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, distinguishing human bone marrow mesenchymal stem cells (hMSCs) from human fibroblasts, have recently been reported. Messenger RNA levels of tissue factor pathway inhibitor (TFPI)-2, MHC-DR-α, MHC-DR-β, and neuroserpin are higher in hMSCs than human fibroblasts. However, the hMSCs express less mRNA of apolipoprotein D 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 which distinguish hMSCs from human fibroblasts, may provide insight into the characteristics of human periodontal ligament cells (HPL cells). In this study, we compared the molecular markers of HPL cells with those of hMSCs and human gingival fibroblasts (HGF).

Methods: The mRNA expression of the molecular gene markers was analyzed with real-time PCR. Statistical differences were determined with the two-sided Mann- Whitney *U* test.

Results: Messenger RNA levels of MHC-DR-α and MHC-DR-β mRNAs were lower and higher respectively, in HPL cells than in hMSC or HGF. HPL cells showed the lowest mRNA levels of apolipoprotein D among the three types of cells.

Conclusions: HPL cells may be distinguished from hMSCs and HGF by the genes for apolipoprotein D, MHC-DR-α, and MHC-DR-β.

Introduction

Bone marrow mesenchymal stem cells (MSCs), also called plastic-adherent marrow cells or bone marrow stromal cells, can differentiate into osteoblasts, chondorocytes, adipocytes, tenocytes, and muscle cells *in vitro* and *in vivo* (1-3). A recent study has identified several molecular marker genes which distinguish human MSCs (hMSCs) from human fibroblasts (4). MHC-DR-α, MHC-DR-β, tissue factor pathway inhibitor (TFPI)-2, and neuroserpin mRNA levels were all higher in hMSCs than in fibroblasts. On the other hand, adrenomedullin, apolipoprotein D, C-type lectin superfamily member-2, collagen type XV a1, 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 the hMSCs than fibroblasts. Thus, the identified marker genes may be useful for regenerative medicine with MSCs (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 osteoprogeniter cells and fibroblasts (13-16). The identification of molecular markers which distinguish periodontal ligament cells from MSCs as well as from gingival fibroblasts may aid in the characterization of periodontal ligament cells.

In the present study, we compared the characteristics of HPL cells with those of hMSCs and human gingival fibroblasts (HGF) by examining the expression of molecular gene markers distinguishing hMSCs from human fibroblasts.

Materials and Methods

Preparation of HPL cells and HGF

HPL 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 from four patients undergoing orthodontic treatment, with their informed consent. HGF 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, NY) with Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, South Logan UT), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 1 μg/ml of amphotericin B (Medium A). When the HPL cells or the HGF formed a confluent monolayer, they were harvested and seeded on a 100-mm culture dish (Corning) in the presence of medium A. HPL cells at the 6^{th} passage or HGF at the 4^{th} passage were used for the experiments.

Preparation of hMSCs

hMSCs-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 (Hiroshima, Japan) 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 (FGF)-2 (Kaken pharmaceutical Co.,Ltd. Tokyo, Japan). FGF-2 was added every other day (17). Passages were performed when the cells became subconfluent. hMSCs at the 4^{th} passage were used for the experiments.

RNA preparation

HPL cells-1, -2, -3, and -4 at the 6^{th} passage, HGF-1, -2, -3, and -4 at

the 4th passage, or hMSCs-1, -2, -3, and -4 at the 4th passage were harvested, seeded at the a density of 7 x 10^4 cells/60-mm culture dish coated with type I collagen, and maintained in 5 ml of medium A. After 10 days of culture, the confluent cells were washed three times with phenol red-free Hank's solution (pH7.4). 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 PCR

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

Statistical analysis

The statistical differences between HPL cells and hMSCs, and HPL cells and HGF, were determined with the two-sided Mann- Whitney U test. Differences with $P<0.05$ were considered significant.

Results

Messenger RNA levels of apolipoprotein D were lower in HPL cells than in either hMSCs or HGF (Table 2). HPL cells also had lower levels of neuroserpin than the hMSCs, though not the HGF (Table 2). Messenger RNA levels of MHC-DR-α and MHC-DR-β were lower and higher respectively, in HPL cells than in the hMSCs or HGF (Table 2). HPL cells had higher levels of TFPI-2 mRNA than did the HGF but not the hMSCs (Table 2). No significant differences between HPL cells and hMSCs, or between HPL cells and HGF 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 HPL cells than in HGF, although no significant difference was found between HPL cells and hMSC in the expression of these mRNAs (Table 2). The findings regarding the expression of the ten genes in hMSCs compared with HGF are consistent with a previous report (4).

Discussion

Since HPL cells, hMSCs, and HGF are spindle-like cells, HPL cells have not been characterized by their morphology. The present study, for the first time, demonstrated that the genes for apolipoprotein D, MHC-DR-α, and MHC-DR-β are candidates for molecular markers distinguishing HPL cells from hMSCs and HGF.

In the present study, mRNA expressions of MHC-DR-α and -β, and TFPI-2 are lower in HGF than HPL cells. On the other hand, apolipoprotein D, CUG triplet repeat RNA-binding protein, C-type lectin, and MMP-1 are higher in HGF than HPL cells. Regarding the MMP-1 expression, the present finding is consistent with the previous report with 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 suggests that HGF show the highest mRNA levels of apolipoprotein D among HGF, hMSCs, and HPL cells. hMSCs can differentiate into neurons (19). However, to our knowledge, there is no report about the involvement of 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.

TFPI-2 is thought to play an important role in the regulation of extracellular matrix is 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 due to increased TFPI-2 levels.

In conclusion, the genes for apolipoprotein D, MHC-DR-α, and MHC-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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Table-1

Primers and probes for real-time PCR

gene name		
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 RNA-binding protein 2	Forward	5'-CATGAATGCTTTACAGTTGCAGAA-3'
	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'

Table-2

Comparison of gene expressions between HPL cells and hMSCs and between HPL cells and HGF

Cells / Genes	HPL cells		hMSC			HGF		
	-1	-2	-1	-2		-1	-2	
	-3	-4	-3	-4		-3	-4	
apolipoprotein D	0.44	0.63	1.21	1.03	*	81.31	7.83	¶
	0.11	0.03	1.19	0.54		159.9	175.6	
neuroserpin	0.31	0.72	0.78	1.21	*	0.16	0.56	
	0.16	0.32	1.33	0.66		0.18	0.45	
MHC-DR-α	0.12	0.03	0.95	0.48	*	0.01	0.03	¶
	0.18	0.14	1.55	10.64		0.001	0.001	
MHC-DR-β	0.18	0.01	1.34	0.65	*	0.01	0.01	¶
	0.27	0.08	6.89	63.25		0.001	0.001	
TFPI-2	0.50	0.70	1.52	0.29		0.03	0.09	¶
	0.19	0.31	1.30	0.87		0.11	0.12	
adrenomodullin	0.45	1.34	0.66	1.49		0.38	1.96	
	0.31	0.23	1.13	0.69		10.79	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	0.42	0.52	0.81		13.59	11.69	¶
	1.79	0.55	1.65	0.01		9.30	18.77	
collagen type XV α1	0.63	11.06	0.19	1.95		2.43	4.62	
	3.43	0.07	6.28	0.85		74.15	81.31	
MMP-1	14.7	22.32	16.99	0.76		4218	1306	¶
	2.97	10.29	5.96	1.23		99.57	80.75	

Values are arbitrary ratios of each mRNA to GAPDH mRNA.

*Significantly different between HPL cells and hMSC; $P<0.05$.

¶Significantly different between HPL cells and HGF; $P<0.05$.