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Human periodontal ligament: a niche of neural crest stem cells

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Background and Objective: The periodontal ligament is a specialized connective tissue, derived from dental follicle and originated from neural crest cells. Recently it has been suggested, based on animal models, that periodontal ligament could be a niche for neural crest stem cells. However, there is still little knowledge on this subject. The identification of neural crest adult stem cells has received much attention based on its potential in tissue regeneration. The objective of the present work was to verify the human periodontal ligament as a niche for neural crest stem cells.

Material and Methods: Cells from human periodontal ligament were isolated from 10 teeth of seven individuals (periodontal ligament pool group) and also from four teeth of one individual (periodontal ligament single group), after enzymatic digestion. The cells were cultured in specific inductive medium. Analyses of protein and gene expression were performed through immunocytochemistry and reverse transcription–polymerase chain reaction techniques, respectively.

Results: Mesodermal phenotypes (adipogeneic, osteogenic and myofibroblastic) were identified after culture in inductive medium. Immunocytochemistry analyses showed the presence of the nestin marker of neural stem cells and also markers of undifferentiated neural crest cells (HNK1, p75). When cultured in inductive medium that allowed neural differentiation, the cells showed markers for β-tubulin III, neurofilament M, peripherin, microtubule-associated protein 2 and protein zero. The results were similar between the two study groups (the periodontal ligament pool group and the periodontal ligament single group).

Conclusion: This research provides evidence that human periodontal ligament, in addition to its mesodermal derivatives, produces neural crest-like cells. Such features suggest a recapitulation of their embryonic state. The human periodontal ligament revealed itself as a viable alternative source for possible primitive precursors to be used in stem-cell therapies.

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The periodontal ligamentis a specialized, vascular and highly cellular connective tissue that plays an important role in supporting tooth function, maintaining homeostasis and repairing tissue damage caused by periodontal disease or mechanical trauma (1). Periodontal ligament tissue originates from neural crest-derived ectomesenchyme (2,3) and contains a heterogeneous population of cells, including periodontal fibroblasts, cementoblasts, osteoblasts, endothelial cells and epithelial cells (4). Recently, it has been suggested that human periodontal ligament contains mesenchymal stem cells (5). In addition, multipotent stem cells with mesodermal and neural potentiality have been identified in rat and human periodontal ligament (6,7). However, the mechanisms that regulate the development of periodontal ligament lineages have not been fully elucidated (6). Periodontal diseases are

responsible for a substantial worldwide public health burden (8). The regeneration of periodontal tissue is a major challenge for periodontal therapy (9). On the basis of recent advances in adult stem-cell biology, this research investigates the periodontal ligament tissue as a niche for neural crest stem cells.

Material and methods

Cell culture

A total of 10 normal impacted third molars were collected from seven healthy subjects (18–25 years of age). In addition, four premolars were extracted from one patient (14 years of age), following guidelines approved by the Ethical Committee of the Federal University of Santa Catarina (number 309/06). Periodontal ligament was gently separated from the middle root surface and then digested in a solution containing pancreatin (Sigma, Saint Louis, MO, USA) and α-minimal essential medium supplemented with antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin; Invitrogen, Carlsbad, CA, USA) for 25 min at 37°C. Then, trysin was added to a final concentration of 0.25% and incubation continued for a further 10 min. The trypsin reaction was stopped by the addition of standard medium containing α-minimal essential medium supplemented with 20% fetal bovine serum (Cultilab, Sao Paulo, Brazil) and antibiotics. The cell suspension was centrifuged (500 *g*, 10 min, 22°C) and plated in 25-cm^2 flasks in standard medium. Periodontal ligament samples from different individuals were pooled and are referred to hereafter as the periodontal ligament pool group. The periodontal ligament sample collected from one individual is referred to as the periodontal ligament single group. Cells were grown in standard medium, at 37°C in 5% CO_2 with the medium changed every 4 d, until they reached confluence. Analyses of the periodontal ligament pool group were performed in passages 8–9, and analyses of the periodontal ligament single group were performed in passages 3–4.

Adipogenic differentiation

To induce adipogenic differentiation, 1×10^4 cells/well were seeded on 24-well culture plates and cultured in α-minimal essential medium supplemented with 10% fetal bovine serum, 10^{-6} M dexamethasone (Sigma), 50 μM indomethacin (Sigma) and 10 μg/mL of insulin (Sigma). The medium was changed every 3 d. Control cells were cultured in standard medium. After 30 d, the cells were fixed and stained with fresh Oil-red-O solution (Sigma).

Osteogenic differentiation

To induce osteogenic differentiation, 1×10^4 cells/well were seeded on 24-well culture plates and cultured in α-minimal essential medium supplemented with 10% fetal bovine serum, 10^{-7} M dexamethasone, 50 μg/mL of L-ascorbic acid (Sigma) and 20 mM β-glycerophosphate (Sigma). The medium was changed every 3 d. Control cells were cultured in standard medium. After 30 d, the cells were fixed and subjected to Von Kossa staining.

Inductive medium for the growth of neural crest-derived cells

The potential of periodontal ligament to differentiate into neural crest-derived cells was evaluated using culture conditions previously described for the growth of neural crest (10,11). Briefly, cells were cultured at low density (100 cells/well in 24-well plates) in α-minimal essential medium supplemented with 10% fetal bovine serum, 2% chicken embryo extract, 10 mg/mL of transferrin, 0.1 mg/mL of hydrocortisone, 0.01 ng/mL of glucagon, 1 ng/mL of insulin, 0.4 ng/mL of triiodotironine, 0.1 ng/mL of epidermal growth factor, 1 ng/mL of fibroblastic growth factor 2, 100 μ/ml of penicilin and 100 μg/ml of streptomycin (all from Sigma). The culture medium was changed every 3 d. After 15 d, cells were analyzed by immunocytochemistry and reverse transcription–polymerase chain reaction (RT-PCR), as described below.

RT-PCR

The total cellular RNA was isolated using Trizol reagent (Invitrogen). The extracted RNA was reversed transcribed according to the conventional protocols described below. Isolated RNA was used as a template for the synthesis of cDNA, which was prepared using a ThermoScript RT-PCR first-strand cDNA synthesis kit (Invitrogen). PCR reactions were performed using the following human-specific sense and antisense primers: nestin (sense, 5′-CAGCTGGCGCACCTCAAGATG-3′; antisense, 5′-AGGGAAGTTGGGCTCAGGA-CTGG-3′), β-tubulin III (sense, 5′-AGATGTACGAAGACGACGAGGAG-3′; antisense, 5′-GTATCCCCGAAAATATAAACACAAA-3′), neurofilament M (sense, 5′-GAGCGCAAAGACTACCTGAAGA-3′; antisense, 5′-CAGCGATTTCTATATCCAGAGCC-3′), peripherin (sense, 5′-ATGGCCGAGGCCCTCACCCAAGAG-3′; antisense, 5′-TAGGCGGGACAGAGTGGCGTCGTC-3′), microtubule-associated protein 2 (sense, 5′-CCATTTGCAACAGGAAGACAC-3′; antisense, 5′-CAGCTCAAATGCTTTGCAACTAT-3′), protein zero (sense, 5′-GCCCTGCTCTTCTCTTCTTT-3′; antisense, 5′-CCAACACCACCCCATACCTA-3′), glial fibrillary acidic protein (sense, 5′-GTGGGCAGGTGGGAGCTTGATTCT-3′; antisense, 5′-CTGGGGCGGCCTGGTATGACA-3′) and α-smooth muscle actin (sense, 5′-CGATAGAACACGGCATCATC-3′; antisense, 5′-CATCAGGCAGTTCGTAGCTC-3′), according to the manufacturer's recommendations (Integrated DNA Technologies, Inc., Coralville, IA, USA). Amplification reactions were performed for 35 cycles (denaturation at 94°C for 1 min; annealing for 1 min and extension at 72°C for 1.5 min) with an initial denaturation at 94°C for 2 min and a final 7-min extension at 72°C. The annealing temperatures were 64°C (nestin), 58.5°C (β-tubulin III), 56°C (neurofilament M), 68°C (peripherin), 55°C (microtubule-associated protein 2), 57°C (protein zero), 65°C (glial fibrillary acidic protein) and 51°C

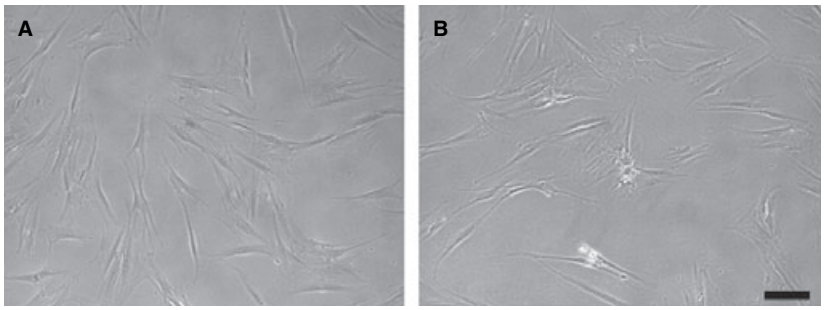

Fig. 1. Morphological analysis of human periodontal ligament cell cultures by phase contrast microscopy. Cell culture was performed, as described in the Material and methods, in standard medium. (A) Periodontal ligament pool group (passage 9). (B) periodontal ligament single group (passage 4). Scale bar = 50 μm.

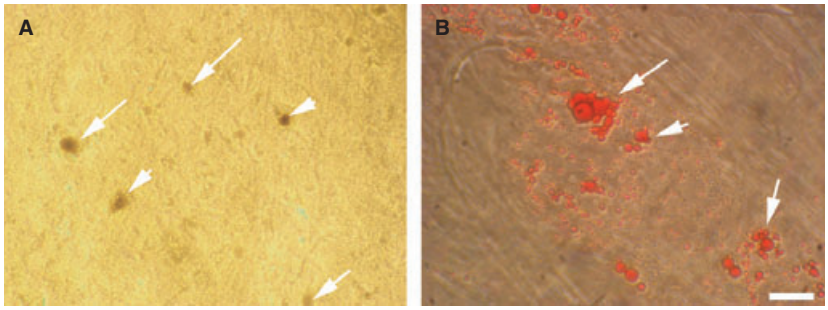

Fig. 2. (A) Osteogenic and (B) adipogenic differentiation of periodontalligament-derived cells. (A) Cultured periodontal ligament cells formed Von Kossa-positive mineralized nodules (arrows) and (B) oil O red-positive lipid clusters (arrows) after 30 d of induction in osteogenic or adipogenic mediim, respectively. Scale bar = 50 μm.

(α-smooth muscle actin). Glyceraldehyde-3-phosphate dehydrogenase expression was used as an internal control of RNA integrity and efficiency of the reverse transcription process. The PCR products were separated by electrophoresis on a 2% agarose gel and were visualized by ultraviolet-induced fluorescence. Two independent experiments were performed.

Immunocytochemistry

The monolayers were fixed in 4% formaldehyde (30 min), washed in phosphate-buffered saline and permeabilized (10 min) with phosphate-buffered saline containing 0.25% Triton X-100 (Sigma, Saint Louis, MO, USA). Cells were incubated with primary antibodies (overnight at 4°C) and then with the secondary antibodies (for 90 min at room temperature–22°C). The following primary antibodies were used: anti-smooth muscle actin (Sigma), anti-β-tubulin III (Promega, Fitchburg, WI, USA), anti-nestin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-p75 (Promega) and anti-HNK-1 (12). Fluorescein isothiocyanate (Invitrogen), Texas red (Santa Cruz Biotechnology), or biotin specific-secondary antibodies were used. The biotin reaction was revealed by the tyramide signal amplification kit (Perkin-Elmer, Boston, MA, USA), according to the manufacturer's instructions. Cell nuclei were stained using 4′6,-diamidino-2-phenylindole (DAPI) (Sigma, Saint Louis, MO, USA). The cells were visualized and photographed using an Olympus BX-40 (Tokyo, Japan) epi-fluorescence microscope. The experiments were performed in duplicate, in two independent experiments.

Results

Cell culture

Cells (periodontal ligament pool and single groups) displayed fibroblastic morphology after 35 d of culture in standard medium. Subconfluence was observed after 8 d, in both experimental groups, suggesting intense cell proliferation. The same cell characteristics were observed in the secondary cultures (Fig. 1A,B).

Osteogenic and adipogenic differentiation

During the 30 d of culture in osteogenic medium, more than 90% of the periodontal ligament cells (pool and single groups) proliferated and produced a dense extracellular matrix characterized by the presence of nodular structures (calcium deposition) when stained using the Von Kossa stain (Fig. 2A). Moreover, after 30 d of culture in adipogenic medium, approximately 20% of cells in both experimental groups displayed an enlarged cell body, as visualized with Oil red O-solution (Fig. 2B).

Gene expression by means of RT-PCR

Table 1 shows the gene expression pattern in human periodontal ligament cells, after 15 d of culture in inductive medium for neural crest cells. Both periodontal ligament groups expressed mRNAs for markers of neural crest (nestin), neurons (β-tubulin III, neurofilament M, microtubule-associated protein 2 and peripherin) and undifferentiated glial cells (protein zero), suggesting ectodermal differentiation. However, the reaction was negative for the differentiated glial cell marker, glial fibrillary acidic protein. The positive expression for peripherin, a specific protein of peripheral neurons, confirmed the neural crest origin of the human periodontal ligament. Moreover, the expression of α-smooth muscle actin was detected, indicating the presence of myofibroblastic cells.

Identification of neural crest-derived phenotypes by immunocytochemistry

At the protein level, cells positive for neural crest-derived phenotypes were indentified in both periodontal

Table 1. Gene expression profile of periodontal ligament (PDL) cells

Markers	PDL pool group	PDL single group
Nestin	+ +	+ +
β-Tubulin III	+ + +	+ + +
Neurofilament M	+ +	+
Microtubule-associated protein 2	+ +	+
Peripherin	+ +	+ +
Protein zero	+	+
Glial fibrillary acidic protein	–	–
α-Smooth muscle actin	+ + +	+ + +

Periodontal ligament cells were cultured in inductive medium for the growth of neural crest cells, as described in the Material and methods. Reverse transcription-polymerase chain reaction was performed as described in the Material and methods using primers for markers of neural stem cells (nestin), neurons (β-tubulin III, neurofilament M, microtubule-associated protein 2 and peripherin), glial cells (protein zero and glial fibrillary acidic protein) and smooth muscle cells/myofibroblasts (α-smooth muscle actin).
PDL pool group: human periodontal ligament cells from seven individuals (passage 9).
PDL single group: human periodontal ligament cells from only one individual (passage 4).
+, weak expression; + +, regular expression; + + +, strong expression; –, negative.

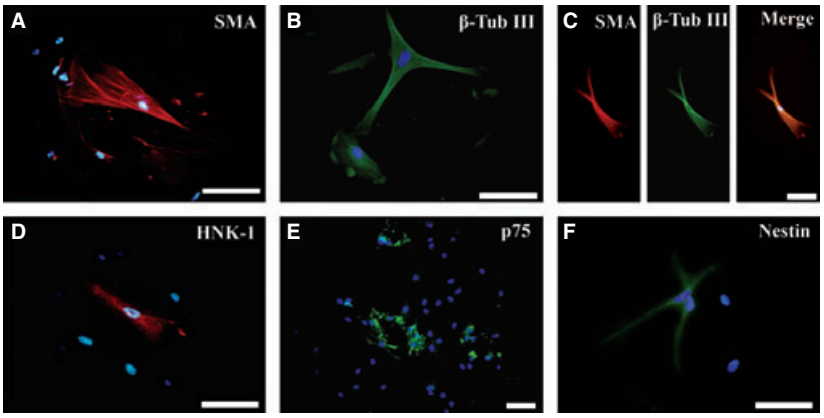

Fig. 3. Differentiation of human periodontal ligament cells in ectodermal and mesodermal derivatives. Cells were cultured in inductive medium for the growth of neural crest cells, as described in the Material and methods. Immunocytochemical analysis was performed to assess the presence of cell-specific protein markers as described in the Material and methods. (A) β-Tubulin III-positive cells (neuronal marker). (B) α-Smooth muscle actin-positive cells (myofibroblast marker). (C) Cells positive for both β-tubulin III (green) and α-smooth muscle actin (red). Cells positive for (D) HNK-1, (E) p75 and (F) nestin (neural crest markers). β-Tub III, β-tubulin III; SMA, α-smooth muscle actin. Scale bar = 50 μm.

ligament groups after 15 d of culture in neural crest inductive medium, corroborating the RT-PCR results. We observed that a high proportion (about 70%) of α-smooth muscle actin-positive cells displayed spread morphology with actin stress fibers, similar to the morphology of neural crest-derived myofibroblasts in culture (Fig. 3A). In addition, about 60% of the cells were positive for β-tubulin III, also displaying spread morphology (Fig. 3B). Interestingly, a small amount of cells (< 20%) were positive for both α-smooth muscle actin and β-tubulin III (Fig. 3C).

Identification of neural crest-like stem cells by immunocytochemistry

Both periodontal ligament groups presented, after 15 d of culture in the same inductive medium, a small proportion (< 10%) of cells positive for the neural crest cell markers HNK-1, p75 and nestin (Fig. 3D,E,F). This suggests the existence of cells with characteristics of undifferentiated neural crest in periodontal ligament cultures, possibly stem cells.

Discussion

During embryogenesis, the periodontal ligament consists of cells derived from the dental follicle which are considered to be neural crest-derived ectomesenchymal cells (13). The neural crest comprises a highly pluripotent cell population that migrates towards the first arch to participate in the formation of the teeth. The neural crest can differentiate into ectodermal and mesodermal cell types (14). In our experiments, using human periodontal ligament cell cultures, we were able to identify, by immunocytochemistry, a small proportion of cells positive for HNK-1 and p75, suggesting the presence of undifferentiated cells with characteristics similar to those of neural crest cells. These markers have been used to identify neural crest stem cells derived from animal embryonic tissues (15,16) and also from human adult tissue (17). Moreover, we observed nestin-positive markers in periodontal ligament cultures by immunocytochemistry and RT-PCR techniques. Nestin corresponds to an intermediate filament protein expressed in neuroectodermal progenitors (18), including neural crest cells (15). Our findings indicate the existence of a small population of neural crest-like cells (< 10%) that express nestin, HNK-1 or p75, possibly corresponding to a population of stem cells or multipotent progenitors residing in the human periodontal ligament.

Previous studies have shown that bone marrow, dental pulp and periodontal ligament tissues contain mesenchymal stem cells, identified by the presence of STRO-1 antigen (5,19,20). STRO-1 was initially associated with the identification of osteogenic precursors isolated from bone marrow and later described as a promising marker for mesenchymal stem cells (21). However, cultured cells progressively lose the expression of this antigen (22). In addition, some studies have reported that STRO-1 is absent in bone marrow mesenchymal stem cells (23) and also in stromal cells derived

from adipose tissue (24). These controversial findings indicate the non-existence of a consensus in validating STRO-1 as a single marker for stem cells. On the other hand, the association of a variety of markers has been suggested as important for the identification of mesenchymal stem cells (25). The objective of our investigation was to identify not only mesenchymal markers in periodontal ligament cultures, but also markers of neural crest or its derivatives. Investigations correlating the expression of STRO-1 and p75 or HNK-1 have been carried out in our laboratory.

Recently, Seo *et al.* (5) and Nagatomo *et al.* (26) demonstrated that periodontal ligament stem cells produce mineralized nodules or Oil-red positive lipidic vacuoles when cultured in osteogenic-inductive or adipogenic-inductive environments, respectively. Under appropriate culture conditions in the present study we observed the formation of calcium deposition after staining with the Von Kossa stain, and noted the presence of adipocite-like cells containing Oil-red-O stained vesicles, suggesting the potential of differentiation to osteoblast and adipocite, respectively. In addition, we observed the presence of α-smooth muscle actin-positive cells, suggesting the capacity for myofibroblastic differentiation. Taken together, these results demonstrate that human periodontal ligament cells exhibit the capacity for differentiation into several mesenchymal lineages, in accordance with previous studies (5,26).

Recently, Techwattanawisal *et al.* (6) and Widera *et al.* (7) identified in periodontal ligament cultures of rats and human, respectively, multipotent adult cells capable of differentiating into neural and mesodermal progenitors, therefore suggesting that the periodontal ligament contains cells with neural crest characteristics. Moreover, cells with the potential for neural differentiation were identified in deciduous and permanent dental pulps (20,27,28). In our experiments, we verified that cells derived from human periodontal ligament produced mesodermal and ectodermal phenotypes. We observed, at the mRNA level, positive gene expression for nestin, β-tubulin III, neurofilament M, microtubule-associated protein 2 and peripherin, suggesting neural differentiation. The expression of nestin and β-tubulin III was also confirmed at the protein level. In addition, the expression of both β-tubulin III and α-smooth muscle actin (Fig. 3C) suggested the presence of bipotent or multipotent progenitors. Alternatively, this effect could be caused by the phenomenon of 'reprogramming' or 'transdifferentiation' of differentiated cells, such as myofibroblasts (α-smooth muscle actin-positive cells), because the neural crest derivatives display great plasticity *in vitro* (11,29). Moreover, we observed the gene expression of protein zero, an earlier marker of glial cells (30). Although the marker of differentiated glial cells (glial fibrillary acidic protein) was not detected, our data indicated that the periodontal ligament-derived cells also showed differentiation potential for the glial phenotype. It is important to emphasize that peripherin is an intermediate filament protein associated with peripheral neurons that are neural crest derived (31). These results corroborate the findings of Techawattanawisal *et al.* and Widera *et al.* (6,7) obtained with rat and human periodontal ligament, respectively, suggesting that human periodontal ligament may be a source of stem cells or of multipotent progenitors with neural crest characteristics.

This study provides further evidence concerning the presence of multipotent cells within the human periodontal ligament. In our cultures we observed the presence of early markers of neural crest cells (HNK-1, p75 and nestin) as well as markers of differentiated ectodermal cells (neurons and glial cells) and mesodermal cells (myofibroblasts, osteoblasts and adipocytes). In addition, we also observed cells with markers for both neuronal-specific (β-tubulin III) and myofibroblast-specific (α-smooth muscle actin) proteins. These results suggest that periodontal ligament cultures contain a heterogeneous population of cells with different levels of maturity, including a small population of multipotent progenitors.

In conclusion, this research shows the human periodontal ligament as a niche for stem cells that are similar to neural crest cells. Because approximately 70% of the human population has impacted third molars (32), periodontal ligament cells could be an accessible alternative source of adult stem cells for use in the regeneration of periodontal tissues. The possibility of obtaining stem cells from cryopreserved adult human periodontal ligament (33) indicates that samples from tissue banks could be viable for future applications. Moreover, the neurogenic potential of periodontal ligament-derived cells suggests that they could also be useful for the treatment of neurodegenerative diseases. However, further research is necessary to confirm the possibility of this clinical application.

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