

Differential Expression of Human Lysyl Hydroxylase Genes, Lysine Hydroxylation, and Cross-Linking of Type I Collagen During Osteoblastic Differentiation In Vitro*

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ABSTRACT

The pattern of lysyl hydroxylation in the nontriple helical domains of collagen is critical in determining the cross-linking pathways that are tissue specific. We hypothesized that the tissue specificity of type I collagen cross-linking is, in part, due to the differential expression of lysyl hydroxylase genes (Procollagen-lysine,2-oxyglutarate,5-dioxygenase 1, 2, and 3 [*PLOD1*, *PLOD2*, and *PLOD3*]). In this study, we have examined the expression patterns of these three genes during the course of in vitro differentiation of human osteoprogenitor cells (bone marrow stromal cells [BMSCs]) and normal skin fibroblasts (NSFs). In addition, using the medium and cell layer/matrix fractions in these cultures, lysine hydroxylation of type I collagen α chains and collagen cross-linking chemistries have been characterized. High levels of *PLOD1* and *PLOD3* genes were expressed in both BMSCs and NSFs, and the expression levels did not change in the course of differentiation. In contrast to the *PLOD1* and *PLOD3* genes, both cell types showed low *PLOD2* gene expression in undifferentiated and early differentiated conditions. However, fully differentiated BMSCs, but not NSFs, exhibited a significantly elevated level (6-fold increase) of *PLOD2* mRNA. This increase coincided with the onset of matrix mineralization and with the increase in lysyl hydroxylation in the nontriple helical domains of α chains of type I collagen molecule. Furthermore, the collagen cross-links that are derived from the nontriple helical hydroxylysine-aldehyde were found only in fully differentiated BMSC cultures. The data suggests that *PLOD2* expression is associated with lysine hydroxylation in the nontriple helical domains of collagen and, thus, could be partially responsible for the tissue-specific collagen cross-linking pattern. (J Bone Miner Res 1999;14:1272-1280)

INTRODUCTION

TYPE I COLLAGEN is a heterotrimeric molecule composed of two $\alpha 1$ chains and one $\alpha 2$ chain. It is the predominant organic matrix molecule in bone and plays a central role in this tissue's support function.⁽¹⁾ The biosynthesis of

the collagen molecule is a long, complicated process involving a number of post-translational modifications.⁽²⁾ The hydroxylation of specific peptidyl lysine (Lys) residues is a modification critical for glycosylation and cross-linking of the collagen molecule.^(3,4) The Lys hydroxylation in the nontriple helical domains of the collagen molecule (both C and N termini) is particularly important in determining the cross-linking pathways/patterns which have been found to differ from tissue to tissue. For instance, the lysine-aldehyde (Lys^{ald})-derived pathways are predominant in the soft connective tissues such as skin and cornea.⁽²⁾ The hy-

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droxylysine-aldehyde (Hyl^{ald})-derived pathways, however, are predominant in skeletal connective tissues such as bone, ligament, and tendon.^(2,5)

Several studies have demonstrated that overhydroxylation of Lys residues in type I collagen occurs in bone diseases such as osteogenesis imperfecta,⁽⁶⁾ osteopenia,⁽⁷⁾ and osteoporosis.^(8,9) Furthermore, changes in collagen cross-link pattern, due in part to an altered Lys hydroxylation at the nontriple helical domains of the collagen molecule, were observed in bones obtained from patients or animals with osteogenesis imperfecta,⁽¹⁰⁾ disuse osteopenia,⁽¹¹⁾ osteogenic osteoblastoma,⁽¹²⁾ vitamin D deficiency,⁽¹²⁾ and osteopetrosis.⁽¹³⁾ These observations and other studies^(5,14) suggest the possibility that lysyl hydroxylase (LH) activity may play a role in modulating the process of bone mineralization.

Due to the difference in the adjacent amino acid sequence of Lys between the triple helical and the nontriple helical domains of collagen, it has long been speculated that there is more than one type of LH.⁽¹⁵⁾ Recently, two new genes that encode isoforms of LH, lysyl hydroxylase 2 and 3 (LH2 and LH3), have been identified and designated as *PLOD2* (Procollagen-lysine,2-oxyglutarate,5-dioxygenase 2) and *PLOD3* (*PLOD1* is the gene for the conventional lysyl hydroxylase 1 [LH1]).⁽¹⁶⁻¹⁸⁾ The functional analyses using a baculovirus expression system showed that the proteins encoded by the cDNA for these new isoforms exhibited LH activity toward the synthetic peptides containing the LH consensus sequence IKGIGKIGK as in the case of the cDNA for *PLOD1*.⁽¹⁸⁾

Type I collagen isolated from skin exhibits a cross-link pattern that is almost exclusively derived from the Lys^{ald} pathways, while cross-links present in bone collagen are predominantly derived from Hyl^{ald} pathway.⁽²⁾ We hypothesized that the tissue-specific collagen cross-linking pattern is, in part, due to the differential expression of these genes in the respective tissues. To study the correlation between the expression of the *PLOD* genes and the patterns of Lys hydroxylation and cross-linking of collagen, we have employed an in vitro system using human osteoprogenitor cells and skin fibroblasts. It has been shown that bone marrow stromal cells (BMSCs) undergo osteoblastic differentiation in culture and that they form bone in vivo when transplanted into immunodeficient mice.^(19,20) Thus, this system offers an excellent model to study events associated with osteoblastic differentiation both in vivo and in vitro. In this study, we have examined and compared the mRNA expression patterns of these *PLOD1*, *PLOD2*, and *PLOD3* genes in human BMSCs and normal skin fibroblasts (NSFs) during the course of their differentiation in vitro. In addition, using the cell layer and medium fractions of these cultures, the hydroxylation of Lys residues in the α chains of type I collagen with and without nontriple helical domains and collagen cross-linking chemistries were analyzed.

MATERIALS AND METHODS

Cells

Human BMSCs were obtained from bone marrow content of normal spine bone fragments that were collected in

the course of corrective surgery (patients' 7-15 years of age) under institutionally approved procedures for the use of human surgical waste. Cultures of the BMSCs were established as described previously^(19,20) and cells from passage 4 obtained from three different patients were used. All BMSC strains used in this study formed bone when transplanted into immunodeficient mice thus confirming their committed osteoblastic phenotype.⁽¹⁹⁾ NSFs (line CRL 1906), passage 11, were obtained from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). Cells were maintained until confluent in 100-mm tissue culture Petri dishes (Costar, Cambridge, MA, U.S.A.) in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (GIBCO BRL Life Technologies, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum (FBS; GIBCO) supplemented with 100 U/ml of penicillin and 100 μ g/ml of streptomycin (GIBCO).

Osteoblastic differentiation in vitro

Cells (BMSCs and NSFs) were released from culture dishes by trypsin-EDTA (GIBCO) treatment, centrifuged at 500g and plated onto 150-mm Petri dishes (Becton Dickinson, Bedford, MA, U.S.A.) at the density of 20,000 cells/cm². Parallel cultures of the same passage cells were also plated into 24-well tissue culture plates (Costar) and used for evaluation of osteoblastic differentiation markers, i.e., alkaline phosphatase (ALP) activity and mineral deposition. Cells were incubated in the growth medium (DMEM/F12 medium containing 10% FBS) until confluent (usually 7-10 days, referred to as the "proliferation stage" hereafter). When cells became confluent, the medium was replaced with the same medium supplemented with 50 μ g/ml of ascorbic acid (Sigma, St. Louis, MO, U.S.A.) and cells were maintained in this "complete" medium for 7 days (early differentiation stage). Following this step, the medium was replaced with "mineralization" medium containing DMEM/F12, 100 U/ml of penicillin, 100 μ g/ml of streptomycin, 10% FBS, 100 μ g/ml ascorbate, 2.5 μ g/ml of insulin-transferrin-selenium (Boehringer-Mannheim, Indianapolis, IN, U.S.A.), 10⁻³ M beta-glycerophosphate (Sigma), and 10⁻⁸ M dexamethasone (Sigma). Cells were then incubated for 16 days under these conditions (late differentiation stage, referred to as the "mineralization stage"). To assess osteoblastic differentiation in vitro, histochemical ALP activity assay, von Kossa staining, and calcium content measurements were performed. ALP activity was evaluated by a commercial kit (kit #86-R; Sigma) according to the manufacturer's protocol. ALP positive cells were counted under a microscope (10 fields/observation), and the activity was evaluated as the percentage of ALP positive. Mineral deposition in the cultures was visualized by the von Kossa staining method. Cells were fixed in 10% (v/v) paraformaldehyde, washed, and stained with 5% AgNO₃ as described.⁽²¹⁾ Calcium content was determined by a commercial kit (Calcium Binding Reagent; Sigma). Briefly, the cultures were washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 5 minutes, and then incubated in 0.5 ml/well of 0.6 N HCl for 24 h. The level of released calcium was de-

terminated by measuring absorbance at 650 nm wavelength by a spectrophotometer (Hitachi U-2001; Hitachi Instruments, Inc., San Jose, CA, U.S.A.), according to the manufacturer's protocol. In parallel with the assay, the amount of DNA obtained from matching samples was measured using TRIzol reagent (GIBCO). The calcium content was normalized to the amount of DNA in each sample.

RNA preparation

Total cellular RNA from BMSCs and NSF s was isolated from the cultures at the end of each stage (proliferation, early differentiation, and mineralization) employing the acid guanidinium thiocyanate-phenol-chloroform method⁽²²⁾ using a RNAGents Total RNA Isolation System (Promega, Madison, WI, U.S.A.). The RNA concentration was determined spectrophotometrically. Poly(A)⁺ RNA was purified from total RNA using the Oligotex mRNA Mini Kit (Qiagen, Chatsworth, CA, U.S.A.) according to the manufacturer's protocol. The samples were stored at -80°C until use.

cDNA synthesis

To create first-strand cDNAs for *PLOD1*, *PLOD2*, and *PLOD3* cDNA probes, 4 µg of total RNA from NSF was used for reverse transcription (RT). Reactions were performed using a First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.) according to the manufacturer's protocol in a final volume of 15 µl. After incubation for 1 h at 37°C, the cDNAs were heated to 90°C for 5 minutes and stored at -20°C until used for amplification by polymerase chain reaction (PCR).

PCR amplification

The specific primers for *PLOD1*, *PLOD2*, and *PLOD3* genes were synthesized based on the published sequences.⁽¹⁶⁻¹⁸⁾ For amplification of cDNAs for *PLOD1*, *PLOD2*, and *PLOD3* genes (GenBank accession #G190073, #G2138313, and #AF046889, respectively), the primer sequences were:

LH1-F: GGAACCTGGCCTATGACACCCT (residues 885-907)

LH1-R: TGCCATGCTGTGCCAGGAACT (residues 1207-1224)

LH2-F: CTAAAGTTTACATTGATCCACT (residues 586-608)

LH2-R: GGCTTCCGCTTGACTTAGAT (residues 1094-1113)

LH3-F: AATGGCTGGACTCCTGAGGGA (residues 1027-1047)

LH3-R: GAGCTGCGGCCAGGAGTCAG (residues 1247-1266).

PCR amplification of cDNAs for *PLOD1*, *PLOD2*, and *PLOD3* was performed in a 100 µl reaction volume containing 50 µl of PCR Master (Boehringer Mannheim), 2 µl of the cDNA obtained from the RT reaction, 2 µl of each of the specific primers (0.5 µg/µl) described above and 44

µl of water. Each cycle of PCR included 30 s of denaturation at 94°C, 45 s of primer annealing at 52°C, and 30 s of extension at 72°C. After 35-40 cycles of the reaction, the PCR products were separated on 1.2% TAE-agarose gel, stained with ethidium bromide, purified from the gel, and subsequently used for generation of the cDNA probes for Northern blot analyses. To confirm the identity of the PCR products, they were cloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced at the UNC-CH Automated DNA Sequencing Facility (University of North Carolina-Chapel Hill, Chapel Hill, NC, U.S.A.). A cDNA for the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was purchased from ATCC.

RNA blot hybridization (Northern) analysis

The cDNAs were labeled with [α -³²P]dCTP using a random primer radiolabeling kit (Boehringer Mannheim) according to the manufacturer's instruction. Purified poly(A)⁺ RNA (2 µg/each lane) was separated on 1% agarose gels containing 2.2 M formaldehyde and transferred onto a Hybond N membrane (Amersham, Arlington Heights, IL, U.S.A.).⁽²³⁾ The RNA was cross-linked to the membrane using UV cross-linker (Stratalinker 1800; Stratagene, Manasha, WI, U.S.A.). The membranes were prehybridized at 42°C in 50% formamide, 5× SSC, 1× Denhardt's solution, and 100 µg/ml sonicated salmon sperm DNA, and hybridized with the radiolabeled probe overnight under the same conditions. Following the wash, blots were exposed to X-ray film (Eastman Kodak, Rochester, NY, U.S.A.) for 2-16 h. After the exposure, the membranes were stripped with 0.1% SDS for 1 h in a boiling-water bath and rehybridized with a GAPDH cDNA probe. The levels of mRNA expression on autoradiographs were determined by scanning X-ray films, and the densitometric analysis of the scanned images was performed on a Power Macintosh 9500/132 computer by employing the National Institutes of Health Image software (Version 1.59; NIH, Bethesda, MD, U.S.A.). The results were normalized as a ratio of each specific mRNA signal to the GAPDH signal within the same RNA sample. Each experiment was repeated at least twice.

Analysis of Lys hydroxylation of collagen

Cell layer and medium fractions from the end of each culture stage (proliferation, early differentiation, and mineralization stage; see above) were collected. The medium fractions were dialyzed extensively against cold water and lyophilized. Cells together with the deposited, insoluble matrix were washed three times with cold PBS and lysed with 1× lysis buffer (0.1 M Tris-HCl, 0.125 M NaCl buffer containing 1% Triton X-100, 0.1% SDS, 1% deoxycholate [Sigma] and a cocktail of protease inhibitors including 0.5 mM phenylmethanesulfonyl fluoride, 5 mM benzamide, 2 mM pepstatin A, and 1 mM leupeptin) for 30 minutes at room temperature. Plates were then scraped with a cell scraper (Costar), and the material collected was transferred into 15-ml plastic tubes and incubated overnight at 4°C on

a rotating platform. The lysates were centrifuged at 2000g for 30 minutes at 4°C and the insoluble residue was washed with PBS (three times) and demineralized with Decalcifying Solution (Stephens Scientific, Riverdale, NJ, U.S.A.) overnight. After ultracentrifugation (25,000g for 30 minutes at 4°C), the precipitates were washed three times with cold distilled water and then lyophilized. Most noncollagenous soluble proteins and procollagen were removed by this extraction/wash procedure.

For the Lys hydroxylation within the triple helical portion of the molecule, the medium and cell layer/matrix fractions were pooled from both cell types at each culture stage to collect sufficient amounts of the material for the following analyses. The following preparation was done at 4°C. Two milligrams of the sample was suspended in 0.5 N acetic acid and treated with pepsin (20% w/w; Worthington, Freehold, NJ, U.S.A.) for 24 h. Following the digestion, the residue was removed by ultracentrifugation (25,000g for 45 minutes) and NaCl was added to the supernatant to a concentration of 0.7 M and stirred for 16 h. The precipitate was collected by ultracentrifugation (25,000g for 45 minutes), dialyzed against cold distilled water for 16 h, and then lyophilized. Fifty micrograms of the dried material was resuspended in the gel sample buffer (Novex, San Diego, CA, U.S.A.), separated on a 5% SDS-PAGE under nonreducing condition and the proteins were then transferred onto an Immobilon-P^{SO} Transfer Membrane (Millipore, Bedford, MA, U.S.A.) in the transfer buffer (10 mM 3-[Cyclohexylamino]-1-propanesulfonic acid [Sigma], 10% methanol, pH 11.0).⁽²⁴⁾ The membrane was stained with 0.5% Coomassie Brilliant Blue (Sigma); the bands corresponding to the type I collagen $\alpha 1$ and $\alpha 2$ chains were excised from the membrane and, after flushing with N₂, hydrolyzed with 6 N HCl in vacuo at 105°C for 22 h by the method reported recently.⁽²⁵⁾ The hydrolysates were then analyzed for their amino acid compositions on a Varian 9050 liquid chromatograph (Varian Associate, Walnut Creek, CA, U.S.A.) configured as an amino acid analyzer using ninhydrin for color development at 135°C in a stainless steel reaction coil.⁽²⁵⁾ The ninhydrin color was monitored at 570 nm wavelength for all amino acids with the exception of hydroxyproline (Hyp) and proline that were monitored at 440 nm. The level of Lys hydroxylation was expressed as hydroxylysine/lysine + hydroxylysine (Hyl/Lys + Hyl).

The levels of Lys hydroxylation in the $\alpha 1$ and $\alpha 2$ chains of type I collagen that include triple helical and nontriple helical domains were analyzed in the following manner. An aliquot of each decalcified sample was extracted by 6 M guanidine/0.05 M Tris-HCl buffer (pH 7.4) for 3 days at 4°C. Following the extraction, the residue was removed by ultracentrifugation (25,000g for 30 minutes), and the supernatant was dialyzed against cold distilled water for 16 h and then lyophilized. The dried sample was subjected to 5% SDS-PAGE, transferred onto an Immobilon-P^{SO} Transfer Membrane (Millipore), stained with 0.5% Coomassie Brilliant Blue (Sigma), and the $\alpha 1$ and $\alpha 2$ chains of type I collagen were excised, hydrolyzed, and subjected to amino acid analyses in the same manner as described above.

The α chains of type I collagen with and without pepsin digestion were identified on the gel/membrane by compari-

son with the respective standards prepared from bovine skin type I collagen.

Collagen cross-link analysis

Two milligrams of each decalcified insoluble sample were suspended in 0.15 M N-trimethyl-2-aminoethanesulfonic acid buffer, pH 7.4, and reduced with standardized NaB³H₄.⁽²⁶⁾ The reduced samples were hydrolyzed with 6 N HCl, and the aliquots were subjected to amino acid analyses in order to determine Hyp content. The hydrolysates containing 300 nM of Hyp were then analyzed for cross-links on a Waters 600E HPLC fitted with an ion exchange column (AA911; Transgenomic, Inc., Omaha, NE, U.S.A.) linked to an on-line fluorescence flow monitor (821-FP; Jasco Spectroscopic Co., Tokyo, Japan) and a liquid scintillation monitor (Flo-one Beta; Packard Instrument, Co., Meriden, CT, U.S.A.) as described previously.⁽¹⁴⁾ The reducible cross-links (deH-DHLNL, dehydro-dihydroxylysionorleucine; deH-HLNL, dehydro-hydroxylysionorleucine; and deH-HHMD, dehydrohistidinohydroxymero desmosine) were identified and quantitated as their reduced forms (DHLNL, HLNL, and HHMD, respectively). The reducible cross-links as well as the nonreducible, fluorescent cross-links (Pyr, pyridinoline; d-Pyr, deoxypyridinoline) were quantitated as mol/mol of collagen as described previously.⁽²⁷⁾

RESULTS

In vitro cell differentiation assay

After 16 days of incubation in the mineralization medium, the BMSC cultures differentiated into mature osteoblasts as evidenced by an increase in ALP activity and mineral deposition within the cultures (Figs. 1 and 2). The NSF cultures showed only a minimal level of ALP activity and the cultures did not mineralize throughout the course of this experiment.

Expression patterns of the LH genes

To assess the expression of *PLOD1*, *PLOD2*, and *PLOD3* genes during cellular differentiation in vitro, RNA blot-hybridization (Northern blot analysis) with PCR-generated cDNA probes was employed. The ³²P-labeled cDNAs of *PLOD1*, *PLOD2*, and *PLOD3* hybridized specifically with mRNA of 4.2, 3.4, and 2.75 kb, respectively, which were in agreement with the mRNA sizes reported in the literature (Fig. 3).⁽¹⁶⁻¹⁸⁾ Steady-state levels of *PLOD1* and *PLOD3* mRNAs were observed in both cell types under all conditions tested (Fig. 3). In contrast, the *PLOD2* gene was highly expressed by BMSCs only at the "mineralization stage" (i.e., after 16 days of incubation in the mineralization medium; Fig. 3). When normalized to the GAPDH mRNA signal, the expression of *PLOD2* message in the mineralizing BMSC cultures was about 6-fold higher than those in the proliferation and early differentiation stages. NSF cultures showed a low level of *PLOD2* message

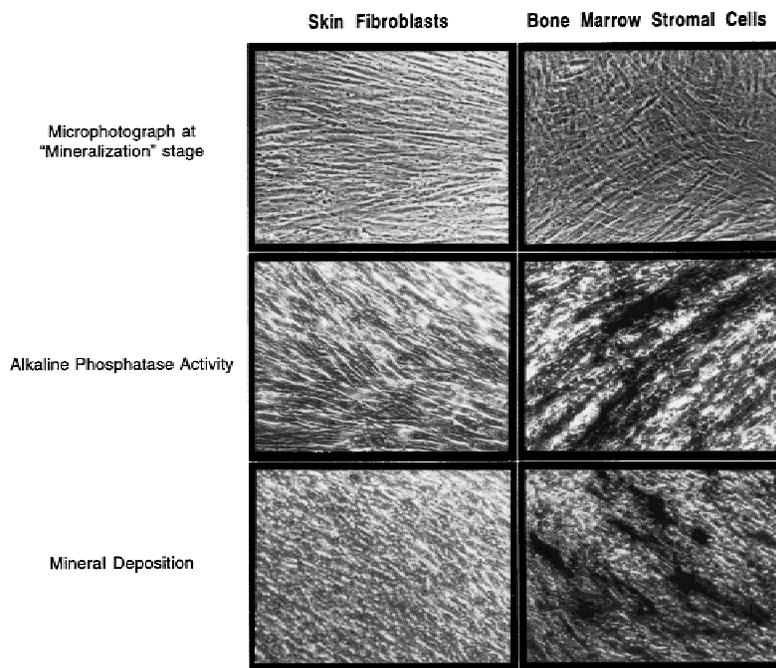


FIG. 1. The morphology, ALP activity, and mineral deposition via von Kossa staining of BMSCs and skin fibroblasts after 16 days in culture in mineralization medium. Osteoblastic differentiation of BMSC confirmed by high numbers of cells stained for ALP and by extensive mineral deposition. Original magnification $\times 100$.

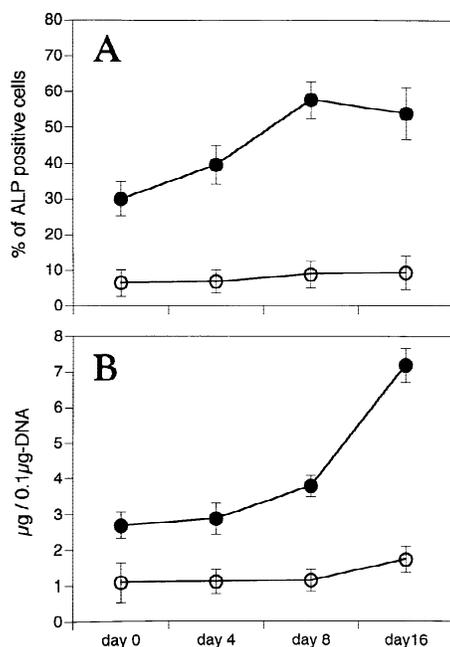


FIG. 2. ALP activity and calcium content in BMSCs and skin fibroblasts in culture. After incubation in “complete” medium in a 24-well plate (day 0), cells were incubated for 16 days in mineralization medium (see Materials and Methods). At the indicated time points, the percentage of ALP positive cells (A) and the calcium content (B) are shown. Values are the average of at least 10 measurements \pm SD. Filled circle, BMSCs; open circle, NSFs.

throughout the culture period, slightly lower but comparable to the levels observed in less differentiated BMSC (proliferation and early differentiation stages). These re-

sults were further confirmed by semiquantitative RT-PCR (data not shown). All BMSCs obtained from three patients yielded identical results in these assays.

Lysine hydroxylation

Amino acid analysis as well as SDS-PAGE indicated that very little collagen was present in the medium and cell layer/matrix fraction obtained from cells at the “proliferation stage” (fewer than 5 residues of Hyp per 1000); therefore, no further analysis for Lys hydroxylation was performed for this material. The amino acid analysis of the cell layer/matrix fractions obtained from early differentiation and mineralization stages showed that they were essentially composed of collagen (more than 80 residues of Hyp per 1000). To determine the level of Lys hydroxylation in the triple helical portion of type I collagen that was secreted during the *in vitro* cell differentiation, the cell layer/medium fractions obtained from these stages were digested with pepsin. The $\alpha 1$ and $\alpha 2$ collagen I chains were separated by SDS-PAGE and each chain was analyzed for amino acid composition.⁽²⁵⁾ The medium and cell layer/matrix fractions prepared from all cultures examined was virtually solubilized by pepsin digestion and the SDS-PAGE analysis of the digest revealed no appreciable protein bands other than $\alpha 1$ and $\alpha 2$ chains of type I collagen (Fig. 4, lane B). This indicates that type I collagen is the predominant component in this fraction and that it is not highly cross-linked (i.e., no β , γ , or other higher molecular weight components on the gel). In both cell types, the Lys hydroxylation in the triple helical domains of both $\alpha 1$ and $\alpha 2$ chains of the secreted type I collagen showed no significant difference between the early differentiation and mineralization stages (Table 1). The $\alpha 1$ and $\alpha 2$ chains of type I collagen obtained from guanidine-HCl extract migrated on the SDS-PAGE

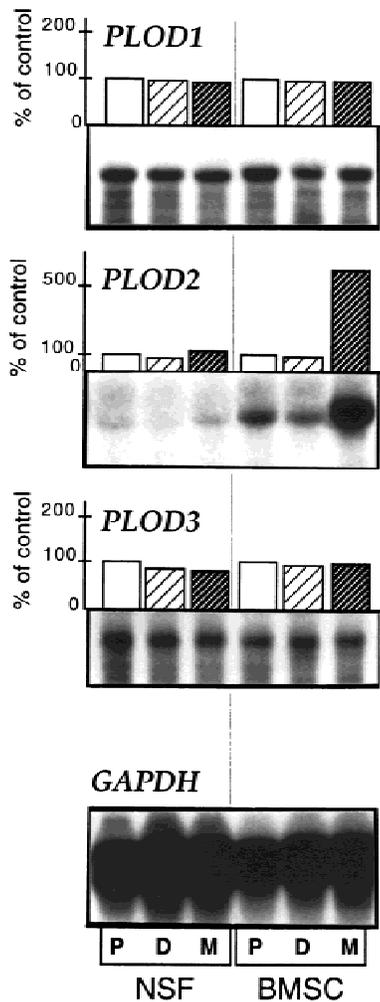


FIG. 3. Northern blot analyses of *PLOD1*, *PLOD2*, and *PLOD3* gene expressions in BMSCs and NSFs in the course of differentiation in vitro. Poly(A)⁺ RNA (2 μg/lane) isolated from BMSC and skin fibroblast (NSF) at the end of each stage was probed with *PLOD1*-, *PLOD2*-, and *PLOD3*-specific cDNAs (bottom panels). Values obtained from densitometric analysis for each mRNA were first normalized to GAPDH mRNA levels and then expressed as the percentage of the values obtained for the proliferation (P) stage for each cell type (top panels). P, proliferation stage; D, early differentiation stage; M, mineralization stage.

slightly slower than those obtained from pepsin digested collagen (Fig. 4, lane A). The α1 and α2 chains with intact nontriple helical domains that were obtained from the BMSC cultures at the mineralization stage showed a significant increase in Lys hydroxylation (calculated by Hyl/Lys + Hyl). No such increases were observed in the samples obtained from any stage of NSF cultures or less differentiated BMSC cultures. This suggests that the degree of Lys hydroxylation in the nontriple helical domains of collagen correlates with the expression level of the *PLOD2* mRNA (Table 1 and Fig. 3).

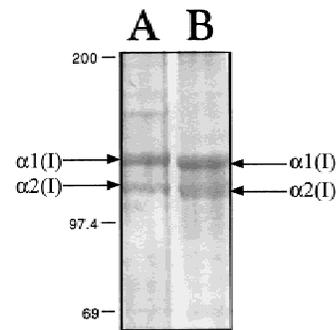


FIG. 4. Typical SDS-PAGE profiles of the α1 and α2 chains of type I collagen isolated from the cell layer/matrix fraction of the cultures. Lane A, guanidine-HCl extract; lane B, pepsin digest. Samples (50 μg) were applied to each lane and separated by electrophoresis on 5% SDS-PAGE and stained with Coomassie brilliant blue. Note that the α chains obtained from guanidine-HCl extract (lane A) migrate slightly slower than those obtained from the pepsin digested sample (lane B) due to the presence of the nontriple helical domains.

Quantitative collagen cross-link analysis

Figure 5 shows the typical chromatographic profiles of reducible and nonreducible collagen cross-links in the cell layer fractions obtained from BMSC cultures at early differentiation (Fig. 5A) and mineralization (Fig. 5B) stages. In the early differentiation stage, only the typical Lys^{ald}-derived cross-links (HLNL and HHMD) were detected. However, the profile from fully differentiated BMSCs showed significant amounts of Hyl^{ald}-derived cross-links including reducible (DHLNL) and nonreducible mature cross-links (Pyr and d-Pyr). NSFs did not show any significant amounts of cross-links with the exception of HHMD, one of the major Lys^{ald}-derived cross-links during the course of the study (data not shown). Table 2 summarizes the results of the collagen cross-link analyses.

DISCUSSION

The present study demonstrates that the expression of *PLOD2* gene, which is a member of the human LH gene family, is specifically up-regulated during the late stage of osteoblastic differentiation in vitro. In vitro, an elevation of Lys hydroxylation in the nontriple helical domains of type I collagen appears to coincide with the higher expression of *PLOD2* mRNA. Furthermore, only fully differentiated BMSC cultures expressing the elevated levels of *PLOD2* mRNA exhibited the collagen cross-links (deH-DHLNL, Pyr, and d-Pyr) that are derived from the hydroxylated Lys residues in the nontriple helical domains.⁽²⁷⁾ Less differentiated BMSCs, as well as NSFs in all stages tested, which were expressing basal, low levels of *PLOD2* mRNA showed the collagen cross-link patterns that were typical for the Lys^{ald}-derived pathways.

Interestingly, the levels of proline hydroxylation (Hyp/

TABLE 1. LYSINE HYDROXYLATION OF TYPE I COLLAGEN OBTAINED FROM BMSC AND NSF CULTURES AT DIFFERENT STAGES OF DIFFERENTIATION

Cell	Culture stages	Lysine hydroxylation (%)*			
		Helical domain		Helical + nonhelical domains	
		$\alpha 1$ (I)	$\alpha 2$ (I)	$\alpha 1$ (I)	$\alpha 2$ (I)
BMSC	proliferation	ND [†]	ND	ND	ND
	early differentiation	20.5	25.6	21.3	25.4
	mineralization	21.8	26.1	26.7	29.6
NSF	proliferation	ND	ND	ND	ND
	early differentiation	13.7	15.1	13.4	14.8
	mineralization	14.2	14.8	13.7	15.2

*{Hyl/(Lys + Hyl)} × 100 (%).

† ND, not detectable.

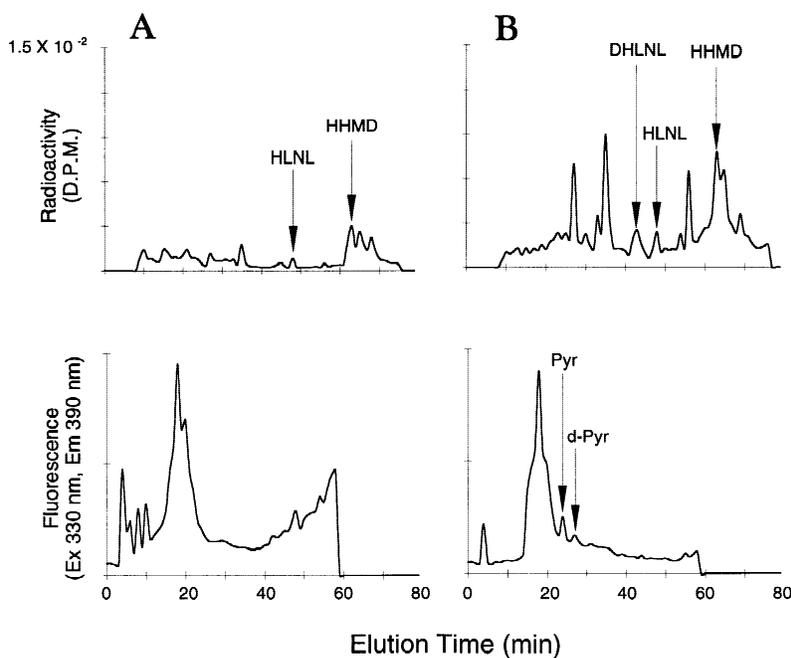


FIG. 5. Chromatographic profiles of reducible and nonreducible collagen cross-links determined for BMSC at the end of early differentiation (A) and mineralization stage (B). The upper panels show reducible and the lower panels nonreducible fluorescent collagen cross-links. Arrows indicate the elution positions of the relevant cross-links. DHLNL, dihydroxylysinoxonorleucine; HLNL, hydroxylysinoxonorleucine; HHMD, histidinohydroxymerodesmosine; Pyr, pyridinoline; d-Pyr, deoxypyridinoline.

TABLE 2. QUANTIFICATION OF COLLAGEN CROSS-LINKS IN THE CELL LAYER/MATRIX FRACTION OBTAINED FROM BMSC AND NSF AT THE MINERALIZATION STAGE

Cell	Cross-links*				
	Reducible			Fluorescent	
	DHLNL	HHMD	HLNL	Pyr	d-Pyr
NSF	—	0.13	—	—	—
BMSC	0.058	0.35	0.02	0.009	0.006

* The content of cross-links is expressed in mol/mol of collagen.

proline + Hyp) on both $\alpha 1$ and $\alpha 2$ chains of type I collagen obtained from cultures of BMSC and NSF were very constant (44–45%) and showed no difference between these cultures during the course of experiment.

Barnes and coworkers, in their early report,⁽¹⁵⁾ predicted

that there could be more than one mechanism for Lys hydroxylation of collagen, one for the helical and the other for the nontriple helical domains of the molecule. Their assumptions were based on the fact that the amino acid sequences around the Lys residues in the respective domains of the molecule were different from one another. Gerriets et al. have shown that, in hypertrophic tendon, there is an increased Lys hydroxylation at the N and C nontriple helical domains of the type I collagen molecule with no change in the triple helical domain.⁽²⁸⁾ This study also suggests that more than one type of LH is involved in the Lys hydroxylation of collagen.

Several studies have shown that *PLODI* mRNA is expressed in numerous tissues and cells in vitro and the level of expression does not vary significantly among various tissues and cells. This is consistent with the relatively steady level of Lys hydroxylation of the triple helical domain of the type I collagen molecule.^(29,30) These observations were

further corroborated by our current study. Therefore, it appears that LH1 is an enzyme responsible for the hydroxylation of Lys residues within the triple helical domain of the collagen molecules but not for the nontriple helical domains. The results obtained from the current study and several others^(29,30) support this hypothesis.

In contrast to the *PLOD1* gene, the mRNA expressions of the isoforms (*PLOD2* and *PLOD3*) appear to be more tissue specific.^(16,17) The tissue distribution of each mRNA (placenta, heart muscle and pancreas) appears to correlate with the tissue distribution of Hyl^{ald}-derived collagen cross-links (no data is available as yet regarding the expressions of *PLOD2* and *PLOD3* in bone in vivo). However, the expression pattern of *PLOD3* mRNA did not show any change during osteoblastic differentiation in vitro, while *PLOD2* mRNA expression was dramatically up-regulated. Thus, *PLOD2* but not *PLOD3* may play an important role in differentiated osteoblasts. Similar *PLOD2* gene up-regulation was also observed during differentiation of human tooth cementum-derived cells while the *PLOD1* and *PLOD3* levels remained unchanged (unpublished data). In fact, the tooth cementum exhibits a collagen cross-link pattern that is similar to that of bone.⁽³¹⁾

It has been proposed that deposition of a proper collagen fibrillar matrix, i.e., molecular conformation,⁽³²⁾ molecular packing arrangement,⁽³³⁾ and dynamics of intermolecular cross-linking,^(2,27) is important for subsequent normal mineralization therefore in a broader sense, for the bone function as a weight-bearing organ.^(14,32,33) Our previous studies on mineralizing collagen have indicated that, at the premineralizing stage, collagen molecules are packed in the fibril in a stereospecific manner and are laterally connected to one another mainly by the Hyl^{ald}-derived bifunctional cross-links.⁽⁵⁾ This connected segment in the fibril would form a template to facilitate the orderly precipitation of mineral at the early stage of mineralization. With further mineralization, some of these bifunctional cross-links rupture by physical force induced by mineralization releasing free Hyl^{ald}.^(14,27) This diminished molecular connection would then allow the fibril to deform easily to encase the large mass of mineral. In this context, the striking up-regulation of *PLOD2* mRNA seen in BMSCs at the mineralization stage of culture could be of biological relevance, although this still awaits further proof. It is conceivable that the up-regulated expression of the *PLOD2* gene in osteoblastic cells may be necessary to secrete collagen with properly hydroxylated Lys residues in the nontriple helical domains that will initiate a series of cross-linking reactions prior to during the mineralizing process.

In conclusion, the results presented in this report suggest that LH2 encoded by the *PLOD2* gene is involved in Lys hydroxylation at the nontriple helical domains of bone type I collagen; therefore, it partly modulates this tissue's cross-linking pattern.

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