Functional Characteristics of Gingival and Periodontal Ligament Fibroblasts

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Abstract. In periodontal surgery, healing after Guided Tissue Regeneration (GTR) may be explained by differences in functional activities of gingival and periodontal ligament fibroblasts (GF and PDLF). Several studies in vitro have supported this hypothesis, but much remains to be defined. In the present work, gingival and periodontal ligament fibroblasts derived from five healthy subjects were isolated and compared in vitro. The morphology of the cells was observed under scanning electron microscopy (SEM). Several extracellular matrix components (ECM) were studied to compare the effects on fibroblast attachment, proliferation, and protein synthesis. Several biochemical markers were examined in both cellular extract (CE) and conditioned medium (CM). We also examined the muscle differentiation markers alpha-smooth muscle actin, desmin, and smooth-muscle myosin. Finally, we studied the effects of epithelial cells on the proliferation and protein synthesis of the two types of fibroblasts. GF and PDLF appeared identical under the SEM. All ECM components enhanced attachment; however, while collagen types I and IV promoted the attachment of GF, gelatin, laminin, and vitronectin promoted that of PDLF. Most ECM components increased the proliferation rate of GF and the biosynthetic activity of PDLF. The biochemical markers were similarly distributed between the two cell types, except for alkaline phosphatase, which was detected only in the CE of PDLF. Both GF and PDLF strongly expressed alpha-smooth-muscle actin and were negative for desmin; only PDLF were positive for smooth-muscle myosin. Epithelial cells increased the proliferation of both GF and PDLF but had no effect on their biosynthetic activity. These in vitro results may better explain the in vivo functional differences between GF and PDLF.

Key words: fibroblasts, attachment factors, biomechanical markers, cytoskeletal proteins.

Introduction

Fibroblasts account for about 5.6% of the total volume of gingival and periodontal ligament connective tissue (900 et al., 1973). In case of injury or after periodontal surgery, these cells are activated: they proliferate, migrate into the wound site, and synthesize new matrix components until the defect has been corrected (for review, see Narayanan and Page, 1983). Several studies have shown that when gingival connective tissue is allowed, after surgery, to come into contact with the root surface, root resorption will occur. On the contrary, when periodontal ligament cells are selectively allowed to come into contact with the root surface, regeneration of a functional periodontium can be observed histologically (Melcher, 1976; Boyko et al., 1988; Meyer, 1986; Engelberg, 1987). Based on such observations, recent surgical techniques have focused on regenerating the periodontal ligament (Niemann et al., 1982, 1987; Gottlow et al., 1986; Cuisnier et al., 1989). By the use of membranes of various kinds, such techniques of guided tissue regeneration can limit the apical migration of gingival epithelial cells and favor the establishment of a new attachment by periodontal fibroblasts.

Such histological and clinical findings anticipate important differences between fibroblasts from gingival connective tissue (GF) and fibroblasts from periodontal ligament (PDLF). Several investigators have shown that the morphology and growth rates of both types of fibroblasts are similar (Ohshima et al., 1988; Somerman et al., 1988; Hou and Nyberg, 1993). However, the functional properties of GF and PDLF have rarely been studied and compared. For instance, it is known that, as compared with GF, PDLF contain high levels of alkaline phosphatase (Ohshima et al., 1988; Somerman et al., 1988), but the contents of other biochemical markers have, to our knowledge, not been studied and compared. Whereas the GF and PDLF to different types of biological attachment factors or to the presence of co-cultured epithelial cells—as well as their content and the distribution of cytoskeletal proteins (Beertsen et al., 1974; Ponder and McCulloch, 1991)—have received little attention (Somerman et al., 1988; Hou et al.,
The aim of the investigation presented here was to study several of these functional characteristics.

**Materials and methods**

**Isolation and culture**

Connective tissue cells were obtained from the periodontal ligament of premolar teeth and adjacent healthy gingiva or interdental papilla from five individuals. The patients were all white males, with a mean age of 22.5 years (range, 25 to 30 years), undergoing tooth extractions for orthodontic reasons. Prior to extractions, informed consent was obtained from the patients or their guardians. After three washes in Dulbecco's modified Eagle medium (DMEM, Fakoiso) supplemented with 10 mM L-penicillin and streptomycin (Gibco), the gingival and periodontal ligament tissues were cut into small pieces, plated in Petri dishes, and incubated in DMEM supplemented with 10% heat-inactivated fetal calf serum (Gibco) at 37°C in humidified air containing 5% CO₂. When confluent, the cells surrounding the explants were transferred to tissue culture flasks by 0.05% trypsin (Gibco) and were designated as "first transfer cells" (P₁). Two pairs of GF and PDLF mass cultures were obtained from each patient, and the two cell populations were compared at the same passage between the first and the fifth transfers. The statistical significance of the results was evaluated with Student's t-test.

**Morphological characteristics**

Gingival and periodontal ligament fibroblasts (GF and PDLF) grown on glass coverslips were observed by scanning electron microscopy (SEM). Briefly, the cells were rinsed in 0.1% cetylpyridinium chloride and fixed with 3% glutaraldehyde in the same buffer for 1 h. They were then stained with aqueous osmium tetroxide for 30 min, dehydrated with graded alcohols, critical-point-dried, and sputter-coated with gold palladium. The morphology of the cells was observed after 3 hrs under the SEM. Random fields were recorded at different magnifications on Polaroid films.

**Effects of extracellular matrix components on the attachment, proliferation, and protein synthesis**

The following putative attachment agents were used: collagen type I (Sigma C 8914), at a concentration of 40 µg/mL; collagen type IV (Sigma C 5313) (40 µg/mL); fibronectin (Sigma F 2068) (20 µg/mL); and laminin (Sigma L 2020) (10 µg/mL), and vitronectin (Sigma V 8379) (20 µg/mL).

Uncoated bacteriological 96-well plates were conditioned and incubated overnight at 4°C in 0.1 mL of the above putative attachment factors. In addition, fresh serum-free medium was added to the cells to render them quiescent. The following day, the cells were rinsed with PBS. Ten pairs of GF and PDLF, from five healthy patients and belonging to the third passage, were added to the wells at a concentration of 3 x 10⁴ in 0.1 mL of DMEM.

The attached cells were counted by the method of Finlay et al. (1984) after incubation periods increasing from 40 min to 6 to 24 hrs. After each incubation time, the cells were stained with methylene blue (5 g/L in 50% ethanol), rinsed, air-dried, and solubilized in PBS. Absorbance at 620 nm was measured. Preliminary experiments showed good agreement between cell counts obtained by this method and those performed with a hemocytometer. Results were expressed as the percentage of cells attached representing the maximum number attached.

The effects of attachment agents on GF and PDLF were studied after an incubation period of 6 h. The following putative attachment agents were used: 2 µg/mL rat IgG, 1-methyl thymine phosphorylase (METP), 1000 U/mL hyaluronidase (1000 U/mL), 1 mg/mL bovine serum albumin (BSA), and 100 µg/mL protease with respect to each well for 6 hrs. The medium was then removed and replaced with PBS. The cells were dissociated with 100 µg/mL EDTA, pipetted into counting vials containing 100 µL of 0.5% trypsin (0.5%), and then counted in a Coulter counter (Packard), and the radioactivity counted in a scintillation fluid.

**Biochemical markers**

Several enzymes and inhibitors were examined. The conditioned medium (CM) and cellular extracts of GF and PDLF belonging to P₁, P₃, P₄, and P₅ (passage number) were examined for the activity of the enzyme by the free portion of the inhibitor. The enzyme was inhibited by 50% macroglobulin (Salvesen et al., 1988; Gianopoulos et al., 1992), which was measured with an hemoglobin substrate. The activity of the inhibitor was measured with the fluorescence substrate Meso-Suc-Ala-Ala-Pro-methylcoumarin (MW 527.69) (Bachem, Switzerland) (Castillero et al., 1979); Batsch, phosphatase activity was measured with the use of SDS-Gelin-PAGE gels. Finally, the use of SDS-microglobulin and 1-antitrypsin was measured with the use of SDS-Gelin-PAGE gels. Finally, the use of SDS-microglobulin and 1-antitrypsin was measured with the use of SDS-Gelin-PAGE gels.

**Expression of cytoskeletal proteins**

Three major cytoskeletal proteins were examined: immunofluorescence: α-smooth muscle actin, smooth-muscle myosin. For the determination of α-smooth muscle actin and desmin, GF and PDLF of first, third, and fifth passages were obtained from five individuals grown on tissue culture dishes (Nunc) as described earlier. Before becoming

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The gingival tissue was rinsed with antibiotics and digested in Dispase. The digested cells were obtained from the epithelium by dispersing on 35 x 10 mm Primaria dishes and incubated in a keratinocyte growth medium (37°C in humidified air containing 5% CO₂). Filter-feeding experiments, epithelial cells of the first culture, were seeded at a density of 40,000 cells on Transwell inserts prepared with 0.5 mm pores in diameter, 0.4 μm pore size, and a 1 mm-high insert provided by Costar. Cells were loaded with 6.15 μCi of 0.15 μCi/ml [3H] thymidine (NEN-Du Pont, Boston, MA) for 4 h and then harvested using the solvent 0.1 N HClO₄. The DNA was dissolved in 0.1 N HClO₄ and the radioactivity was counted in a liquid scintillation counter (Beckman, Model LS 6000). The percentage of incorporation of [3H] thymidine into DNA of the cells was calculated according to the following formula:  

\[
\text{Percentage of Incorporation (API)} = \frac{\text{Experimental Counts per Minute (CPM)}}{\text{Control CPM}} \times 100
\]

The results were subjected to t-test (Student's t-test) and were expressed as mean ± standard error of mean (SEM) in triplicate. Data were considered significant at the level of 0.05 by Student's t-test.

**Results**

**Isolation and culture**

By day 20, gingival and periodontal ligament fibroblasts were observed extending from almost all explants. By days 30 to 35, they were confluent and ready to be trypsinized and transferred. Generally, in the primary cultures, the proliferation of GF was faster than that of PDLF, but such differences in growth rates were not statistically significant (data not shown). Comparison of the growth rate of passage cultures also indicated no differences between GF and PDLF (data not shown). Trypan blue exclusion after trypsinization showed that 98% of the cells were viable.

**Morphological characteristics**

Under the SEM, GF and PDLF were similar. They appeared rounded, with a spherical nucleus in the center and typical prolongations (Fig. 1).

**Effects of extracellular matrix proteins**

When seeded onto plastic surfaces, less than 40% of GF and PDLF cells attached within 90 min (Fig. 2a). On the contrary, when plastic surfaces were coated with collagen type I and IV, almost 85% of GF and more than 50% of PDLF cells attached within 90 min. Collagen type I and type IV were more effective in promoting GF attachment, as compared with PDLF. With fibronectin, both GF and PDLF demonstrated the same degree of enhanced attachment. Gelatin, laminin, and vitronectin were more effective in promoting PDLF adherence. The same tendency was observed after an incubation period of 6 hrs (Fig. 2b), with, of course, many more cells attached. After 24 hrs, GF and PDLF attached similarly on all plastic- and protein-covered surfaces (data not shown).
When cells were incubated in the presence of matrix proteins, the incorporation of 3H-thymidine was significantly increased for GF but not for PDLF (Fig. 3). Such increase varied from 1.5- to 2.3-fold, respectively. Results are expressed as percentage, with 100% representing the mean ± standard deviation of the mean. The highest degree of attachment was obtained at 6 hrs. The highest degree of attachment was observed with GF on collagen type I and PDLF derived from different patients.

Chemical markers

Among biochemical parameters, only the activity of alkaline phosphatase was significantly different between GF and PDLF. ALPase could be routinely detected in the cellular extract of PDLF, while GF contained only traces (data not shown). Enzyme activities, plasminogen activators, matrix metalloproteinases, and the inhibitor α2M were similarly distributed in GF and PDLF of the same passage (data not shown). No α1AT could be detected in either GF or PDLF. An increase in the expression of these markers was observed for all pairs of GF and PDLF with each increasing passage (data not shown).

Expression of cytoskeletal proteins

In PDLF of the first passage, the percentage of positive cells was higher than in GF. SM-actin-positive cells increased in number of passages in both GF and PDLF. The fifth passage were positive for actin and smooth muscle, while PDLF were positive for desmin and smooth muscle. The mean number of positive cells was significantly higher for PDLF than for GF. Only PDLF at passage strongly expressed smooth muscle. PDLF of early passages were SM-actin negative.

Co-culture of fibroblasts with epithelial cells

Epithelial cells significantly stimulated the growth of fibroblasts in both GF and PDLF. On the contrary, no significant effect was observed on the biosynthetic activity of either GF or PDLF with each increasing passage (data not shown).

Discussion

Our investigation confirmed that GF and PDLF were different under the scanning electron microscopy in the proliferation of cells. The growth patterns for both GF and PDLF isolations from the same donor were similar. In contrast, the growth patterns for the cells from different donors were different. In conclusion, these results suggest that PDLF may serve as a valuable source of epithelial cells for the study of oral diseases.