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Identification of marker genes distinguishing human periodontal ligament cells from human mesenchymal stem cells and human gingival fibroblasts

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Background and Objective: Molecular gene markers, which can distinguish human bone marrow mesenchymal stem cells from human fibroblasts, have recently been reported. Messenger RNA levels of tissue factor pathway inhibitor-2, major histocompatibility complex-DR-α, major histocompatibility complex-DR-β, and neuroserpin are higher in human bone marrow mesenchymal stem cells than in human fibroblasts. However, human bone marrow mesenchymal stem cells express less apolipoprotein D mRNA than human fibroblasts. Periodontal ligament cells are a heterogeneous cell population including fibroblasts, mesenchymal stem cells, and progenitor cells of osteoblasts or cementoblasts. The use of molecular markers that distinguish human bone marrow mesenchymal stem cells from human fibroblasts may provide insight into the characteristics of human periodontal ligament cells. In this study, we compared the molecular markers of human periodontal ligament cells with those of human bone marrow mesenchymal stem cells and human gingival fibroblasts.

Material and Methods: The mRNA expression of the molecular gene markers was analyzed using real-time polymerase chain reaction. Statistical differences were determined with the two-sided Mann–Whitney *U*-test.

Results: Messenger RNA levels of major histocompatibility complex-DR-α and major histocompatibility complex-DR-β were lower and higher, respectively, in human periodontal ligament cells than in human bone marrow mesenchymal stem cells or human gingival fibroblasts. Human periodontal ligament cells showed the lowest apolipoprotein D mRNA levels among the three types of cells.

Conclusion: Human periodontal ligament cells may be distinguished from human bone marrow mesenchymal stem cells and human gingival fibroblasts by the genes for apolipoprotein D, major histocompatibility complex-DR-α, and major histocompatibility complex-DR-β.

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Bone marrow mesenchymal stem cells, also called plastic-adherent marrow cells or bone marrow stromal cells, can differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes, and muscle cells *in vitro and in vivo* (1–3). A recent study has identified several molecular marker genes that distinguish human bone marrow mesenchymal stem cells from human fibroblasts (4). The mRNA levels of major histocompatibility complex-DR-α, major histocompatibility complex-DR-β, tissue factor pathway inhibitor-2, and neuroserpin were all higher in human bone marrow mesenchymal stem cells than in fibroblasts. On the other hand, the mRNA levels of adrenomedullin, apolipoprotein D, C-type lectin superfamily member-2, collagen type XV α1, CUG triplet repeat RNA-binding protein, matrix metalloproteinase (MMP)-1, protein tyrosine kinase-7 and Sam68-like phosphotyrosine protein/T-STAR levels were lower in human bone marrow mesenchymal stem cells than in fibroblasts. Thus, the identified marker genes may be useful for regenerative medicine with bone marrow mesenchymal stem cells (4).

The periodontal ligament is a connective tissue between two mineralized tissues – alveolar bone and cementum. Periodontal ligament cells are a heterogeneous cell population, containing fibroblasts and progenitor cells, which can differentiate into osteoblasts and cementoblasts, and have osteoblast-like properties, such as high levels of alkaline phophatase activity and production of bone-associated proteins (5–9). The gene expression pattern of periodontal ligament cells is different from that of gingival fibroblasts (10–12). Periodontal ligament tissue has recently been found to contain mesenchymal stem cells, in addition to osteoprogenitor cells and fibroblasts (13–16). The identification of molecular markers, which distinguish periodontal ligament cells from bone marrow mesenchymal stem cells, as well as from gingival fibroblasts, may aid in the characterization of periodontal ligament cells.

In the present study, we compared the characteristics of human periodontal ligament cells with those of human bone marrow mesenchymal stem cells and human gingival fibroblasts by examining the expression of molecular gene markers distinguishing human bone marrow mesenchymal stem cells from human fibroblasts.

Material and methods

Preparation of human periodontal ligament cells and human gingival fibroblasts

Human periodontal ligament cells-1, -2, -3, and -4 were obtained separately by the explant culture of healthy periodontal ligament from the mid-root of four premolars extracted (after obtaining informed consent) from four patients undergoing orthodontic treatment. Human gingival fibroblasts were obtained separately from four healthy gingival tissue explants from four different volunteers. Informed consent was obtained under a protocol approved by the Ethics Committee of the Hiroshima University (Hiroshima, Japan) Faculty of Dentistry. Periodontal ligament tissue and human gingival tissue were cut into small pieces and plated in 35-mm culture dishes (Corning Inc., Corning, NY, USA) containing Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 1 μg/ml of amphotericin B (Medium A). When the human periodontal ligament cells or the human gingival fibroblasts formed a confluent monolayer, they were harvested and seeded on a 100-mm culture dish (Corning) in the presence of medium A. Human periodontal ligament cells at the sixth passage, or human gingival fibroblasts at the fourth passage, were used in the experiments.

Preparation of human bone marrow mesenchymal stem cells

Human bone marrow mesenchymal stem cells-1, -2, -3, and -4 were obtained from the iliac crest of four patients. Informed consent was obtained under a protocol approved by the Ethics Committee of the Hiroshima University Faculty of Dentistry. Bone marrow cells, including erythrocytes, were seeded at a density of 0.1 ml of aspirate per 35-mm tissue culture dish and maintained in 2 ml of medium A. Three days after the seeding, floating cells were removed and the medium was replaced with fresh medium A. Thereafter, attached cells were fed with fresh medium A supplemented with 1 ng/ml of fibroblast growth factor-2 (Kaken Pharmaceutical Co., Ltd. Tokyo, Japan). Fibroblast growth factor-2 was added every other day (17). Passages were performed when the cells became subconfluent. Human bone marrow mesenchymal stem cells at the fourth passage were used for the experiments.

RNA preparation

Human periodontal ligament cells-1, -2, -3, and -4 at the sixth passage, human gingival fibroblasts-1, -2, -3, and -4 at the fourth passage, or human bone marrow mesenchymal stem cells-1, -2, -3, and -4 at the fourth passage were harvested, seeded at a density of 7×10^4 cells per 60-mm culture dish coated with type I collagen, and maintained in 5 ml of medium A. After 10 d of culture, the confluent cells were washed three times with phenol red-free Hank's solution (pH 7.4). Total RNA was extracted from each cell using ISOGEN® (Wako Pure Chemical Industries, Osaka, Japan) and quantified by spectrometry at 260 and 280 nm.

Real time polymerase chain reaction

First-strand DNAs were synthesized with 1 μg of total RNA using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Real-time polymerase chain reaction (PCR) with the cDNAs was performed using an ABI 7900 system (Applied Biosystems, Tokyo, Japan). The TaqMan probe, sense primers, and antisense primers used for detection are listed in Table 1. A commercially available human glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems) was used for quantitative PCR.

Table 1. Primers and probes for real-time polymerase chain reaction

Gene name	Primer	
TFPI-2	Forward	5′-GGCAACGCCAACAATTTCTAC-3′
	Reverse	5′-CAAACTTTGGGAACTTTTTCTATCCT-3′
	Probes	5′-CTGGGAGGCTTGCGACGATGC-3′
Neuroserpin	Forward	5′-TGGGTGGAGAATAACACAAACAA-3′
	Reverse	5′-CCAGATAAGTGGCAGCATCAAA-3′
	Probes	5′-CTGGTGAAAGATTTGGTATCCCCAAGGG-3′
MHC-DR-α	Forward	5′-GCCCAGGGAAGACCACCTT-3′
	Reverse	5′-CAGTCGTAAACGTCCTCAGTTGA-3′
	Probes	5′-TCCGCAAGTTCCACTATCTCCCCTTCCT-3′
MHC-DR-β	Forward	5′-GGCTGAAGTCCAGAGTGTCCTT-3′
	Reverse	5′-GCTGGGCCTGCTCTTCCT-3′
	Probes	5′-CCTGAAGTAGATGAACAGCCCGGCC-3′
Apolipoprotein D	Forward	5′-TGAGAAGATCCCAACAACCTTTG-3′
	Reverse	5′-TGATCTTTCCGTTTTCCATTAGTG-3′
	Probes	5′-ATGGACGCTGCATCCAGGCCAACTA-3′
Adrenomedullin	Forward	5′-GGTTTCCGTCGCCCTGAT-3′
	Reverse	5′-GAGCCCACTTATTCCACTTCTTTC-3′
	Probes	5′-ACCTGGGTTCGCTCGCCTTCCTAG-3′
CUG triplet repeat	Forward	5′-CATGAATGCTTTACAGTTGCAGAA-3′
RNA-binding protein 2	Reverse	5′-GCGCTGCTCGTGGTAGAGA-3′
	Probes	5′-CTCAGCCACCAGCACCAATGCAAAC-3′
C-type lectin	Forward	5′-ATCCATTTTCTTTCGGTGTAACATCTA-3′
	Reverse	5′-CATGAGAGGGAGTGAAGGATGTG-3′
	Probes	5′-CTGTTGCTGCACCATCATCGCTGAG-3′
Collagen type XV α1	Forward	5′-CCAGCAACCCACATCAGCTT-3′
	Reverse	5′-ATGCAGAGCAGGCTTCTCATAAT-3′
	Probes	5′-TGCCTCCACCAAACCCTATTTCAAGTGC-3′
MMP-1	Forward	5′-GATGGACCTGGAGGAAATCTTG-3′
	Reverse	5′-CCGCAACACGATGTAAGTTGTACT-3′
	Probes	5′-TCATGCTTTTCAACCAGGCCCAGGTATT-3′

MMP, matrix metalloproteinase; MHC-DR-α, major histocompatibility complex-DR-α; MHC-DR-β, major histocompatibility complex-DR-β; TFPI-2, tissue factor pathway inhibitor.

Statistical analysis

The statistical differences between human periodontal ligament cells and human bone marrow mesenchymal stem cells, and between human periodontal ligament cells and human gingival fibroblasts, were determined with the two-sided Mann- Whitney *U*-test. Differences with a p-value of < 0.05 were considered significant.

Results

Messenger RNA levels of apolipoprotein D were lower in human periodontal ligament cells than in either human bone marrow mesenchymal stem cells or human gingival fibroblasts (Table 2). Human periodontal ligament cells also had lower levels of neuroserpin than human bone marrow mesenchymal stem cells, but not human gingival fibroblasts (Table 2). Messenger RNA levels of major histocompatibility complex-DR-α and major histocompatibility complex-DR-β were lower and higher, respectively, in human periodontal ligament cells than in human bone marrow mesenchymal stem cells or human gingival fibroblasts (Table 2). Human periodontal ligament cells had higher levels of tissue factor pathway inhibitor-2 mRNA than did human gingival fibroblasts but not human bone marrow mesenchymal stem cells (Table 2). No significant differences between human periodontal ligament cells and human bone marrow mesenchymal stem cells, or between human periodontal ligament cells and human gingival fibroblasts, were observed in the mRNA levels of Type XV collagen and adenomedullin (Table 2). On the other hand, CUG triplet repeat RNA-binding protein, C-type lectin, and MMP-1 mRNA levels were lower in human periodontal ligament cells than in human gingival fibroblasts, although no significant difference was found between human periodontal ligament cells and human bone marrow mesenchymal stem cells in the expression of these mRNAs (Table 2). The findings, regarding the expression of these 10 genes in human bone marrow mesenchymal stem cells compared with human gingival fibroblasts, are consistent with those of a previous report (4).

Discussion

Because human periodontal ligament cells, human bone marrow mesenchymal stem cells, and human gingival fibroblasts are spindle-like cells, human periodontal ligament cells have not been characterized by their morphology. For the first time, the present study demonstrated that the genes for apolipoprotein D, major histocompatibility complex-DR-α, and major histocompatibility complex-DR-β are candidates for molecular markers distinguishing human periodontal ligament cells from human bone marrow mesenchymal stem cells and human gingival fibroblasts.

In the present study, the mRNA expressions of major histocompatibility complex-DR-α and -β, and tissue factor pathway inhibitor-2 were lower in human gingival fibroblasts than in human periodontal ligament cells. On the other hand, the mRNA expressions of apolipoprotein D, CUG triplet repeat RNA-binding protein, C-type lectin, and MMP-1 were higher in human gingival fibroblasts than in human periodontal ligament cells. Regarding MMP-1 expression, the present finding is consistent with the previous report on DNA array analysis (10).

Apolipoprotein D is known to participate in maintenance and repair within the central and peripheral nervous systems (18). The present study found that human gingival fibroblasts show the highest mRNA levels of apolipoprotein D among human gingival fibroblasts, human bone marrow mesenchymal stem cells, and human periodontal ligament cells. Human bone marrow mesenchymal stem cells can differentiate into neurons (19). However, to our knowledge, there is no report regarding the involvement of

Table 2. Comparison of gene expressions between human periodontal ligament (HPL) cells and human bone marrow mesenchymal stem cells (hMSC) and between HPL cells and human gingival fibroblasts (HGF)

	Cells							
	HPL cells		hMSC			HGF		
Genes	-1 -3	-2 -4	-1 -3	-2 -4		-1 -3	-2 -4	
Apolipoprotein D	0.44 0.11	0.63 0.03	1.21 1.19	1.03 0.54	*	81.31 159.9	7.83 175.6	**
Neuroserpin	0.31 0.16	0.72 0.32	0.78 1.33	1.21 0.66	*	0.16 0.18	0.56 0.45	
MHC-DR-α	0.12 0.18	0.03 0.14	0.95 1.55	0.48 10.64	*	0.01 0.001	0.03 0.001	**
MHC-DR-β	0.18 0.27	0.01 0.08	1.34 6.89	0.65 63.25	*	0.01 0.001	0.01 0.001	**
TFPI-2	0.50 0.19	0.70 0.31	1.52 1.30	0.29 0.87		0.03 0.11	0.09 0.12	**
Adrenomodullin	0.45 0.31	1.34 0.23	0.66 1.13	1.49 0.69		0.38 10.79	1.96 14.38	
CUG triplet repeat	0.09	0.39	0.19	1.52		4.53	0.73	**
RNA-binding protein 2	0.52	0.91	1.06	0.40		11.88	18.41	
C-type lectin	6.06 1.79	0.42 0.55	0.52 1.65	0.81 0.01		13.59 9.30	11.69 18.77	**
Collagen type XV α1	0.63 3.43	11.06 0.07	0.19 6.28	1.95 0.85		2.43 74.15	4.62 81.31	
MMP-1	14.7 2.97	22.32 10.29	16.99 5.96	0.76 1.23		4218 99.57	1306 80.75	**

Values are arbitrary ratios of each mRNA to glyceraldehyde-3-phosphate dehydrogenase mRNA.
*Significantly different between human periodontal ligament cells and hMSC; $p < 0.05$.
**Significantly different between human periodontal ligament cells and human gingival fibroblasts; $p < 0.05$.
MMP, matrix metalloproteinase; MHC-DR-α, major histocompatibility complex-DR-α; MHC-DR-β, major histocompatibility complex-DR-β; TFPI-2, tissue factor pathway inhibitor.

fibroblasts in the functioning of neurons. Therefore, the higher levels of expression suggest a new role for apolipoprotein D in the functioning of gingival fibroblasts.

Tissue factor pathway inhibitor-2 is thought to play an important role in the regulation of extracellular matrix in digestion and remodeling (20,21). Periodontal ligament tissue is thought to be more actively remodeled than gingival tissue. The active remodeling of periodontal ligament tissue may be a result of increased levels of tissue factor pathway inhibitor-2.

In conclusion, the genes for apolipoprotein D, major histocompatibility complex-DR-α, and major histocompatibility complex-DR-β are suggested to be molecular markers characterizing periodontal ligament cells. The role of the markers in periodontal ligament needs to be studied further.

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