Gene Expression of Small Leucine-Rich Proteoglycans during Differentiation of Human Mesenchymal Stem Cells toward Osteoblasts

Hiroshi Hashimoto

Department of Maxillofacial Surgery, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

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Abstract

Human mesenchymal stem cells (hMSC) have the capacity to differentiate into osteoblasts. To identify genes involved in the commitment of hMSC to osteoblasts, total RNA from hMSC cultured in osteogenic induction medium was isolated on days 0 and 10 and used to create gene expression profiles with oligonucleotide microarrays. Seven SLRPs were expressed in hMSC during osteoblast differentiation. Asporin, which is a small leucine-rich proteoglycan (SLRP), was the most up-regulated extracellular matrix protein in the mineralization stage. The expression levels of these SLRPs genes were compared in hMSC cultured in osteogenic induction medium and hMSC cultured in MSC growth medium on days 0, 1, 3, and 10. The mRNA levels of asporin and osteomodulin were drastically increased in hMSC cultured in osteogenic induction medium as compared to MSC growth medium. The expression levels of asporin, osteomodulin (OMD), proline arginine rich end leucine rich repeat protein, and decorin on day 10 were significantly increased in osteogenic induction medium culture as compared to MSC growth medium culture. These SLRPs are ECM components in bone and articular cartilage. Thus, these SLRPs, particular OMD and ASPN, may be important matrix molecules during differentiation into osteoblasts and/or mineralization.

Keywords :

mesenchymal stem cells, osteoblastic differentiation, DNA microarray, small leucine-rich proteoglycans

Introduction

Human mesenchymal stem cells (hMSC) from bone marrow can differentiate into osteogenic (1), chondrogenic (2), or adipogenic (3) lineages dependent on the conditions of the medium in which they are cultured. hMSC cultured in the presence of dexamethasone, ascorbic acid, and β -glycerophosphate differentiate into osteoblasts and form mineralized matrix (1, 4). Many studies have highlighted the complexity of using hMSC to identify genes associated with bone cell lineage progression and mineralized matrix formation (1, 5, 6), and the underlying mechanisms at the cellular and molecular levels are still not well understood.

Recent advancements have been attained in techniques to analyze and compare differentially expressed genes. DNA Microarrays are increasingly used for random screening of mRNA transcripts from cells and tissues. The technology is

based on the principle that the biology of an organism corresponds with its gene expression profile, disclosing preferentially expressed genes by comparative analysis (1, 3-6).

Small leucine-rich proteoglycans (SLRPs), which have a protein folding motif composed of 20-30 amino acids with leucines in conserved positions, play vital roles in a variety of tissues and extracellular matrices (7), by binding and regulating collagen fibrils (8, 9). SLRPs have multiple functions within mineralized tissues, including cell regulation, matrix assembly and mineral deposition (10).

The present study examined gene expression profiles during the osteogenic differentiation of hMSC, and focus on the expression patterns of SLRPs.

Materials and Methods

Cell culture

Poietics™ Human Mesenchymal Stem Cells (Lonza, Walkersville, MD, USA) were cultured in hMSC growth

Correspondence to :

Hiroshi Hashimoto

E-mail : dentist@abiko-clinic.co.jp

medium [MSCGM; consisting of Mesenchymal Stem Cell Basal Medium (440ml) and SingleQuots™ growth supplements, which contained fetal bovine serum (50ml), L-glutamine (10ml) and penicillin/streptomycin (1ml); Lonza, Walkersville, MD, USA] in a CO₂ incubator (Sanyo, Japan) in the presence of 95% air and 5% CO₂ at 37°C. To induce osteogenic differentiation, hMSC from the 5th to 7th doubling passages were seeded at 3.1×10^3 cells/cm² in MSCGM; then, after 24 h (day 0), the media was changed to hMSC osteogenic induction medium [MSCOIM; consisting of osteogenic basal medium (170ml), osteogenic SingleQuots™ that contained fetal bovine serum (20ml), L-glutamine (4ml), penicillin/streptomycin (2ml), dexamethasone (1ml), ascorbate (1ml) and beta-glycerophosphate (1ml); Lonza]. The media was changed twice a week.

Total RNA extraction

hMSC cultured with MSCOIM or MSCGM were homogenized by using TRIZOL Reagent (Life Technologies, Gaithersburg, MD, USA) and the FastPrep FP120 Instrument (BIO 101, Vista, CA, USA). Total RNA was isolated by using the acid guanidinium thiocyanate-phenol-chloroform method.

GeneChip expression and data analysis

For gene expression profiling, we used the Affymetrix GeneChip Human Genome U133A Array (Affymetrix, Santa Clara, CA, USA) according to Affymetrix protocols. Briefly, double-stranded cDNA was generated from 7 µg of total RNA by using a T7-oligo (dT) primer and the Superscript Choice System kit (Invitrogen, Carlsbad, CA). Biotin-labeled cRNA was synthesized by in vitro transcription by using the BioArray high-yield RNA transcript labeling system (ENZO, Farmingdale, NY) and then was hybridized to the Human Genome U133A Array (22,283 genes) for 16 h at 45°C. Arrays were subjected to washing and staining with R-phycoerythrin streptavidin (SAPE, Molecular Probes) by using the GeneChip Fluidics Station 400 (Affymetrix). After the arrays were scanned with an Affymetrix scanner, the oligonucleotide hybridization data were exported for gene expression value analysis by using Affymetrix Microarray Suite (version 5.0).

The data from the GeneChips were loaded into GeneSpring software (version 6.2; Agilent Technologies, Waldbronn, Germany) and extracted for further analysis.

Real-time PCR

cDNA was synthesized by using a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT). Real-time PCR was performed by using a DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The PCR mixture contained 20 pmol of forward and reverse primers and 2 µl of cDNA. Amplification was performed in a DNA Engine Opticon 1 (BioRad, Hercules, CA, USA), with preheating at 95°C for 10min, followed by 40 cycles of 94°C for 15s, 57°C for 30s and 72°C for 30s. The relative gene expressions were calculated as the values obtained from the ratio of culture day 0 versus each culture points calibrated by the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta CT}$ method as described previously (11). The statistical analysis was done in triplicate by independent experiments. The primer sequences are listed in Table 1.

Statistics

Data from real time-PCR are expressed as the mean $\pm$ SD. Differences between groups were analyzed by Student's *t*-test.

Results

Small leucine-rich proteoglycan expression by microarray analysis

The gene expression profile was examined in hMSC cultured with MSCOIM on days 0 and 10. Of the 22,283 total genes sets, 13,069 genes were present in one or more samples on days 0 and 10 (data not shown).

Asporin (ASPN), which is a small leucine-rich proteoglycan (SLRP), was the most up-regulated matrix protein on day 10 as compared to day 0. I focused on the genes encoding 12 SLRP members. Seven SLRPs, ASPN, osteomodulin (OMD, also called osteoadherin; OSAD), biglycan (BGN), decorin (DCN), fibromodulin (FMOD), proline arginine-rich end leucine-rich repeat protein (RPELP), and lumican (LUM), were expressed in hMSC during differentiation toward osteoblasts (Table 2). After 10 days of culture in osteogenic induction medium, the expression levels of ASPN and OMD were enhanced by 254-fold and 106-fold, respectively, as compared to day 0. The expression of 5 other SLRPs, keratocan, mimecan, dermatan sulfate proteoglycan 3, chondroadherin, and nyctalopin, was not detected in hMSC by oligonucleotide microarray analysis (Table 2).

Table 1 DNA Primers for SLRPs

Gene name		Primers	products bp
ASPN	F	5'-ctgggcctaggaacaacaa-3'	205
	R	5'-ttggcactgttgacagaag-3'	
OMD	F	5'-catcttcttctgcttccctca-3'	193
	R	5'-gtcaaagtgcccttctgctc-3'	
BGN	F	5'-gcttcgtactccaagctgt-3'	203
	R	5'-caccactttggtgatgttg-3'	
DCN	F	5'-gaagggagaagacattggtttg-3'	193
	R	5'-gcagaggggtaaattgaaaca-3'	
LUM	F	5'-cctggttgagctggatctgt-3'	211
	R	5'-catatccggtggaagactgg-3'	
PRELP	F	5'-ctttccaatcttgccttca-3'	200
	R	5'-tctgggttccgttgatttc-3'	
FMOD	F	5'-gtaatgttgctggggctta-3'	236
	R	5'-ctggtttggctttgtggat-3'	

ASPN, asporin; OMD, osteomodulin/osteoaderin; BGN, biglycan; DCN, decorin; LUM, lumican; PRELP, proline arginine-rich end leucine-rich repeat protein; FMOD, fibromodulin

Table 2 GeneChip data of SLRPs

SLRP	GeneBank ID	Fold ^a	Day 0		Day 10	
			Raw signal Intensity	Flag ^b	Raw signal intensity	Flag ^b
ASPN	NM_017680	253.5	3.1	A	913.9	P
OMD	NM_005014	106.2	4.5	A	555.8	P
PRELP	NM_002725	17.9	32.7	A	679.8	P
FMOD	NM_002023	3.8	104.5	P	461.1	P
BGN	NM_001711	3.3	987.4	P	3765.9	P
LUM	NM_002345	2.5	430.7	P	1232.0	P
DCN	NM_001920	1.0	1801.3	P	5517.2	P
CHAD	NM_001267	1.4	4.1	A	6.6	A
OGN	NM_014057	0.8	2.0	A	1.9	A
KERA	NM_007035	0.5	17.2	A	10.8	A
NYX	AF254868	0.5	60.4	A	34.5	A
DSPG3	NM_004950	0.4	22.5	A	9.3	A

ASPN, asporin; OMD, osteomodulin/osteoaderin; PRELP, proline arginine-rich end leucine-rich repeat protein; FMOD, fibromodulin; BGN, biglycan; LUM, lumican; DCN, decorin; CHAD, chondroadherin; OGN, osteoglycin/mimecan; KERA, keratocan; NYX, nyctalopin; DSPG3, dermatan sulfate proteoglycan 3/epipycan.

^aFold, raw signal intensity of day 10/raw signal intensity of day 0

^bFlag, P is present on gene expression and A is not present on gene expression

The kinetic expression levels of the 7 SLRPs were confirmed by using real-time PCR to compare hMSC cultured in MSCOIM to hMSC cultured in MSCGM (Fig.1). ASPN and OMD were up-regulated in MSCOIM cultures as compared to the MSCGM cultures. The gene expression of

OMD was enhanced from day 1 in osteogenic medium culturer. In contrast, ASPN was up-regulated on day 10. The expression levels of PRELP and DCN were slightly increased in MSCOIM cultures, whereas those of FMOD, LUM, and BGN were slightly higher in MSCGM cultures.

Fig. 1 Time course of SLRP gene expression in hMSC cultured with osteoblastic induction medium, MSCOIM (●), and growth medium, MSCGM (■). The mRNA levels of each SLRP were examined by real time-PCR and normalized by using the mRNA levels of GAPDH. ASPN, asporin; OMD, osteomodulin/osteoaderin; PRELP, proline arginine-rich end leucine-rich repeat protein; DCN, decorin; BGN, biglycan; FMOD, fibromodulin; LUM, lumican.

Fig. 2 SLRP gene expression levels were statistically examined between MSCGM culture and MSCOIM culture on day 10 by using real time-PCR. ASPN, asporin; OMD, osteomodulin osteoadherin; PRELP, proline arginine-rich end leucine-rich repeat protein; DCN, decorin; BGN, biglycan; FMOD, fibromodulin; LUM, lumican. $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Next, I performed a statistical analysis of the gene expression levels of SLRPs in hMSC on day 10. The expression levels of ASPN, OMD, PRELP, and DCN were significantly increased in MSCOIM culture as compared to MSCGM culture.

Discussion

In the present study, I assessed gene expression profiling during the differentiation of hMSC into osteoblasts. Oligonucleotide microarray analysis was performed on days 0 and 10 of culture in MSCOIM. It have already reported increased alkaline phosphatase (ALP) activity on day 3 and increased alizarin red S staining on day 10 of osteoblastic differentiation of hMSC(12). The gene expression profiling indicated the differentiation state of the hMSC at approximate time points.

Microarray analysis revealed that ASPN, a SLRP member (13), was the most up-regulated matrix protein during differentiation toward osteoblasts. Since SLRPs play vital roles in a variety of tissues and extracellular matrices (7) related to the binding and regulation of collagen fibrils(8, 9), I focused on the expression data for SLRPs in the microarray analysis. The expression of 7 SLRPs was detected in hMSC during differentiation. Although microarray analysis was performed on MSCOIM cultures only, the expression of the 7 SLRPs was confirmed by real-time PCR comparison of MSCOIM cultures and MSCGM cultures. The expression of ASPN and OMD was enhanced in MSCOIM cultures as compared to MSCGM cultures and was increased for 10 days in a time-dependent manner.

ASPN contains a unique aspartic acid repeat in its N-terminal region(13), and polymorphisms in this repeat are significantly associated with osteoarthritis(14, 15). ASPN is expressed in the perichondrium/periosteum of long bones and the periodontal ligament (PDL) (16, 17). It has been reported that PDL cells have increased levels of ASPN during the formation of mineralized tissue regulated by BMP-2 (17); however, overexpression of ASPN interfered with both naturally and BMP-2-induced mineralization of PDL cells (18). It was suggested that ASPN negatively regulates chondrogenesis and bone mineralization by binding to BMP-2 and TGF- β (18, 19). However, the expression of ASPN was enhanced in hMSC and periodontal ligament cells during mineralization. ASPN might have crucial roles in the regulation of mineralization. ASPN may be necessary to modulate the molecular functions of BMP-2

and TGF- β and to trap BMP-2 and TGF- β in ECM for subsequent remodeling processes in the bone.

OSAD/OMD, OSAD is the OMD precursor, has been detected in mineralization tissues such as bone (20) and teeth (21, 22). The expression of OMD is regulated by transcription factors Runx and Smads and cytokines TGF- β and BMP-2 (23). The distribution pattern of OMD is strikingly similar to that of bone sialoprotein (BSP) (20), which suggests that OMD is critical for the mineralization process. Recently, it has been reported that OMD is likely an osteoblast maturation marker that is induced by osteoclast activity. In addition, OMD in teeth may mediate odontoblast binding to the surrounding matrices of predentin, dentin, and pulp through integrin receptor $\alpha v \beta 3$ (24).

The expression of BGN, DCN, FMOD, PRELP, and LUM was also detected in hMSC. DCN and PRELP were significantly increased in MSCOIM culture as compared to MSCGM culture, although the expression levels of these genes increased only slightly during differentiation into osteoblasts. BGN, FMOD, and LUM were not significantly increased in hMSC during osteoblast differentiation. All of these SLRPs are ECM components in bone and articular cartilage(10, 25-27), and the aberrant expression of these genes is implicated in the etiology of osteoporosis or osteoarthritis (28-32). In the present study, BGN was abundantly expressed constitutively during long-term culture of hMSC. Recently, BGN-deficient mice exhibited a mild osteoporosis-like phenotype and/or osteoarthritis (29-32). It has been suggested that the smaller size of collagen fibrils in deficient mice may predispose the mice to osteoporosis and/or osteoarthritis. On the other hand, BGN within mineralized tissues is predominately substituted with chondroitin sulphate, while BGN in soft connective tissues predominantly carries the isomeric dermatan sulphate(10). BGN is an important matrix component not only in mineralized tissues but also other tissues, and the functions of BGN may be dictated by conjugated glycosaminoglycans.

In conclusion, the analysis of global gene expression patterns provides the molecular basis for beginning to understand the regulation of the differentiation of hMSC toward osteoblasts. The data from this approach revealed that the expression of ASPN and OMD was greatly upregulated and that 7 SLPRs were detected in hMSC during the differentiation into osteoblasts. These results suggest that these SLPRs may be important matrix components for interaction with fibrillar collagen and

modulation with TGF- β and BMP-2.

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References

- Kulterer B, Friedl G, Jandrositz A, Sanchez-Cabo F, Prokesch A, Paar C, Scheideler M, Windhager R, Preisegger K-H, Trajanoski Z: Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics*, 8: 70, 2007.
- Djouad F, Delorme B, Maurice M, Bony C, Apparailly F, Louis-Pence P, Canovas F, Charbord P, Noël D, Jorgensen C: Microenvironmental changes during differentiation of mesenchymal stem cells towards chondrocytes. *Arthritis Res Ther*, 9: R33, 2007.
- Hung S-C, Chang C-F, Ma H-L, Chen T-H, Ho L L-T: Gene expression profiles of early adipogenesis in human mesenchymal stem cells. *Gene*, 340: 141-150, 2004.
- Qi H, Aguiar D J, Williams SM, La Pean, A, Pan W, Verfaillie CM: Identification of genes responsible for osteoblast differentiation from human mesodermal progenitor cells. *Proc Natl Acad Sci USA*, 100: 3305-3310, 2003.
- Hishikawa K, Miura S, Marumo T, Yoshioka H, Mori Y, Takato T, Fujita T: Gene expression profile of human mesenchymal stem cells during osteogenesis in three-dimensional thermoreversible gelation polymer. *Biochem Biophys Res Commun*, 317: 1103-1107, 2004.
- Beck Jr GR, Zerler B, Moran E: Gene array analysis of osteoblast differentiation. *Cell Growth Differ*, 12: 61-83, 2001.
- Kresse H, Schönherr E: Proteoglycans of the extracellular matrix and growth control. *J Cell Physiol*, 189: 266-274, 2001.
- Kalamajski S, Oldberg Å: The role of small leucine-rich proteoglycans in collagen fibrillogenesis. *Matrix Biol*, 29: 248-253, 2010.
- Rühland C, Schönherr E, Robenek H, Hansen U, Iozzo RV, Bruckner P, Seidler DG: The glycosaminoglycan chain of decorin plays an important role in collagen fibril formation at the early stages of fibrillogenesis. *FEBS J*, 274: 4246-4255, 2007.
- Waddington RJ, Roberts HC, Sugars RV, Schönherr E: Differential roles for small leucine-rich proteoglycans in bone formation. *Eur Cell Mater* 6: 12-21, 2003.
- Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25: 402-408, 2001.
- Ogura N, Kawada M, Chang W-J, Zhang Q, Lee S-Y, Kondoh T, Abiko Y: Differentiation of the human mesenchymal stem cells derived from bone marrow and enhancement of cell attachment by fibronectin. *J Oral Sci*, 46: 207-213, 2004.
- Lorenzo P, Aspberg A, Önnérjörð P, Bayliss MT, Neame PJ, Heinegård D: Identification and characterization of asporin. *J Biol Chem*, 276: 12201-12211, 2001.
- Kizawa H, Kou I, Iida A, Sudo A, Miyamoto Y, Fukuda A, Mabuchi A, Kotani A, Kawakami A, Yamamoto S, Uchida A, Nakamura K, Notoya K, Nakamura Y, Ikegawa S: An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis. *Nat Genet*, 37: 138-144, 2005.
- Iida A, Kizawa H, Nakamura Y, Ikegawa S: High-resolution SNP map of ASPN, a susceptibility gene for osteoarthritis. *J Hum Genet* 51: 151-154, 2006.
- Kou I, Nakajima M, Ikegawa S: Expression and regulation of the osteoarthritis-associated protein asporin. *J Biol Chem*, 282: 32193-32199, 2007.
- Yamada S, Murakami S, Matoba R, Ozawa Y, Yokokoji T, Nakahira Y, Ikegawa K, Takayama S, Matsubara K, Okada H: Expression profile of active genes in human periodontal ligament and isolation of PLAP-1, a novel SLRP family gene. *Gene*, 275, 279-286, 2001.
- Yamada S, Tomoeda M, Ozawa Y, Yoneda S, Terashima Y, Ikegawa K, Ikegawa S, Saito M, Toyosawa S, Murakami S: PLAP-1/Asporin, a novel negative regulator of periodontal ligament mineralization. *J Biol Chem*, 282: 23070-23080, 2007.
- Nakajima M, Kizawa H, Saitoh M, Kou I, Miyazono K, Ikegawa S: Mechanisms for asporin function and regulation in articular cartilage. *J Biol Chem*, 282: 32185-32192, 2007.
- Ramstad VE, Franzén A, Heinegård D, Wendel M, Reinholt FP: Ultrastructural distribution of osteoadherin in rat bone shows a pattern similar to that of bone sialoprotein. *Calcif Tissue Int*, 72: 57-64, 2003.
- Petersson U, Hulténby K, Wendel M: Identification, distribution and expression of osteoadherin during tooth formation. *Eur J Oral Sci*, 111: 128-136, 2003.
- Couble M-L, Bleicher F, Farges J-C, Peyrol S, Lucchini M, Magloire H, Staquet M-J: Immunodetection of osteoadherin in murine tooth extracellular matrices. *Histochem Cell Biol*, 121: 47-53, 2004.
- Rehn AP, Chalk AM, Wendel M: Differential regulation of osteoadherin (OSAD) by TGF- β 1 and BMP-2. *Biochem Biophys Res Commun*, 349: 1057-1064, 2006.
- Lucchini M, Couble M-L, Romeas A, Staquet M-J, Bleicher F, Magloire H, Farges J-C: $\alpha v \beta 3$ integrin expression in human odontoblasts and co-localization with osteoadherin. *J Dent Res*, 83: 552-556, 2004.

25. Raouf A, Ganss B, McMahon C, Vary C, Roughley PJ, Seth A: Lumican is a major proteoglycan component of the bone matrix. *Matrix Biol*, 21: 361-367, 2002.
26. Grover J, Chen X-N, Korenberg JR, Recklies AD, Roughley PJ: The gene organization, chromosome location, and expression of a 55-kDa matrix protein (PRELP) of human articular cartilage. *Genomics*, 38: 109-117, 1996.
27. Gori F, Schipani E, Demay MB: Fibromodulin is expressed by both chondrocytes and osteoblasts during fetal bone development. *J Cell Biochem*, 82: 46-57, 2001.
28. Boskey AL, Young MF, Kilts T, Verdelis K: Variation in mineral properties in normal and mutant bones and teeth. *Cell Tissues Organs*, 181: 144-153, 2005.
29. Ameye L, Aria D, Jepsen K, Oldberg A, Xu, T, Young MF: Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. *FASEB J*, 16: 673-680, 2002.
30. Corsi A, Xu T, Chen XD, Boyde A, Liang J, Mankani M, Sommer B, Iozzo RV, Eichstetter I, Robey PG, Bianco P, Young MF: Phenotypic effects of biglycan deficiency are linked to collagen fibril abnormalities, are synergized by decorin deficiency, and mimic Ehlers-Danlos-like changes in bone and other connective tissues. *J Bone Miner Res*, 17: 1180-1189, 2002.
31. Chakravarti S: Functions of lumican and fibromodulin: Lessons from knockout mice. *Glycoconjugate J*, 19: 287-293, 2003.
32. Xu T, Bianco P, Fisher LW, Longenecker G, Smith E, Goldstein S, Bonadio J, Boskey, A, Heegaard A-M, Sommer B, Satomura K, Dominguez P, Zhao C, Kulkarni AB, Robey PG, Young MF: Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. *Nat Genet*, 20: 78-82, 1998.