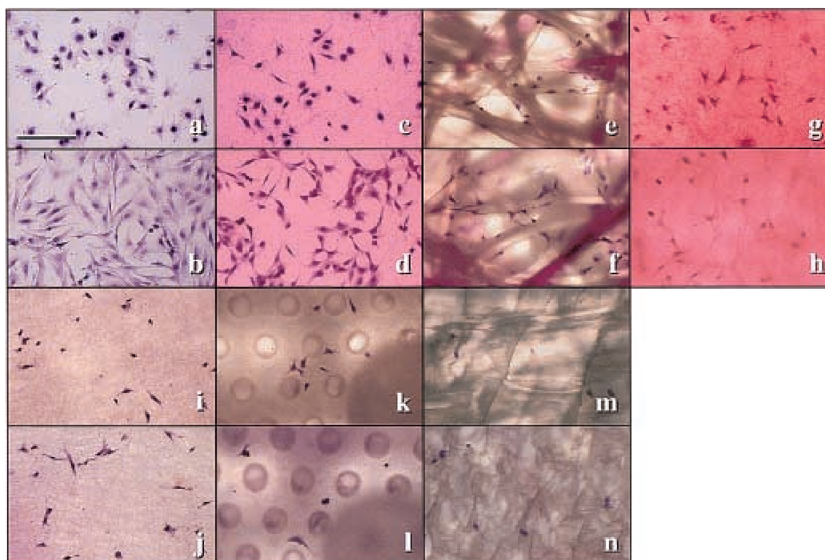

Fig. 1. Light microscope view of osteoblasts attached to various barriers at 1.5 and 24 h (Bar = 200 μm). Culture dish (CD) (a, b), Millipore filter (MP) (c, d), Resolut (RL) (e, f), BioMend (BM) (g, h), EpiGuide (EG) (i, j), Guidor (GD) (k, l), Gore-Tex (GT) (m, n). Figures a, c, e, g, i, k and m are at 1.5 h and figures b, d, f, h, j, l and n are at 24 h.

Discussion

The data from this study demonstrated osteoblast initial attachment and morphology when exposed to different GBR barrier membranes. How factors such as membrane constituents, morphology, adherence ability, protein-binding capacity, substances released during degradation, surface textures, size of perforations and duration of barrier function may influence GBR outcomes has not yet been completely understood. The data from this study demonstrate the ability of osteoblast initial attachment when exposed to different GBR barrier membranes. For a barrier membrane to be successful, initial cell attachment to the materials is essential, since cell replication begins only after the cell has absorbed glycoproteins, contacted, attached and spread on the substrates (4). To allow this action to occur, materials used should have no deleterious effects on cells, plus they should possess the capacity to encourage cell spreading and proliferation.

Under the conditions of this *in vitro* experiment, the mean number of attached osteoblasts was greatest over MP, followed by RL, then BM and EG, with the least amount of attachment noted on GD and GT membranes. The mean numbers of attached osteoblasts over all tested barrier materials were significantly less than those on the CD as the control. This implies that GBR barriers may limit early osteoblast attachment. This may be attributed by influence of different components or structures noted in each membrane. For example, MP showed the greatest mean cell attached. This can be attributed by its cellulose ester

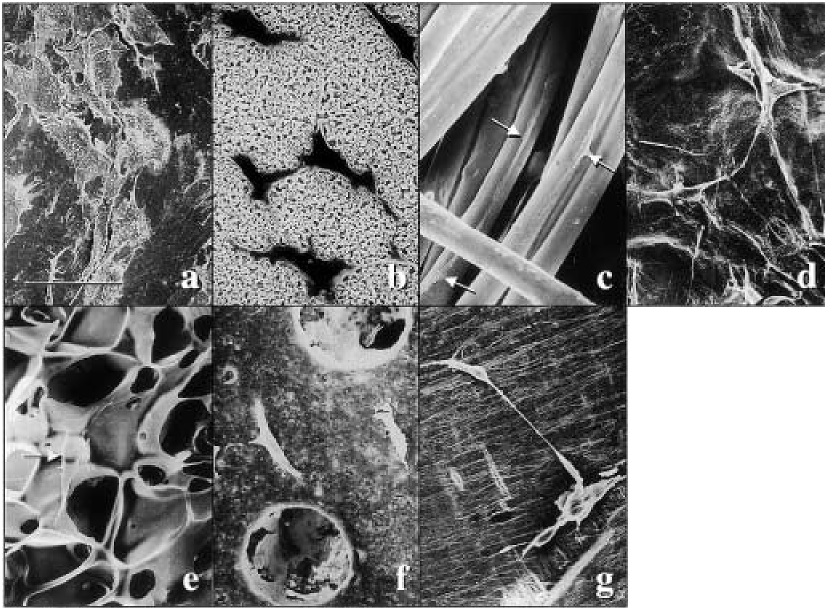

Fig. 2. Scanning electron microscope view of cell morphology attached to various barriers at 24 h (Bar = 5 μm). Culture dish (CD) (a), Millipore filter (MP) (b), Resolut (RL) (c), Bio-Mend (BM) (d), EpiGuide (EG) (e), Guidor (GD) (f), Gore-Tex (GT) (g). Arrows show attached cells.

(i.e. mixture of cellulose acetate and nitrocellulose) component, a higher glycoprotein binding capacity, which promotes cell attachment. Furthermore, cells attached to RL may be influenced by the porous structure of glycolide polymer fiber, since it was a common phenomenon that cells attached on the fibers and migrated on them. However, Payne *et al.* reported opposite findings (8). They reported very limited number of cell attachment and fibroblasts cultured on the glycolide and lactide copolymer. In addition, abnormal flattened 'fired egg' cell appearance was also noted. Similar findings were also illustrated by Simain-Sato *et al.*, who reported that fibroblasts cultured on RL showed rounded oval cells and cell fragments (9). The difference noted in these results may be explained by two different cell types being used; Simain-Sato *et al.* (9) and Payne *et al.* (8) used gingival fibroblasts (from rat and human), whereas MC3T3-E1 osteoblasts were used in the present study. In addition, the acids released from these polymers may affect cell attachment, spreading and migration and further influence the clinical outcomes as speculated by Hammerle (1). However, we did not notice the effect of these acids in this experiment. Further study is definitely needed to clarify whether acid released from the degradation of lactide and glycolide influencing cell culture environment as well as its impacts upon clinical healing/results.

The unique feature of geometry of EG may account for the results and spindle-shaped morphology observed in the study. EG is designed with internal void spaces and is similar to the tooth enamel structure that supports a developing blood clot (fluid) to further promote the invasion of cells into its matrix. The different modifiers, acetyltributylcitrate for EG and glycolide polymer for RL, may account for the minor difference noted. These modifiers, as well as the surface topography and spatial structures of the membranes, may cause the different cellular responses as suggested by Warrer *et al.* (12).

The results observed in BM collagen membrane may be attributed to the collagen molecular structure, which is known to modulate various cell behaviors such as adhesion, spreading and the ability to attracting cells. This is in part agreement with Nagahara *et al.*, who utilized collagen membrane cultured with osteoblastic cells *in vitro* to promote calcification formation *in vivo* (13). Similar results were also found by Locci *et al.* (14). They reported extracellular matrix, which contains primary of collagen and chondroitin-4-sulphate, was the most suitable device to stimulate both cellular proliferation and extracellular macromolecule accumulation. These attempts imply that collagen membrane when placed *in vitro* may facilitate cell attachment and then promote formation of a thin osteoblastic cell layer to eventually enhance bone regeneration. Further confirmation of this hypothesis is required.

Data from this study indicated that GT had minimal cell adherence. This is in agreement with Salonen and Persson, who also found significantly less cell attachment on GT membranes as compared to MP (7). Similar findings have also been reported (8, 9, 14). The lack of adherence may be due to the decreased wettability, the surface roughness created by the overlapping fibrils, and/or the low protein binding capacity (7, 15). The minimal tissue integration to GT may be an advantage for membrane retrieval. However, this may also create potential problems for initial clot formation, wound stabilization and membrane stability, and thus may interfere with wound healing. Nonetheless, its ability to create space may add a tremendous advantage in GBR procedure when compared to other barriers, since one of the most important factors influencing GBR outcomes is the ability of membrane to maintain and create space that is needed for the new bone to grow. Hence, the advantages of minimal cell attachment of GT during GBR remain to be discovered. Further study in this area is needed to find out what is the true effect of minimal cell attachment (i.e. GT) and ability of space maintaining in the clinical setting.

Results from this study showed that GD had minimal osteoblast attachment when compared to other barriers except when compared to GT. Several possible reasons may account for this finding. A slow release of ethylene oxide to the medium may be toxic to the cells. Double layered structure of the GD may trap the cells within the

rectangular pores. The acid component of these materials may be detrimental. However, further studies are needed to confirm these possibilities.

In the present study, there was no increase of cell number from 1.5 h to 24 h for BM, EG, GD and GT, while CD, MP and RL showed two to three times increase of cell attachment. The increased number of attached cells in above-mentioned materials may largely be due to new cells attached, because we observed no cell proliferation during the first 24 h after plating the cells on CD in our previous studies (16, 17). However, the significance of these new cell attachments in clinic remains to be determined.

One of the main regulators of proliferation rate in anchorage dependent cells is shape (18, 19). Cells in a rounded configuration divide at a lower rate than those flattened and well spread on a substratum (18, 19). When attached cell morphology was examined under scanning electron microscope, RL, BM and EG showed flattened morphology that exhibited numerous cytoplasmic extensions, while GT and GD had a rounded appearance. This indicates that GD and GT had a lower proliferative rate than RL, BM and EG.

Cell culture systems serve as excellent models for examination of these events in relation to barrier materials. However, one must be cautious in interpreting results obtained by using an *in vitro* experimental model, since it can not recreate the complex interactions of cells *in vivo*. Although MC3T3-E1 osteoblast cells are a mouse osteoprogenitor cell line, well characterized and grow well in tissue culture, possible differences may occur between these cells and orally derived human osteoblasts.

Results from this limited *in vitro* study suggest that MP, RL, BM, and EG appears to have the best ability to promote initial osteoblast cell attachment. However, future studies are needed to clarify the true clinical benefits of the results observed in this study.

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