GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Gene expression analysis of demineralized bone matrix-induced osteogenesis in human periosteal cells using cDNA array technology

S. Honsawek[1], P. Bumrungpanichthaworn[1], T. Thitiset[1] and L. Wolfinbarger Jr.[2]

[1]Department of Biochemistry, Faculty of Medicine,
King Chulalongkorn Memorial Hospital, Chulalongkorn University,
Thai Red Cross Society, Bangkok, Thailand
[2]Old Dominion University and BioScience Consultants, LLC, Norfolk, VA, USA

Corresponding author: S. Honsawek
E-mail: Sittisak.H@chula.ac.th

Genet. Mol. Res. 10 (3): 2093-2103 (2011)
Received February 15, 2011
Accepted July 12, 2011
Published September 16, 2011
DOI http://dx.doi.org/10.4238/vol10-3gmr1329

ABSTRACT. Demineralized bone matrix (DBM) has been widely investigated as a biomaterial to promote new bone formation and is utilized clinically for bone repair and regeneration. We investigated gene expression patterns of osteogenic differentiation in human periosteal (HPO) cells cultured with demineralized bone matrix, using cDNA array technology. Osteogenic differentiation of HPO cells was determined using alkaline phosphatase assay. In order to examine differential gene expression during osteogenic differentiation, total RNA was isolated from HPO cells in the absence or presence of DBM on day seven and analyzed using osteogenesis cDNA gene array. The selected genes were verified using reverse transcriptase (RT)-PCR analysis. Human periosteal cells differentiated along an osteogenic lineage after treatment of DBM. The alkaline phosphatase activity assay showed that HPO cells differentiated into an osteogenic lineage. Gene expression of HPO cells treated with DBM for seven days was analyzed with cDNA array and RT-PCR analyses. Expression of biglycan, TGF-β1, and TGF-βR1 was upregulated, whereas collagen14A1 expression was downregulated, as confirmed by RT-PCR. Human periosteal cells

expressed osteogenesis genes when treated with DBM. These findings provide new insight into the capability of demineralized bone matrix to modulate the osteogenic differentiation of human periosteal cells.

Key words: cDNA array; Demineralized bone matrix; Osteogenesis; Gene expression; Human periosteal cells

INTRODUCTION

Demineralized bone matrix (DBM) is a promising candidate for bone tissue engineering composite scaffolds due to its similar relationship in structure and function with autologous bone (Honsawek et al., 2010). The ability of DBM to promote osteogenic differentiation of mesenchymal stem cells has been recently documented and is believed to be attributable to the interaction of osteoprogenitor cells with these matrix-contained osteoinductive proteins, which can stimulate mesenchymal stem cells into becoming osteoblasts (Honsawek et al., 2007). It is presumed that these osteoinductive proteins, which are mainly bone morphogenetic proteins (BMP), are capable of activating osteogenesis (Zhang et al., 1997; Honsawek et al., 2005).

The potential of DBM to induce osteogenic differentiation contributes to the application of DBM in biomaterials for treatment of bone defects. In addition, osteoprogenitor cells can be derived from human periosteum (Hutmacher and Sittinger, 2003). Previous studies have demonstrated that human mesenchymal stem cells derived from Wharton's Jelly comprise pluripotent or multipotent stem cells that are able to differentiate into osteoblasts (Honsawek et al., 2006). However, there have been limited studies and there is little data regarding the osteoinductive activity of DBM to activate osteoblast differentiation in human periosteal (HPO) cells. Consequently, we examined the osteoinductive potential of DBM to induce HPO cells to differentiate into bone-forming cells and investigated gene expression profiling of osteogenic differentiation in HPO cells using cDNA array and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses.

MATERIAL AND METHODS

Preparation of demineralized bone matrix

Freeze-dried human cortical and cancellous bone was ground by impact fragmentation and separated using sized sieves. Ground bone matrix (particle size less than 1000 microns) was demineralized by exposure to diluted hydrochloric acid. Briefly, ground bone matrix was exposed to 0.5 N HCl (100 mg DBM in 10 mL 0.5 N HCl) and DBM of variable calcium content was obtained by removing bone matrix from the acid for 8 h. Then, DBM was washed, freeze dried, and stored at -80°C. All DBM used in this study contained approximately 2% residual calcium.

Cell line initiation

Initiation of HPO cells was accomplished as previously described (Zhang et al., 1997), with some modifications. Periosteum from a girl's (8 years old) tibia was obtained during the

course of limb amputation with informed consent. The periosteal tissues were washed three times with alpha-minimal essential medium (α-MEM, Gibco BRL, Gaithersburg, USA) containing 200 U/mL penicillin and 100 µg/mL streptomycin, cut into small fragments, 1.0 x 1.0 mM pieces, and placed with the internal stratum osteogenicum layer facing toward the surface of the T-25 flasks. The preparation was cultured in α-MEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)/streptomycin (50 µg/mL) in a 5% CO_2 incubator at 37°C. The outgrowing cells were combined and transferred into T-75 flasks by detachment with a 0.025% trypsin and 0.05% EDTA solution and cultured in the same medium and incubator. When periosteal cells reached confluence, they were split into new T-75 flasks at a split ratio of 1:4. Cells were continually passaged until sufficient numbers of cells had been generated to provide an opportunity to create a cell bank, in which cells of a uniform passage number were cryopreserved and stored for use in subsequent studies.

Cell culture

Human periosteal cells were thawed and cultured to sufficient cell numbers and seeded into T-25 flasks at 5.0 x 10^5 cells per flask (or 2.0 x 10^4 cells/cm^2). These cells were maintained in α-MEM supplemented with 10% FBS and penicillin (100 U/mL)/streptomycin (50 µg/mL), until reaching confluence. The α-MEM with 10% FBS was then changed to α-MEM supplemented with 2% FBS in the absence (as a control group) or presence (as a DBM group) of 5 mg DBM. Alkaline phosphatase activities were subsequently analyzed at days 0, 3, 5, 7, and 10.

Alkaline phosphatase activity

Human periosteal cells were plated in culture and treated as described above. Alkaline phosphatase staining assay was performed using the Sigma Diagnostics Alkaline Phosphatase kit (Catalog No. 86-2, Sigma Diagnostics, St. Louis, USA), as directed by the manufacturer. Briefly, cell layers were rinsed with phosphate-buffered saline (PBS), fixed with citrate-acetone-formaldehyde fixative solution at room temperature for 30 s, and then stained with alkaline-dye mixture at room temperature for 15 min. Naphthol AS-BI alkaline solution and fast red violet B alkaline solution were utilized for determining enzyme activity. Slides were then counterstained with hematoxylin solution, gill No. 3 and evaluated using light microscopy.

Alkaline phosphatase activity was measured using a modification of a published procedure (Wolfinbarger Jr. and Zheng, 1993). Briefly, treated cells were quickly rinsed twice with 3 mL deionized water, scraped with a cell scraper (Fisher Scientific, Pittsburgh, USA) in 3 mL deionized water and sonicated at 30% intensity for 30 s. Next 200-µL aliquot samples were transferred to each well of a 96-well plate and were mixed with 40 µL 100 mol p-nitrophenyl phosphate in 0.15 M 2-amino-2-methyl-1-propanol buffer, pH 10.4, and incubated for 20 min at 37°C. The reaction was stopped by the addition of 10 µL 10 N NaOH into each well and absorbance determined at 405 nm, using a microplate reader (Multiskan Ascent, Labsystems, Franklin, USA). Alkaline phosphatase activity (expressed as pmol converted p-nitrophenol/min) was normalized by total protein content. Protein concentrations of the samples were determined by using the BCA protein assay (Pierce, Rockford, USA). The alkaline phosphatase activities were expressed as pmol p-nitrophenol.min^{-1}.µg $protein^{-1}$).

RNA isolation and cDNA array hybridization

Total RNA was prepared from HPO cells after seven days of culture with and without DBM, using the Rneasy Mini Kit (Qiagen, Valencia, USA), according to instructions provided by the manufacturer. The purity and amount of isolated RNA were assessed by spectrophotometric measurement at 260 and 280 nm.

A cDNA probe was synthesized from total RNA isolated as described above. Equal amounts of total cellular RNA (5 µg) were reverse transcribed to cDNA at 42°C for 90 min in a volume of 20 µL containing the following reagents: 3 µL GEA primer mix (Buffer A); 4 µL 5X GEA labeling buffer (Buffer BN); 1 µL RNase inhibitor (Promega, Madison, USA), 2 µL 1 mM Biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany), and 50 U MMLV reverse transcriptase (Promega). After terminating the reaction by adding 2 µL 10X stop solution (Buffer C), the biotin-labeled cDNA probes were denatured by heating at 94°C for 5 min and chilled quickly on ice. Human osteogenesis GEArray Q series cDNA expression array (SuperArray Bioscience, Frederick, USA) nylon membranes, containing over 96 cDNA fragments from genes associated with bone development, were hybridized with the biotin-labeled cDNA probes. The membranes were washed, scanned, and analyzed using the ScanAlyze and GEArray Analyzer software. Genes with ratio number above 2 or under 0.5 (2-fold induction or repression) were selected and subjected to further analysis. Each signal was normalized against the signal of a housekeeping gene to compare data from two different arrays. The relative specific intensity within each signal was calculated as the ratio of the density of each signal in the DBM treatment divided by the density of corresponding signal in the control.

RT-PCR analysis

RNA was isolated from HPO cells that were cultured with or without DBM for seven days and was reverse transcribed to cDNA at 42°C for 60 min in a volume of 20 µL containing the following reagents: 0.5 mM dNTP mix; 1X ImProm-II Reaction Buffer; 6 mM $MgCl_2$, and 20 U Recombinant RNasin Ribonuclease Inhibitor. The reactions were then terminated at 70°C for 15 min. Aliquots of the cDNA were amplified in 100 µL of a PCR mixture that contained 1 µM primer sets, 1X thermophilic polymerase reaction buffer (Bio-Rad, Hercules, USA), and 5 U iTaq DNA polymerase (Bio-Rad). Using a thermocycler, DNA amplification included an initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and a final extension at 72°C for 2 min. The final cycle included 5 min for extension. Glyceraldehyde phosphate dehydrogenase (GAPDH) primers were added in all reactions as an internal control. cDNAs were also amplified using specific primers by RT-PCR to generate products corresponding to mRNA encoding for biglycan, collagen14A1, and GAPDH after seven days of culture. Primers for biglycan, collagen14A1, and GAPDH were prepared based on previously described sequences as follows: biglycan (sense: 5'-CCCTCTCCAGGTCCATCCGC-3' and antisense: 5'-GAGCTGGGTAG GTTGGGCGGG-3'; Chen et al., 2002); collagen14A1 (sense: 5'-TGGACAGAACCAGC TACAACCATAGT-3' and antisense: 5'-GGAGACGCTGACGCCTTCGCCATCCGT-3'; Meirowitz et al., 2002); and GAPDH (sense: 5'-ACCACAGTCCATGCCATCAC-3' and antisense: 5'-TCCACCACCCTGTTGCTGTA-3'; Locklin et al., 2001).

Detection of PCR products

The PCR products were analyzed on 2% agarose gels and visualized with ethidium bromide. The density of each band was quantified using the NIH Image software. Relative gene expression was determined by dividing the densitometric value of treatment by that of the control.

Statistical analysis

Data are reported as means with error bars representing standard deviation. Analysis of variance (ANOVA) was used to determine the significant differences among treatment groups. The unpaired *t*-test was performed to check for significant differences between control and experimental groups. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Morphologic and histochemical studies of the effects of DBM on HPO cells

In monolayer cultures, adherent periosteal cells exhibited fibroblast-like morphology. HPO cells without DBM treatment were used as a control. After seven days of incubation, cell morphology was observed and alkaline phosphatase-staining assay was performed. The control cells (without DBM treatment) possessed a spindle shape, and there was very little extracellular matrix (Figure 1A). In contrast, the DBM-treated cells appeared shortened and flattened, and they were surrounded by extracellular matrix (Figure 1B). Furthermore, the control cells were stained blue (Figure 1A), suggesting low levels of alkaline phosphatase activity. On the other hand, in the DBM-treated cells, a strong red and purple color appeared in all the areas (Figure 1B), indicating very high alkaline phosphatase activity. These histochemical studies confirmed that DBM has a strong stimulatory effect on the alkaline phosphatase activities of HPO cells, a very early marker of cell differentiation into the osteogenic lineage.

Additionally, alkaline phosphatase activity assay was performed after 3, 5, 7, and 10 days of incubation. Alkaline phosphatase activity in the DBM-supplemented cultures was significantly higher than in the comparable control cell cultures on days 7 and 10 (Figure 2).

Gene expression profiling of HPO cells during osteogenic differentiation

Figure 3 represents typical hybridization results, when the cDNA probe was obtained from untreated cells and from DBM-treated cells. Using a cutoff value of 2-fold, three genes were found to be upregulated more than 2-fold. Only one gene appeared to be downregulated more than 2-fold during the differentiation process. The highly upregulated genes were biglycan, TGF-β1, and TGF-βR1. Among downregulated genes, collagen14A1 displayed a more than 2-fold decrease in the level of transcript.

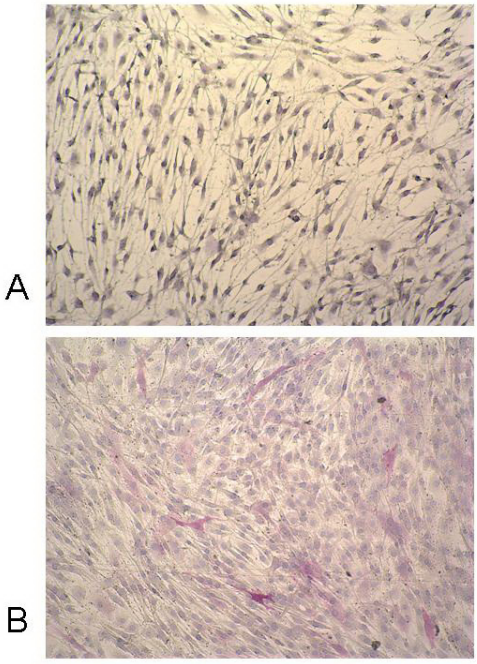

Figure 1. Alkaline phosphatase staining of human periosteal cells. **A.** Untreated cells are negative staining for alkaline phosphatase. **B.** Demineralized bone matrix-treated cells display positive staining for alkaline phosphatase.

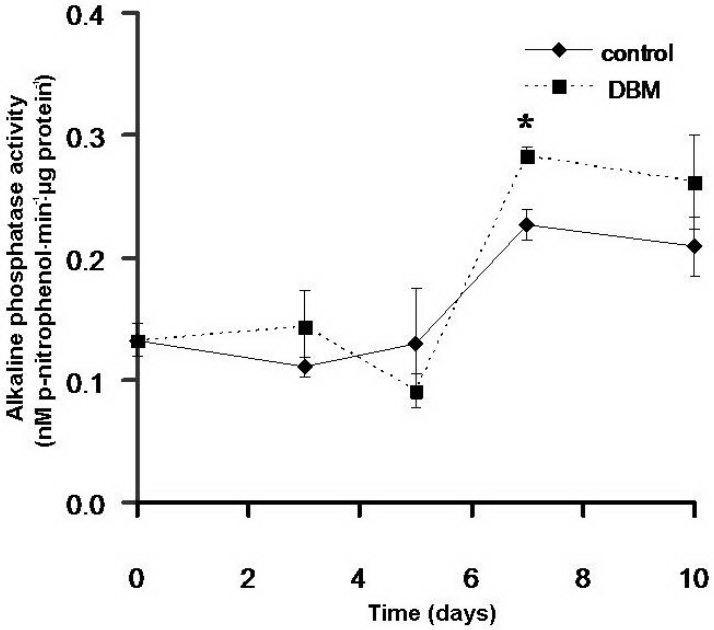

Figure 2. Alkaline phosphatase activity of human periosteal cells cultured for 10 days in the absence or presence of 5 mg demineralized bone matrix. All data are reported as means ± standard deviation. *P value <0.05.

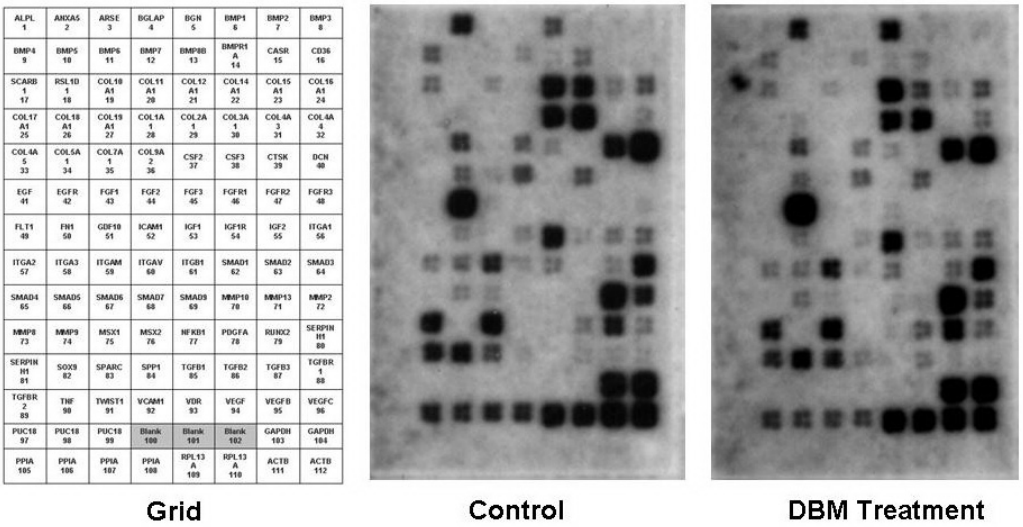

Figure 3. Comparison of gene expression between human periosteal cells in the absence or presence of demineralized bone matrix (DBM) treatment using cDNA array analysis.

Verification of expression of selected genes using RT-PCR analysis

To verify the cDNA array results, RT-PCR analysis was performed using RNA prepared under the same conditions as used in the cDNA array studies. Among upregulated genes, biglycan found to be highly upregulated was selected. The gene encoding for collagen14A1 was chosen as the downregulated gene. A housekeeping GAPDH gene, whose expression was unchanged during osteogenic differentiation, was chosen to serve as an internal control. RT-PCR analysis of parallel samples was consistent with the cDNA array data in that there was an increase in the level of biglycan expression after day seven of DBM treatment (Figure 4). In accordance with the cDNA array analysis, expression of the gene encoding for collagen14A1 decreased after day seven of DBM addition (Figure 4).

To examine the temporal expression of biglycan and collagen14A1 in HPO cells untreated or treated with DBM, RNA was purified for RT-PCR analysis at days 0, 4, 7, 10, and 14 in the absence or presence of DBM. Figure 5A illustrates the time course of biglycan mRNA expression relative to that of GAPDH mRNA in HPO cells with and without DBM treatment. Biglycan mRNA expression level in the DBM-treated cells was approximately 2-fold higher than that of untreated cells at every time. In downregulated genes, the time course of collagen14A1 expression relative to that of GAPDH mRNA is demonstrated in Figure 5B. The results revealed that gene expression of collagen14A1 in the DBM-treated cells decreased over time when compared with that in the untreated cells. The repression level of collagen14A1 was greater than 2-fold on days 4, 7, and 14.

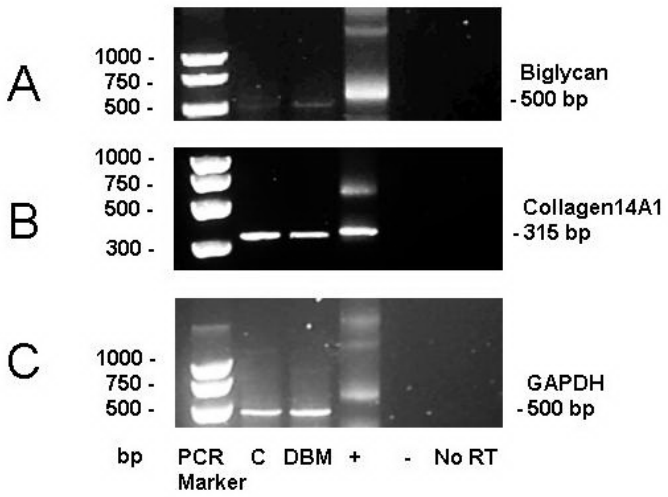

Figure 4. RT-PCR analysis during osteogenic differentiation of human periosteal cells. **A.** Biglycan. **B.** Collagen14A1. **C.** GAPDH. C = control (human periosteal cells without demineralized bone matrix (DBM)); DBM = DBM treatment (human periosteal cells with DBM); + = positive control; - = negative control; no RT = no reverse transcriptase addition in RT-PCR.

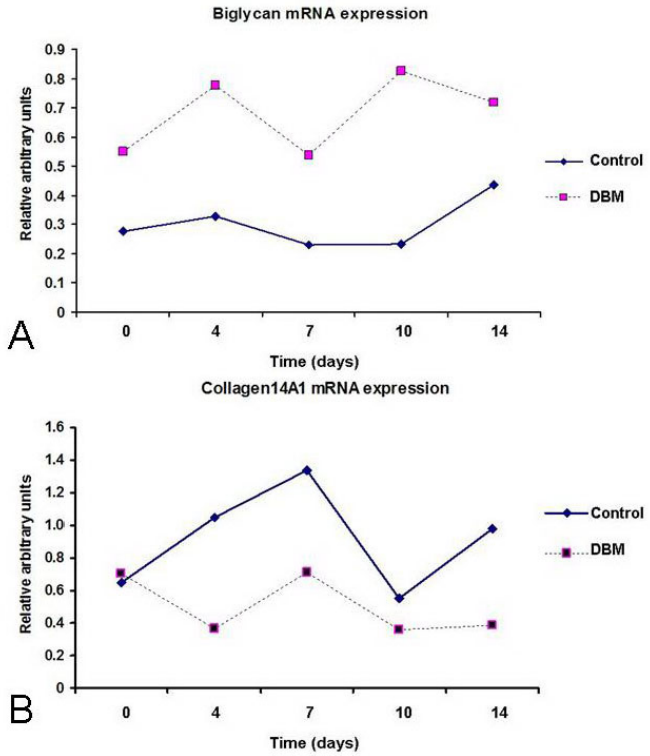

Figure 5. Comparison of gene expression by RT-PCR analysis from time course study of human periosteal cells. **A.** Biglycan. **B.** Collagen14A1. DBM = demineralized bone matrix.

DISCUSSION

Demineralized bone matrix derived from human cortical bone are prepared by removing the minerals while allowing the active bone inductive growth factors, collagenous proteins and other organic constituents to remain (Honsawek et al., 2005). DBM should be used in conjunction with a transplant or implant displaying mechanical strength. DBM can augment cortical grafts used for bridging gaps or defects and lengthening procedures by increasing the connectivity of the structural graft with the host bone. Another useful application is providing a biological boost to patients who have less-than-ideal physiology. Because DBM has higher concentrations of available bone morphogenic proteins, it can aid in the incorporation of other grafts. Other uses include delayed unions, non-unions, packing joints for arthrodesis, filling resected cysts, and filling gaps of debrided infected bone. DBM is a potentially attractive scaffold for use in tissue engineering since DBM is able to support and promote osteogenesis of matrix-incorporated osteoprogenitors *in vivo*.

The development of cDNA gene expression array technology allows the analysis of the expression levels of hundreds to thousands of genes simultaneously, providing an effective tool for large-scale gene expression studies (Schena et al., 1996). Our current research used gene array technology to screen alterations in gene expression during differentiation of human periosteal cells to an osteoblast-like phenotype. The expression of 96 genes in human periosteal cells in response to DBM was examined using a human osteogenesis gene array. The results obtained from the cDNA gene expression array analysis were validated on HPO cells using RT-PCR.

Our study revealed that expression of biglycan, TGF-β1, and TGF-βR1 is upregulated whereas the expression of collagen14A1 is downregulated in DBM-treated cultures compared with untreated cultures. Among the upregulated genes during osteogenic differentiation, biglycan coding for extracellular matrix protein is a prominent osteoblastic marker that has previously been associated with the development and function of osteoblasts and has been shown to play an important role in bone formation (Xu et al., 1998). Interestingly, TGF-β1 involved in the enhancement of the formation of extracellular matrix and inhibition of matrix degradation was also upregulated, including its receptor, TGF-βR1. In contrast, expression of the gene encoding for collagen14A1 was downregulated. This decrease might have implications during the process of bone development because collagen14A1 occurs in virtually every collagen I-containing tissue (periosteum, tendon, skeletal muscle, cardiac muscle, and nerve) (Walchli et al., 1994).

Although bone is a highly specialized tissue, osteoblasts are very similar to fibroblasts in terms of gene expression (Ducy et al., 2000). A characteristic feature of osteoblasts is the secretion and processing of extracellular matrix. The strongest modulations were observed in the cDNA array experiments for extracellular matrix genes, with particularly striking upregulation of the small leucine-rich proteoglycan, biglycan. Proteoglycans play a role in matrix assembly and can promote *in vitro* matrix mineralization (Boskey et al., 1997). The result of the time course study of biglycan is in agreement with the model of osteoblast differentiation, in which osteoblast differentiation is followed by matrix deposition and matrix maturation. This cDNA array study thus may extend current knowledge about the mechanisms whereby osteoblasts deposit bone matrix.

In addition, other genes, such as alkaline phosphatase, Runx2, insulin-like growth

factor-1 (IGF-1), and vascular endothelial growth factor (VEGF), were slightly upregulated. Their induction level on the cDNA array was less than 2-fold and therefore not included in RT-PCR analysis. However, unknown relevant osteoblast-specific genes might not be present on the cDNA arrays that we used.

The data derived from this type of approach are broadly descriptive. However, the analysis that we present offers a screening method and a dynamic picture of gene expression during osteogenesis. Although other reports have examined gene expression during osteogenic differentiation by using gene expression array technology (Beck et al., 2001; Locklin et al., 2001), accurate comparison of these studies is difficult for several reasons: 1) the cDNA array used in our study differs from the arrays used in other studies; therefore, different genes are screened; 2) studies are performed with different cell lines; 3) different agents or inducers of osteogenic differentiation such as β-glycerophosphate, ascorbic acid, BMP, and conditioned media are used, and 4) expression analysis is performed at different times. The ability to compare data collected on hundreds of genes under one set of conditions with data from other system results in overall understanding of the molecular basis of osteogenesis. These data are valuable not just for a better understanding of osteogenesis but also for comparison with other tissue types.

We demonstrated that DBM induced gene expression of osteogenic differentiation in HPO cells using cDNA gene array analysis. Expression of biglycan was highly upregulated whereas the expression of collagen14A1 was downregulated in the DBM-treated cultures, compared with the untreated cultures. Further studies are essential to better understand new bone-related genes, which may play essential roles in osteoblast development, and to provide insight into the biological process of osteogenic differentiation in human periosteal cells.

ACKNOWLEDGMENTS

The authors acknowledge financial support from the Research, Development and Engineering (RD & E) Fund through the National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Thailand. This investigation was also supported in part by the Thailand Research Fund (#DBG4980017), the National Research Council of Thailand, the National Research University Project of CHE and the Ratchadaphiseksomphot Endowment Fund (#HR 1155A) and LifeNet Research Fund. The authors also thank Chulalongkorn Medical Research Center (Chula MRC) and Research Core Facility at Department of Biochemistry for kindly providing facilities.

REFERENCES

Beck GR Jr, Zerler B and Moran E (2001). Gene array analysis of osteoblast differentiation. *Cell Growth Differ.* 12: 61-83.

Boskey AL, Spevak L, Doty SB and Rosenberg L (1997). Effects of bone CS-proteoglycans, DS-decorin, and DS-biglycan on hydroxyapatite formation in a gelatin gel. *Calcif. Tissue Int.* 61: 298-305.

Chen WB, Lenschow W, Tiede K, Fischer JW, et al. (2002). Smad4/DPC4-dependent regulation of biglycan gene expression by transforming growth factor-beta in pancreatic tumor cells. *J. Biol. Chem.* 277: 36118-36128.

Ducy P, Schinke T and Karsenty G (2000). The osteoblast: a sophisticated fibroblast under central surveillance. *Science* 289: 1501-1504.

Honsawek S, Powers RM and Wolfinbarger L (2005). Extractable bone morphogenetic protein and correlation with induced new bone formation in an *in vivo* assay in the athymic mouse model. *Cell Tissue Bank.* 6: 13-23.

Honsawek S, Dhitiseith D and Phupong V (2006). Effects of demineralized bone matrix on proliferation and osteogenic

differentiation of mesenchymal stem cells from human umbilical cord. *J. Med. Assoc. Thai.* 89 (Suppl 3): S189-S195.
Honsawek S, Dhitiseith D and Phupong V (2007). Gene expression characteristics of osteoblast differentiation in human umbilical cord mesenchymal stem cells induced by demineralized bone matrix. *Asian Biomed.* 1: 383-391.
Honsawek S, Bumrungpanichthaworn P and Thanakit V (2010). Osteoinductive potential of small intestinal submucosa/demineralized bone matrix as composite scaffolds for bone tissue engineering. *Asian Biomed.* 4: 913-922.
Hutmacher DW and Sittinger M (2003). Periosteal cells in bone tissue engineering. *Tissue Eng.* 9 (Suppl 1): S45-S64.
Locklin RM, Riggs BL, Hicok KC, Horton HF, et al. (2001). Assessment of gene regulation by bone morphogenetic protein 2 in human marrow stromal cells using gene array technology. *J. Bone Miner. Res.* 16: 2192-2204.
Meirowitz NB, Smulian JC, Hahn RA, Zhou P, et al. (2002). Collagen messenger RNA expression in the human amniochorion in premature rupture of membranes. *Am. J. Obstet. Gynecol.* 187: 1679-1685.
Schena M, Shalon D, Heller R, Chai A, et al. (1996). Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. *Proc. Natl. Acad. Sci. U. S. A.* 93: 10614-10619.
Walchli C, Koch M, Chiquet M, Odermatt BF, et al. (1994). Tissue-specific expression of the fibril-associated collagens XII and XIV. *J. Cell Sci.* 107: 669-681.
Wolfinbarger L Jr and Zheng Y (1993). An *in vitro* bioassay to assess biological activity in demineralized bone. *In Vitro Cell Dev. Biol. Anim.* 29A: 914-916.
Xu T, Bianco P, Fisher LW, Longenecker G, et al. (1998). Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. *Nat. Genet.* 20: 78-82.
Zhang M, Powers RM Jr and Wolfinbarger L Jr (1997). A quantitative assessment of osteoinductivity of human demineralized bone matrix. *J. Periodontol.* 68: 1076-1084.