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CASE REPORT

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Compatibility of resorbable and nonresorbable guided tissue regeneration membranes in cultures of primary human periodontal ligament fibroblasts and human osteoblast-like cells

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Abstract The purpose of this study was (a) to evaluate the cytocompatibility of three resorbable and nonresorbable membranes in fibroblast and osteoblast-like cell cultures and (b) to observe the growth of those cells on the various barriers by scanning electron microscopy (SEM). Primary human periodontal ligament fibroblasts (HPLF) and human osteoblast-like cells (SAOS-2) were incubated with nonresorbable polytetrafluoroethylene (ePTFE) barriers and resorbable polylactic acid as well as collagen membranes. Cytotoxic effects were determined by XTT (mitochondrial metabolic activity) and sulforhodamine B assays (cellular protein content). In addition, HPLF and SAOS-2 grown for 21 days on the investigated barriers were evaluated by SEM. Data were analyzed statistically by ANOVA using the Wilcoxon-Mann-Whitney test ($P<0.05$). No changes were established in the periodontal ligament fibroblasts and human osteoblast-like cells after incubation with the collagen membrane. Cytotoxic effects, however, were induced by the polylactic acid barrier which slightly inhibited cell metabolism of the periodontal fibroblasts (XTT: 90.1%±3.6 of control value). Moderate cytotoxic reactions were caused by the nonresorbable ePTFE membrane in HPLF-cultures (XTT: 82.7%±3.5) and osteoblast-like cell monolayers (XTT: 80.0%±0.6%). Mitochondrial activity in both cell cultures was significantly reduced by ePTFE barriers in comparison to nonincubated control cells ($P = 0.028$). SEM analysis of cell behavior on barriers demonstrated the differences between these materials: collagen barriers were densely populated with HPLF and SAOS-2, whereas only few or no cells were seen to adhere to the ePTFE and polylactic acid membranes. Our findings indicate that the collagen barrier investigated is very cytocompatible and may be integrated into connective tissue well. On the contrary, the ePTFE and polylactic acid membranes induced slight to moderate cytotoxic reactions which may reduce cellular adhesion. Thus, gap formation between the barrier surface and the connective tissue may be promoted which may facilitate epithelial downgrowth and microbial accumulation. Consequently, these effects may reduce the potential gain in periodontal attachment.

Keywords GTR membranes · Resorbable · Cytocompatibility · Periodontal fibroblasts · Osteoblast-like cells

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Introduction

In general, two different types of guided tissue regeneration (GTR) barrier are available: nonresorbable and bioresorbable membranes. Various materials are used for the fabrication of those barriers. The first commercial GTR barriers were made of nonresorbable expanded polytetrafluoroethylene (ePTFE) (Gore-Tex). As this type of barrier has to be removed by surgical reentry, bioresorbable membranes are of increasing clinical interest. These barriers are produced with polylactic acid, polyglactin910, type I and III collagen, or other biodegradable components [4, 9]. When using these membranes/barriers, numerous authors have observed new connective tissue attachment as well as new alveolar bone formation depending on the guided tissue regeneration (GTR) technique employed [2, 3, 7, 17, 20, 23, 27]. In addition, GTR has been successfully used for the treatment of gingival recessions [24]. In this study, we used primary human periodontal ligament fibroblasts for connective tissue and human immortal osteoblast-like cells for bone tissue as in vitro models.

It has been reported that exposure and consecutive microbial contamination of the barrier during the early healing period may impair the results of the GTR treatment [12,16,28]. DeSanctis et al. [6] found that bacterial colonization of ePTFE membranes reduced the potential gain in probing attachment following GTR therapy by al-

most 50%. Furthermore, it was observed that microbial accumulation on GTR membranes in-vivo is significantly dependent upon the material. A lower quantity of microbial plaque was present on polylactic acid membranes in comparison to ePTFE or polyglactin 910 barriers 24 h after application [29].

Besides the surgical aspects, specific physical and chemical features of the membranes may influence the reaction and healing of periodontal tissues after GTR therapy e.g., barrier exposure [8–10]. Only very scant findings have been published about the biological behavior of various membrane materials in dental or medical literature. It has been reported that PTFE and polylactic acid membranes impaired the migration and morphology of human gingival fibroblasts in comparison to a calcium sulfate substrate [18]. These in vitro data were corroborated in vivo with ePTFE membranes retrieved 4–6 weeks after application. Only a small number of connective tissue cells was observed on the barriers by scanning electron microscopy [22]. Similar observations have been made in other disciplines, e.g., gynecology and vascular surgery. Haney and Doty reported that ePTFE membranes retrieved from the peritoneal cavities of women 14 days after implantation revealed no adherent mesothelial cells. Oxidized regenerated cellulose membranes, however, implanted into the peritoneal cavities were intensely infiltrated by peritoneal cells [11]. Expanded PTFE grafts in canine carotid arteries were surrounded by a fibrous membrane 90 days after surgery while the histological appearance of small intestine submucosal autografts was comparable with normal vascular tissue [21].

Due to the very limited information available about the cytotoxicity of GTR barriers and the proliferation of periodontal cells adjacent to or on these membranes it was decided (a) to determine the cytocompatibility of three resorbable and nonresorbable membranes in cultures of primary human periodontal ligament fibroblasts and human osteoblast-like cells, and (b) to evaluate the growth of those cells on the various membranes by scanning electron microsocopy (SEM).

Material and methods

Cytotoxicity assays

The GTR membranes investigated in this study were nonresorbable Gore-Tex (GT) (ePTFE, expanded polytetrafluoroethylene; W. L. Gore, Flagstaff, Arizona, USA), as well as the biodegradable barriers Guidor (G) (polylactic acid; Guidor AB, Huddinge, Sweden) and Bio-Gide (BG) (collagen type I and III; Geistlich Biomaterials, Wolhusen, Switzerland).

Human primary fibroblasts (HPLF) were cultured from the periodontal ligament of molar roots and grown in a 10% CO_2 atmosphere at 37°C in DMEM (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS). Human osteogenic sarcoma cells (SAOS-2, DSMZ, German Collection of Microorganisms and Cell Cultures/Dept. Human and Animal Cell Cultures, Braunschweig, Germany) were cultured in a 5% CO_2 atmosphere at 37°C in McCoy's medium (Biochrom) supplemented with 15% FCS.

Cell culture inserts (diameter: 10 mm; pore size: 0.2 µm; Nunc, Roskilde, Denmark) were placed in 24-well microculture dishes (Corning, New York, USA) and immersed in serum-free medium for 5 min. Thereafter, 4×10^4 HPLF/mL (from passages 4, 6, 8) and SAOS-2 (from passages 9, 10, 18) were seeded into the inserts (500 µl/insert). Then, 500 µl medium was added to the wells and the cells were cultured for 24 h.

All barriers were trimmed to an approximate size of 5×5 mm, placed on the floor of other 24-well microculture dishes and immersed in serum-free cell culture medium for 15 min. Thereafter, this medium was replaced by medium supplemented with serum. Then, inserts with cells were transferred into the 24-well microculture dishes containing the membranes. Inserts placed into wells without membranes served as controls.

After 24 h of incubation, the mitochondrial metabolic activity and protein content of the cells were determined with XTT assays (working solution: 2.5 mg XTT/15 ml) medium without phenol red, without serum, and with 0.025 mg phenazine methosulfate; Sigma, Deisenhofen, Germany) and sulforhodamine B (working solution: 0.1% sulfo B in 1% acetic acid; Sigma). The XTT working solution (500 µl/insert) was added by medium change. Inserts without cells but with working solution were used as blanks. Metabolic activity of the mitochondria was measured in a microplate spectrophotometer (Molecular Devices, Sunnyvale, USA). The optical density (OD) was determined at a wavelength of 450 nm. The blanks of the microplates were measured at a wavelength of 690 nm.

Thereafter, the fixation solution (90% ethanol, 5% acetic acid, 5% H_2O) was added (500 µl/inserts) and the cells were fixed for 30 min at 4°C. Then, the fixative was removed completely and sulforhodamine B working solution added to the inserts (500 µl/inserts). The cells were incubated for 30 min at room temperature in the dark, then rinsed twice with tap water. To each insert 500 µl of 10 mM TRIS, pH 10.5, was added. The protein content of the cells was measured in the same microplate reader (wavelength 560 nm). Two inserts for each membrane were used for one assay. In order to ensure reproducibility, all cytotoxicity experiments were run thrice at separate times. Each assay was run in four replicates (i.e., total n for each experiment: 12). Metabolic activity of the mitochondria and protein content of the cells were calculated for all assays and compared to the controls. The results were evaluated statistically for significant differences by ANOVA using the Wilcoxon-Mann-Whitney test ($P<0.05$).

Scanning electron microscopy

Cell culture inserts (diameter: 10 mm, pore size: 3 µm; Nunc) were placed in 24-well microculture dishes.

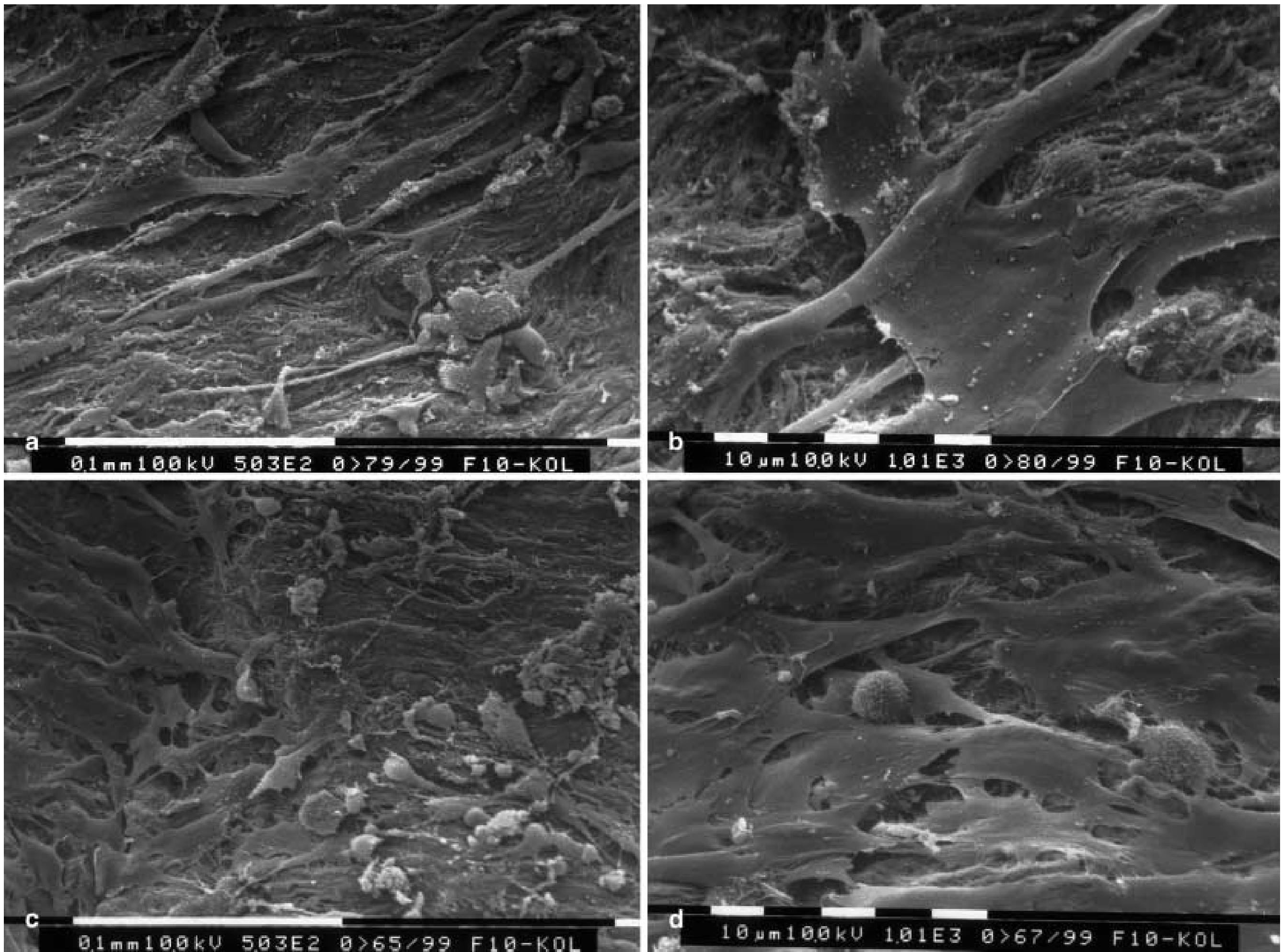

Fig. 1a–d Primary human periodontal ligament fibroblasts (HPLF) and human osteoblast-like cells (SAOS-2) grown on Bio-Gide (BG) barriers; attachment time: 21 days; SEM. **a** HPLF densely grown on the collagen membrane, elongated cells reveal physiologic morphology; original magnification ×500. **b** HPLF cultured on collagen membrane; original magnification ×1000. **c** SAOS-2 cultured on the collagen barrier, numerous adherent cells with physiologic morphology have populated the membrane; original magnification ×500. **d** SAOS-2 densely grown on collagen membrane; original magnification ×1000

Then, 5×10^4 HPLF/ml medium from passage 9 and 5×10^4 SAOS-2 cells/ml medium from passage 25 were seeded onto the backs of the inserts (100 µl). After 6 h attachment time, the inserts were turned upside down and 1 ml medium was added to the wells. After 7 days, the medium was completely replaced by fresh medium. Then, the membranes (5×5 mm) were placed into the inserts and fixed with glass rings. The medium was replaced every second day. After an incubation period of 21 days, each membrane was rinsed gently with 0.1 M buffer to remove nonadherent cells. Thereafter, the barriers were immersed in 2.5% glutaraldehyde with 0.1 M cacodylate buffer at pH 7.4 for 2 h. After fixation, the membranes were prepared for SEM examination by dehydration in a series of graded ethanol solutions ranging from 50% to 100%. Then, the specimens were critical point dried with CO_2. Membranes were sputter-coated with 20 nm of gold-palladium and subsequently examined in a Philips 505 SEM. Photographs were taken at 10 kV. Control membranes were stored for 21 days without cells in medium and then investigated by SEM, too.

Results

Cytotoxicity assays

Mitochondrial metabolic activities and protein contents of the cells are presented in Table 1. The effects of the GT and BG membranes investigated were similar in both cell culture systems. G, however, induced no reduction of metabolic activity of SAOS-2 cells but inhibited metabolism of HPLF slightly.

1. HPLF: Metabolic activity (XTT) of periodontal fibroblasts was significantly reduced by GT to 82.7%±3.5% (P = 0.028). G inhibited mitochondrial activitiy to 90.1%±3.6%, whereas BG caused no effects (99.8%±5%) in comparison to control assays (=100%). No membrane material decreased the protein content of the periodontal fibroblasts.

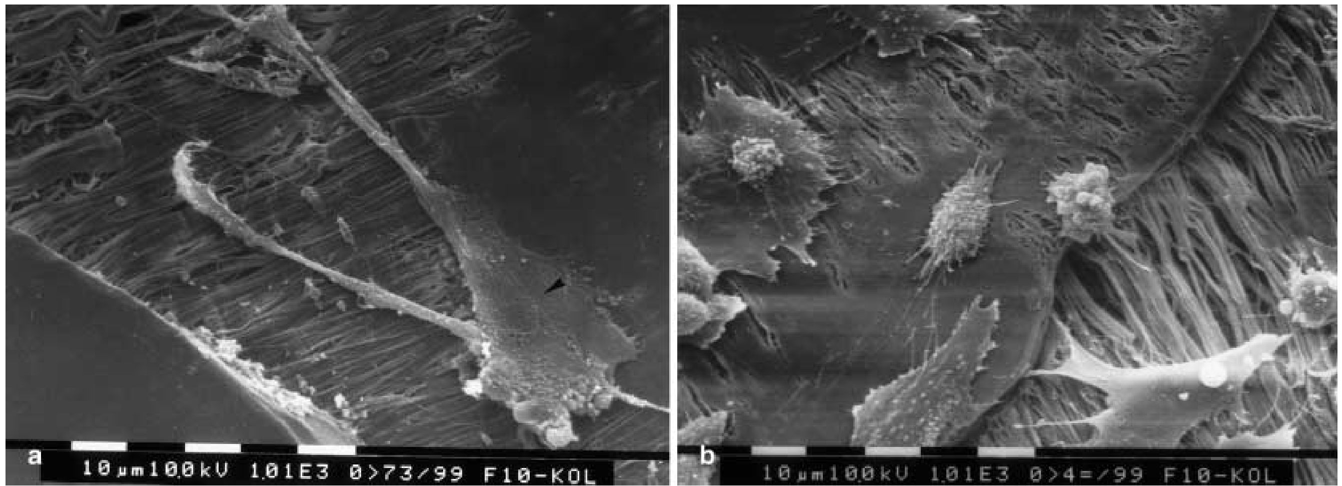

Fig. 2a, b Primary human periodontal ligament fibroblasts (HPLF) and human osteoblast-like cells (SAOS-2) grown on non-resorbable polytetrafluoroethylene (ePTFE) barriers; attachment time: 21 days; SEM; original magnification ×1000. **a** HPLF cultured on the ePTFE membrane, very few flattened cells (*arrowhead*) are visible. **b** SAOS-2 cultured on the ePTFE barrier; few cells are visible

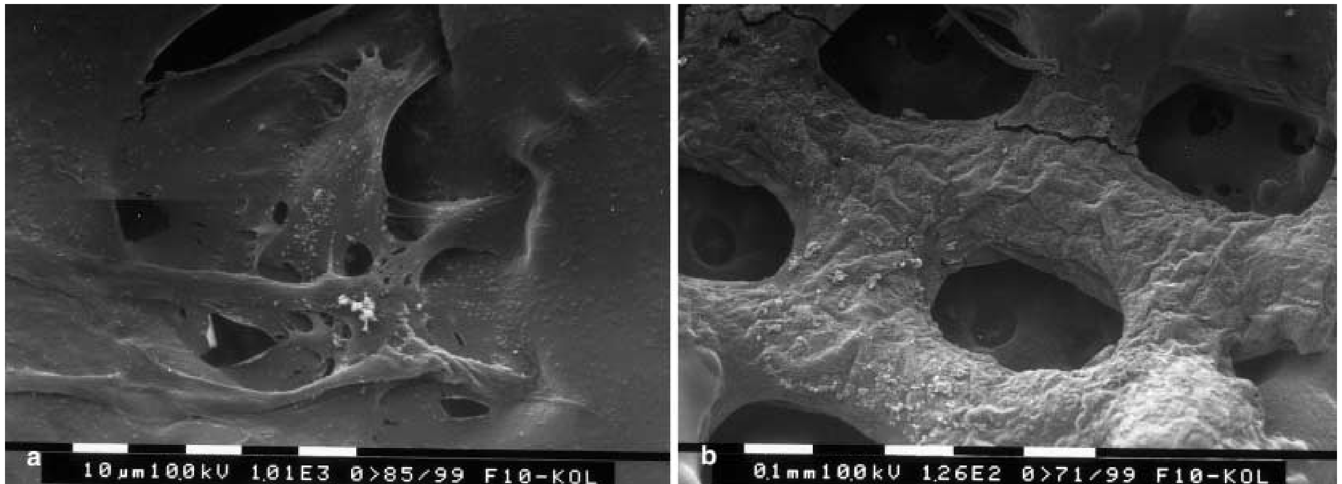

Fig. 3 Primary human periodontal ligament fibroblasts (HPLF) and human osteoblast-like cells (SAOS-2) grown on polylactic acid barriers; attachment time: 21 days; SEM. **a** HPLF grown on the polylactic acid barrier. Few elongated cells adhering to the membrane are visible; original magnification ×500. **b** Polylactic acid membrane 21 days after growth of SAOS-2 cells; even at the lower magnification, which shows a larger area, no adherent cells are visible on the barrier; original magnification ×126

Table 1 Mitochondrial metabolic activity (XTT assay) and cellular protein content (sulforhodamine B assay) of human periodontal ligament fibroblasts (HPLF) and human osteogenic sarcoma cells (SAOS-2) after being treated with the various barrier materials for 24 h (values are expressed in % referred to control assays (100%)±SD; each n=3 with 4 replicates each)

	Gore-Tex		Bio-Gide		Guidor	
	XTT	SULFO B	XTT	SULFO B	XTT	SULFO B
HPLF	82.7±3.5[a]	110.4±16	99.8±5	108.4±4	90.1±3.6	101.2±1
SAOS-2	80.0±0.6[a]	104.3±6	95.8±7.9	95.9±11	105.7±5	100.2±4

[a] Statistically significant reduction in mitochondrial activitiy (XTT) in comparison to control assays (P=0.028).

2. SAOS-2: GT also significantly reduced mitochondrial metabolic activity (XTT) of SAOS-2 cells to 80%±0.6% ($P = 0.028$). BG and G, however, caused no significant decrease in the XTT assay in comparison to the controls. In addition, no significant alteration of protein content was induced by any of the membrane materials investigated.

Scanning Electron Microscopy

SEM examination revealed attachment and physiologic morphology of HPLF and SAOS-2 grown on membranes: generally, HPLF and SAOS-2 exhibited different morphology. Fibroblasts were elongated whereas SAOS-2 exhibited a more stellate-like cell shape. On BG membranes both cell types appeared flat with cytoplasmic extensions and lamellopodia (Fig. 1a–d). The cell density of both culture systems, HPLF and SAOS-2, was significantly higher on BG (Fig. 1a–d) than on GT (Fig. 2a, b) and G (Fig. 3a, b). No significant difference, however, was observed between GT and G. Those barriers were only populated by very few cells (Figs. 2a, b, 3a, b). Furthermore, no adherent human osteoblast-like cells were found on G (Fig. 3b). These observations indicate that adherence of HPLF and SAOS-2 to GT and G is very low.

Discussion

The aims of periodontal GTR are to prevent epithelial apical downgrowth adjacent to the root and promote coronal proliferation of cells derived from the periodontium. Thus, a membrane is placed between the denuded root surface and the repositioned mucogingival flap, providing a secluded space into which fibroblasts and osteogenic cells originating from the healthy apical portion of the periodontium may migrate [3,20]. Apart from the barrier function at the root surface, the membrane must also prevent downgrowth of the epithelium adjacent to the barrier device which might cause exposition and/or microbial colonization of the membrane [19]. These objectives are obtained by application of a biocompatible material integrated into the connective tissue. It was the purpose of our study to determine the compatibility of various barrier materials in human cell cultures which are comparable to the regenerative cells of the periodontium. Primary human periodontal ligament fibroblasts as well as human osteoblast-like cells were used for our experiments. At present, resorbable and nonresorbable membranes with different compositions are employed for the GTR technique. These devices may consist of “classic” nonresorbable ePTFE, synthetic resorbable polylactic acid, natural resorbable type I and III collagen, and other biodegradable, synthetic substances. In order to compare the cytocompatibility of the different types of membrane (biodegradability, composition), used in periodontology and dental implantology, three representative products (Gore-Tex, Guidor, Bioguide) were selected for this investigation.

Our results indicated that the biodegradable collagen membrane investigated exhibited excellent cytocompatibility. No changes in the periodontal ligament fibroblasts and human osteoblast-like cells were found. On the contrary, cytotoxic effects which may be caused by released substances were induced by the polylactic acid barrier. This material reduced the metabolism (XTT) of periodontal fibroblasts slightly. Moderate cytotoxic reactions, however, were caused by the nonresorbable ePTFE membrane. This device significantly reduced the mitochondrial metabolism of human periodontal ligament fibroblasts and human osteoblast-like cells. No comparable studies with these cell types have been reported by other authors. Payne et al. investigated the migration of human gingival fibroblasts over ePTFE and polylactic acid membranes. They found that both barriers inhibited migration and induced cell death [18]. These observations as well as our SEM data indicate that those materials may exhibit impaired tissue integration in vivo in comparison to the collagen membrane tested. Thus, epithelial downgrowth at the surface of ePTFE and polylactic acid membranes may result in barrier exposition and bacterial plaque accumulation, which may impair the outcome of the treatment [19,22,29]. Accordingly, it was found that ePTFE membranes, removed 6 weeks postoperatively, were often populated by epithelial cells, indicating that this barrier cannot totally exclude epithelium [26]. In addition, bacterial colonies were identified extending into the midthird of ePTFE barriers retrieved 4–6 weeks after surgery. Fibroblast-like cells were only seen in the midthird and deep areas of the membranes [22]. Similar findings were reported by Nowzari and Slots [16] and Yoshinari et al. [26]. Their studies revealed that ePTFE membranes are often colonized with periodontal pathogens which may significantly reduce periodontal attachment gain.

Comparable data have been evaluated in other medical disciplines, e.g., vascular surgery and gynecology. Expanded PTFE membranes and degradable oxidized regenerized cellulose barriers (ORC) were retrieved from the peritoneal cavities of women up to 14 days after insertion. It was found that the ePTFE devices were completely enveloped by nonadherent mesothelial cells, forming a “pseudoperitoneum”, while the ORC membranes were infiltrated and resorbed by peritoneal fluid cells [11]. A comparison of the healing of small intestine submucosal (SIS) and ePTFE grafts in canine carotid arteries revealed that a fibrous capsule surrounded the ePTFE membranes while the intestinal autografts had a histological appearance similar to normal arteries after 90 days [21]. These results may be at least partially explained by observations reported by Krause et al. and Wakabayashi et al. who investigated the differential production of inflammatory cytokines (e.g., interleukins 1 and 4) on the surface of surgical biomaterials, including ePTFE barriers for GTR and guided

bone regeneration (GBR) [13,25]. ePTFE induced marked interleukin production indicative of host/foreign body interaction [13].

In addition, it was observed that ePTFE arterial prostheses were significantly less resistant to bacterial infection than autografts constructed of SIS [1]. Fistula formation and multispecies bacterial contamination were found after a myomectomy and implantation of a ePTFE-membrane which was partly inserted into a hole in the anterior wall of the bladder [15]. These studies show that ePTFE may be rapidly and intensely colonized by bacteria when implanted in a milieu with a high concentration of microorganisms. In general, data published in dental and medical literature underscore the prevention of microbial migration onto implanted membranes by intense tissue integration which may be achieved by the application of a highly cytocompatible barrier material.

The SEM observations corroborate our cytotoxicity data as well as the clinical findings of several authors [14, 22]. It must be emphasized that nonadherent fibroblasts and osteoblast-like cells were removed by rinsing the membranes with 0.1 M cacodylate buffer prior to SEM evaluation. Both cell types grew intensely and became attached to the collagen membrane. The polylactic acid and ePTFE barrier, however, only exhibited few or no attached periodontal fibroblasts and osteoblast-like cells after rinsing. These findings indicate that the collagen membrane examined may be tightly integrated into the connective tissue, in contrast to ePTFE and polylactic acid barriers. Thus, gap formation and epithelial downgowth as well as microbial apical migration on the surface of ePTFE and polylactic acid membranes may be facilitated.

In summary, this investigation suggests that the collagen barrier examined is highly cytocompatible and may, therefore, be readily integrated into connective tissue. In contrast, the ePTFE and polylactic acid membranes induced slight to moderate cytotoxic reactions. These effects may impair cellular adhesion and thus may promote gap formation between the barrier surface and the connective tissue which may contribute to reduced attachment gain after GTR treatment.

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