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Expression of lysyl oxidase isoforms in MC3T3-E1 osteoblastic cells

Phimon Atsawasuwan, Yoshiyuki Mochida, Duenpim Parisuthiman, Mitsuo Yamauchi*

CB#7455, Dental Research Center, University of North Carolina at Chapel Hill, NC 27599-7455, USA

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Abstract

Covalent intermolecular cross-linking of collagen is initiated by the action of lysyl oxidase (LOX) on the telopeptidyl lysine and hydroxylysine residues. Recently, several LOX isoforms, i.e., LOX-like proteins 1–4 (LOXL1–4), have been identified but their specific tissue distribution and functions are still largely unknown. In this study, mRNA expression of LOX and LOXL1–4 in MC3T3-E1 osteoblastic cells was screened by RT-PCR and quantitatively analyzed by real-time PCR during cell differentiation and matrix mineralization. The results demonstrated that LOX and all LOXLs, except LOXL2, were expressed in this cell line and that the expression pattern during cell differentiation and matrix mineralization was distinct from one another. This indicates that the expression of LOX and its isoforms is highly regulated during osteoblast differentiation, suggesting their distinct roles in collagen matrix stabilization and subsequent mineralization.

Keywords: MC3T3-E1 cells; Lysyl oxidase; Lysyl oxidase-like proteins; Collagen; Collagen cross-linking; Matrix mineralization

Mineralization is a multifactorial process orchestrated by cells and a number of matrix molecules. In bone, fibrillar type I collagen functions as a three-dimensional template organizing the mineral deposition and growth [1]. For this structural function, the stability and adaptability of the fibril attained by specific covalent intermolecular cross-linking seem to be essential [2–5], and the alterations of collagen cross-linking were indeed observed in numerous bone disorders [2,6–8]. Recently, we have more directly demonstrated that the altered collagen cross-linking has significant effects on collagen fibrillogenesis and mineralization in vitro [9,10].

The process of collagen cross-linking is initiated by the conversion of telopeptidyl lysine and hydroxylysine residues to aldehyde through the action of an enzyme, lysyl oxidase (EC 1.4.3.13, protein-lysine 6-oxidases, LOX) [11,12]. The aldehyde produced then undergoes a series of condensation reactions to form intra- and intermolecular cross-links that are essential for the stability of collagen fibrils [13–15]. Recently several LOX isoforms, i.e., LOX-like proteins 1–4 (LOXL1–4), have been identified and their amine oxidase activities have been demonstrated [16–23]. This indicates that collagen cross-linking may be regulated by a complex mechanism involving multiple LOX isoforms. Such coordination may in part be responsible for the well-known tissue specificity of collagen cross-linking [1,13].

As a first step towards understanding such a potential mechanism in mineralized tissue biology, we examined the mRNA expression of LOX and LOXL1–4, and their changes during the osteoblastic cell differentiation and matrix mineralization.

Materials and methods

All experiments were performed in accordance with the guidelines of the University of North Carolina at Chapel Hill.

* Corresponding author. Fax: +1 919 966 1231.
E-mail address: Mitsuo_Yamauchi@dentistry.unc.edu (M. Yamauchi).

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Cell culture. The mouse calvaria-derived MC3T3-E1 cells, subclone 4 [24], were purchased from American Type Culture Collection (CRL-2593) and maintained in α-minimum essential medium (α-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37 °C. The medium was changed twice a week.

In vitro mineralization assay. Cells were plated on 35 mm plastic dishes at the density of 2×10^5 cells/dish (Falcon) and cultured until confluence. The medium was then replaced with the one supplemented with 50 μg/ml ascorbic acid and 1 mM β-glycerophosphate (mineralization medium), and maintained for up to 4 weeks. In vitro mineralization assay was performed just before the medium was switched to mineralization medium (week 0) and at the end of each week (week 1, 2, 3, and 4). At each time point, cell/matrix layers were washed with phosphate–buffered saline (PBS) twice, fixed with 100% methanol, and stained with 1% Alizarin Red S (Sigma). Three independent experiments were performed to confirm reproducibility.

RT-PCR. For a screening purpose to detect the LOX and LOXL mRNA expression, RT-PCR was performed. Cells were cultured as described above, switched to the mineralization medium, and maintained for up to 4 weeks. At the time points described above (week 0–4), total mRNA was extracted by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA was used for RT and the cDNA was synthesized using the Omniscript RT kit (Qiagen). PCR primers used in this study were designed based on the EMBL/GenBank (Accession Nos.: LOX, NM_010728; LOXL, NM_010729; LOXL2, NM_033325; LOXL3, NM_0135586; LOXL4, NM_053083; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NM_01001978). The primer sequences are shown in Table 1. After appropriate cycles of the reaction, aliquots of the PCR products were separated on a 2.5% TAE (Tris acetate EDTA)–agarose gel, stained with ethidium bromide, and photographed under UV light. As a positive control for LOXL2, ovary tissues of *Harlan Sprague Dawley* mice (Charles River) were homogenized and subjected to RT-PCR as described above.

Quantitative real-time PCR. After the screening by RT-PCR, the mRNA expression levels of LOX, LOXL1, 3, 4, and type I collagen α2 chain (Col1A2) were quantitatively analyzed during the course of cell differentiation and matrix mineralization. As described above, the cells were cultured, switched to mineralization medium, maintained for up to 4 weeks, and total RNA was extracted at the same time points. Two micrograms of the total RNA extract was used for RT as described above, and real-time PCR was performed using sequence specific primers and the ABI Prism 7000 Sequence detection system (Applied Biosystems). Primers used are as follows: LOX (ABI assay No. Mm 00495386_m1), LOXL1 (ABI assay No.174595), LOXL2 (ABI assay No. Mm 00804740_ml), LOXL3 (ABI assay No. Mm 00442953_m1), LOXL4 (ABI assay No. Mm 00446385_m1), COLIA2 (ABI assay No. Mm 00483888_m1), and GAPDH (ABI assay No. 4308313). The analyses were performed in triplicate for two independent experiments to confirm reproducibility of the results. The mRNA expression relative to GAPDH was determined and the fold changes were calculated using the values of week 0 as a calibrator by means of $2^{-\Delta\Delta C_T}$ method [25].

Results

In vitro mineralization assay

The results obtained from three independent experiments were essentially identical and the representative Alizarin Red S staining pattern at week 0–4 is shown in Fig. 1. The initial formation of mineralized nodules was observed at week 2, and the number and size of the nodules were increased thereafter.

The mRNA expression of LOX isoforms by RT-PCR and quantitative real-time PCR

The results of RT-PCR demonstrated that LOX and all LOXL mRNAs except LOXL2 were expressed in MC3T3-E1 cells during the course of cell differentiation and matrix mineralization. The efficiency of the LOXL2 primer used was confirmed by using mouse ovary cDNA

Table 1
Primers of RT-PCR analyses for each enzyme in this study

Name	Forward primer	Reverse primer
LOX	5′CGCCCGCCACTGGTTC3′	5′AGGGCGGCTTGGTAAGAA3′
LOXL1	5′AGGTGCCCGACAACTGGAGAG3′	5′CAAGGCCGTCGGAAGGTT3′
LOXL2	5′GCCCTCCGATGTGGTCAAG3′	5′CCCTCCTTCACCTCCACGTAG3′
LOXL3	5′AAGGAGACCCCTGCCTGTG3′	5′GCTGCCTGCAGGTCCCACTTT3′
LOXL4	5′TTCCAGTAGGCCACAGTCATC3′	5′CCGTCGTACCGTACTTCCA3′
GAPDH	5′ACCACAGTCCATGCCATCAC3′	5′TCCACCACCCTGTTGCTGTA3′

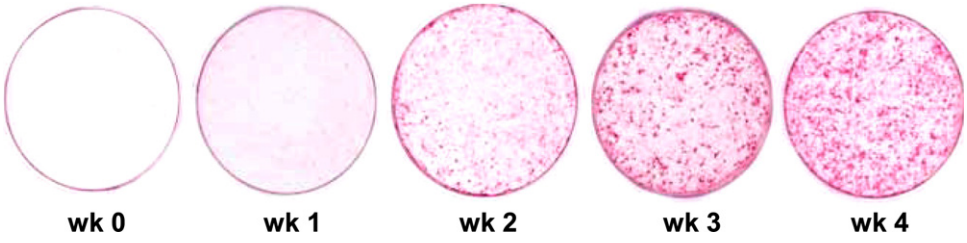

Fig. 1. In vitro mineralization assay. MC3T3-E1 cells were plated in triplicate, cultured for up to 4 weeks and mineralized nodules were stained with Alizarin Red S just before the medium was switched to mineralization medium (week 0) and at the end of each week thereafter (week 1–4). Three independent experiments produced similar results and one representative datum is shown.