Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 299 (2002) 91–97
BBRC
www.academicpress.com

LOX-1 expressed in cultured rat chondrocytes mediates oxidized LDL-induced cell death—possible role of dephosphorylation of Akt

Takefumi Nakagawa,[a] Tadashi Yasuda,[a] Hajime Hoshikawa,[b] Makoto Shimizu,[a] Takumi Kakinuma,[a,b] Mingyi Chen,[c] Tomoh Masaki,[a,c] Takashi Nakamura,[a] and Tatsuya Sawamura[c,*]

[a] *Department of Orthopaedic Surgery, Kyoto University Graduates School of Medicine, Kyoto, Japan*
[b] *Department of Orthopaedic Surgery, Tamatsukuri Koseinenkin Hospital, Shimane, Japan*
[c] *Department of Bioscience, National Cardiovascular Center Research Institute, Suita, Osaka, Japan*

Received 12 October 2002

Abstract

The oxidative changes of lipids in cartilage proceed with ageing and with the grade of osteoarthritis. To clarify the role of oxidatively modified lipids in articular cartilage in osteoarthritis, here, we investigated lectin-like oxidized LDL receptor (LOX-1) in rat cultured articular chondrocytes. LOX-1 expression was detectable in basal culture condition and enhanced by the treatment of oxidized LDL and interleukin-1β. DiI-labeled oxidized LDL was bound and ingested by chondrocytes via LOX-1. Oxidized LDL dose-dependently reduced chondrocyte viability, inducing non-apoptotic cell death, which was again suppressed by anti-LOX-1 antibody treatment. Oxidized LDL reduced the amount of phosphorylated Akt, a substrate of PI3 kinase via LOX-1. Consistently, the PI3 kinase inhibitor, LY294002, decreased cell viability dose-dependently, and the PI3 kinase activator, IGF-I, reversed the effect of oxidized LDL on the cell death. LOX-1 might be involved in the pathogenesis of osteoarthritis, inducing chondrocyte death through PI3 kinase/Akt pathway.

Keywords: LOX-1; Cell death; Chondrocytes; Phosphatidylinositol 3-kinase; Osteoarthrtis; Oxidized LDL

Changes in lipid components associated with ageing have been suggested to be involved in the pathology of osteoarthritis. Ageing increases the content of neutral lipids including cholesterol and triglycerides in the superficial layer of articular cartilage [1,2]. Among the lipid metabolite, lipid peroxidation products are gaining attention for its variety of action on articular cartilage; i.e., activation of arachidonic acid cascade. Supporting the possible involvement of the lipid peroxidation in the pathogenesis of osteoarthritis (OA), the lipid peroxidation inhibitors vitamins C and E inhibit the degradation of the extracellular matrix in chondrocyte monolayer cultures [3], and $n-3$ fatty acid, contained in fish oil, suppresses the degradation of cartilage [4]. The Framingham Knee Osteoarthritis Cohort Study demonstrated that a medium to high intake of antioxidants, such as vitamin C, β-carotene, and vitamin E, also reduces the risk of progressive knee osteoarthritis [5].

Recent studies have shown that inflammation and infection induce the peroxidation of serum LDL in an animal model [6]. In osteoarthritic and rheumatoid arthritic joints, inflammation accelerates vascular porosity, thereby facilitating the invasion of various inflammatory cells and the permeation of biological mediators including oxidized LDL (Ox-LDL) into joints. Ox-LDL has many biological functions and plays an important role in the pathogenesis of atherosclerosis, one of the ageing related disorders [7–9]. Furthermore, both hypercholesterolemia and hypertension are associated with the risk of knee OA, independent of obesity [10]. Taken together, these facts suggest that Ox-LDL plays some role in the pathogenesis of inflammatory arthritic syndromes such as OA.

* Corresponding author. Fax: +81-6-6872-7485.
E-mail address: t-sawamura@umin.ac.jp (T. Sawamura).

0006-291X/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0006-291X(02)02597-4

Receptors for Ox-LDL consist of the structurally distinct diverse member of proteins including scavenger receptors (SR)-AI, -II, CD36, CD68, and LOX-1 [11–14]. Among them, LOX-1 is characterized by the inducible expression by proinflammatory stimuli and the expression in non-phagocytes, i.e., vascular endothelial cells, smooth muscle cells, platelets, and cardiomyocytes [14–17]. The activation of LOX-1 induces various changes in the LOX-1 expressing cells, including activation of NF-κB through the generation of reactive oxygen species [18–20], upregulation of MCP-1 and endothelin-1 expression [21], and induction of apoptosis [17,22].

To address the roles of oxidative lipid metabolism and chondrocyte degeneration, here, we investigated the possible function and the mechanism of action of LOX-1 in chondrocyte, utilizing cultured rat articular chondrocytes.

Materials and methods

Reagents. Recombinant human IL-1β was purchased from Roche Diagnostics (Mannheim, Germany). Fetal bovine serum (FBS), pyrrolidine dithiocarbamate (PDTC), LY294002, RNase A, and staurosporin were purchased from Sigma (St. Louis, MO). Anti-Akt and anti-phospho-Akt (pAkt) antibodies were purchased from Cell Signaling Technology (Boston, MA). MTT was purchased from Calbiochem (La Jolla, CA). Trizol reagent, Superscript II, *Taq* polymerase, and DMEM/Ham's F-12 medium were purchased from Gibco-BRL (Gaithersburg, MD). Vectastain ABC Elite kits were purchased from Vector Laboratories (Burlingame, CA). Trypsin, collagenase, Na_3VO_4, phenylmethylsulfonyl fluoride, sodium fluoride, aprotinin, and leupeptin were obtained from Wako Pure Chemical (Osaka, Japan). Diaminobenzidine (DAB) was purchased from Dojindo (Kumamoto, Japan) and recombinant human insulin-like growth factor (IGF)-I was obtained from Nacalai Tesque (Kyoto, Japan).

Primary culture of rat articular chondrocytes. Chondrocytes were isolated from articular cartilage of 5–6-week-old Wistar rats by sequential enzymatic digestion at 37 °C with 0.1% EDTA/phosphate-buffered saline (PBS; pH 7.4) for 20 min, 0.25% trypsin/PBS for 1 h, and 2 mg/ml collagenase for 6 h. After filtration through nylon mesh to remove debris, cells were seeded on culture plates and cultured in DMEM/Ham's F-12 medium supplemented with 200 U/ml penicillin, 40 μg/ml streptomycin, and 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2 in air. After reaching confluence, cells were cultured in the serum-free culture medium for 12–24 h and then stimulated with various agents in the serum-free medium. For MTT assay, chondrocytes were plated in 96-well flat-bottomed culture plates at 2×10^4 cells/well, cultured for 3–5 days to allow them to attach to the wells, and then stimulated. For the experiments with various inhibitors, chondrocytes were preincubated with these reagents in serum-free medium for 30 min before stimulation.

Preparation of low-density lipoprotein. Human LDL (density 1.019–1.063) was isolated from fresh plasma by sequential ultracentrifugation as described previously [14]. LDL was oxidized at a concentration of 3 mg protein/ml by exposure to 7.5 μM $CuSO_4$ for 20 h at 37 °C. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances (10.7 nmol/mg protein) produced, and their greater mobilities due to increased negative charge on agarose gel electrophoresis were compared with native LDL (NLDL) (relative electrophoretic mobility was 3.25).

Immunocytochemistry. LOX-1 was detected using a specific mouse anti-rat LOX-1 monoclonal antibody (JTX-20) [23]. After stimulation with or without 10 or 40 μg/ml Ox-LDL or 1 ng/ml IL-1β for 48 h, chondrocytes were fixed with acetone for 10 min at 4 °C. The cells were blocked with 1% H_2O_2/methanol and non-immune serum, and then incubated overnight at 4 °C with or without anti-LOX-1 monoclonal antibody (3 μg/ml). After incubation with secondary antibody (1/400 dilution) for 40 min at room temperature, avidin–biotin peroxidase complex (Vectastatin ABC Elite kit) was added and the cells were visualized with 0.025% DAB/PBS and counter-stained with Harris' hematoxylin.

Reverse transcription-polymerase chain reaction. After 12–24 h of serum-starvation, chondrocytes were stimulated with 10 μg/ml Ox-LDL or 1 ng/ml IL-1β in serum-free medium for various time periods (0, 6, 12, 24, or 48 h). Total RNA was extracted from the cells with Trizol reagent. Total RNA (4 μg) was reverse-transcribed into cDNA with Superscript II reverse transcriptase and random hexamers as primers, and resulted cDNA was amplified with *Taq* DNA polymerase using specific primer pairs: 5′-gactggatctggcataaga-3′ and 5′-ccttctt ctgacatatgctg-3′ for rat LOX-1 (361 bp) [24], 5′-tcataggacctaaaggacct-3′ and 5′-tctcgtcaaatcctccagcc-3′ for rat type II collagen (228 bp) [25], 5′-ggcaacctcctgggtgtaag-3′ and 5′-tgggtccgtgggctcacaa-3′ for rat aggrecan (702 bp) [26], and 5′-accacagtccatgccatcac-3′ and 5′-tccaccaccctgttgc tgta-3′ for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (452 bp) [25]. The thermal profiles used were 35 cycles at 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 40 s for LOX-1 and 30 cycles at 94 °C for 40 s, 57 °C for 1 min, and 72 °C for 1 min for type II collagen and aggrecan.

DiI-labeled Ox-LDL binding assay. Chondrocytes were kept in chamber slides. After 12–24 h serum-starvation, 5 μg/ml DiI-labeled Ox-LDL was added into each wells and chondrocytes were incubated for 24 h. Some wells were preincubated with anti-LOX-1 antibody (40 μg/ml) for 30 min and then incubated with DiI-labeled Ox-LDL. To exclude the non-specific binding, unlabeled Ox-LDL (100 or 200 μg/ml) was added simultaneously with DiI-labeled Ox-LDL into some wells. After incubation, chondrocytes were washed with PBS thoroughly and fixed in 4% paraformaldehyde/PBS for 10 min at room temperature. After thorough washes, chondrocytes were counterstained using SYBR green I DNA staining system (Molecular Probes, Eugene, OR) and observed with confocal microscopy (OLYMPUS, Tokyo, Japan).

Preparation of cell lysates for Western blotting. After serum-starvation, chondrocytes were stimulated with 40 μg/ml NLDL, Ox-LDL, or LY294002 (10 nM) as PI3 kinase inhibitor for various time periods (0, 5, 10, 30, or 120 min). Some plates were pre-incubated with anti-LOX-1 antibody (40 μg/ml) or normal mouse IgG_1 as a negative control for 30 min, and then stimulated with Ox-LDL. Some wells were stimulated simultaneously with Ox-LDL and IGF-I (100 ng/ml). Cells were washed with ice-cold PBS and then lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 1 μg/ml aprotinin). After centrifugation at 13,000*g* for 10 min, the obtained supernatants were subjected to immunoblotting. Proteins were quantified using a Coomassie Protein Assay Reagent Kit (Pierce, Rockford, IL) and equal amounts of proteins were subjected to electrophoresis. Cell lysates were separated by 10% SDS–polyacrylamide gel electrophoresis and the proteins were transferred onto a nitrocellulose membrane. After blocking, membranes were incubated overnight at 4 °C with first antibody and then incubated with alkaline-phosphatase conjugated secondary antibody, followed by visualization with NBT/BCIP solution (Roche Diagnostics). The signal intensities, measured using NIH image 1.62 software, between the anti-LOX-1 antibody treated group and the normal mouse IgG_1 treated group ($n = 4$, each) were subjected to statistical analysis (see below).

MTT assay. Chondrocyte viability was assessed by the MTT assay. This procedure uses the pale yellow tetrazolium salt 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), which is cleaved by enzymes present in the endoplasmic reticulum and mitochondria to

form a dark blue formazan product. Cells were stimulated with Ox-LDL, NLDL, or LY294002 (0.03, 0.1, 0.3, and 1 nM) at various concentrations for 20 h at 37 °C. For the analysis of the effects of neutralizing anti-LOX-1 antibody and inhibitors, cells were pre-incubated with 10 or 40 μg/ml anti-LOX-1 antibody and 0–10 μM PDTC for 30 min, and then stimulated with Ox-LDL. IGF-I (100 ng/ml) was added simultaneously into some wells with Ox-LDL and incubated for 20 h. Cells were then incubated with 100 μl DMEM/F-12 supplemented with 10 μg/ml MTT for another 4 h at 37 °C. Dark blue formazan products were dissolved in 100 μl aliquots of 0.04 N HCl/isopropanol and OD_{540} was measured using a microtiter plate reader (Titertek Multiskan MCC, Flow Laboratories, Rockville, MD).

DNA fragmentation assay. After serum-starvation for 12–24 h, the medium was changed to serum-free medium containing NLDL (40 μg/ml), Ox-LDL (40 or 100 μg/ml), and staurosporin (1 μM) for 48 h. Cells were washed with ice-cold PBS and lysed in a buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.3% SDS, and 20 mM EDTA) for 30 min at 4 °C. After incubation with RNase A (20 μg/ml) and proteinase K (0.4 mg/ml), DNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1), dissolved in 30 μl Tris–EDTA buffer, and then subjected to 3% agarose gel electrophoresis. Fragmented DNA was visualized with the SYBR green I DNA staining system.

In situ TdT-mediated dUTP nick end-labeling (TUNEL) assay. Chondrocyte apoptosis was also detected using an in situ TUNEL assay with the in situ Apoptosis Detection Kit (Takara, Kyoto, Japan). After fixation in acetone, cells were treated with permeabilization buffer for 5 min at 4 °C. After blocking with 0.3% H_2O_2/methanol, the cells were incubated in 50 μl terminal dinucleotidyl transferase (TdT)-labeling solution for 90 min at 37 °C. Cells were washed and incubated with biotinylated anti-FITC antibody for 30 min at room temperature. Apoptotic cells were visualized with 0.025% DAB/PBS and counterstained with methyl green. For statistical analysis, the percentages of TUNEL positive cells were counted in four randomly selected fields per slide (original magnification 200×).

Statistical analysis. Results were statistically evaluated using *StatView* software (Abacus Concepts, Berkeley, CA). Individual experiments were performed at least three times. Unpaired Student's *t* tests were used to determine the differences between treated groups. *P* values of less than 0.01 or 0.05 were considered significant. All values are given as means ± SEM of replicate experiments.

Results

Characterization of rat primary chondrocyte cultures

We first confirmed that the cells used in the present study maintained the properties of chondrocytes. RT-PCR showed that the primary cultures of rat chondrocytes without any stimulation constitutively expressed mRNA of both type II collagen and aggrecan gene, which are markers for chondrocytes, suggesting that the cells did not de-differentiate before stimulation (data not shown).

Immunocytochemical detection of LOX-1 in rat chondrocytes

Using anti-rat LOX-1 antibody (JTX-20), we observed some constitutive expression of LOX-1 in primary

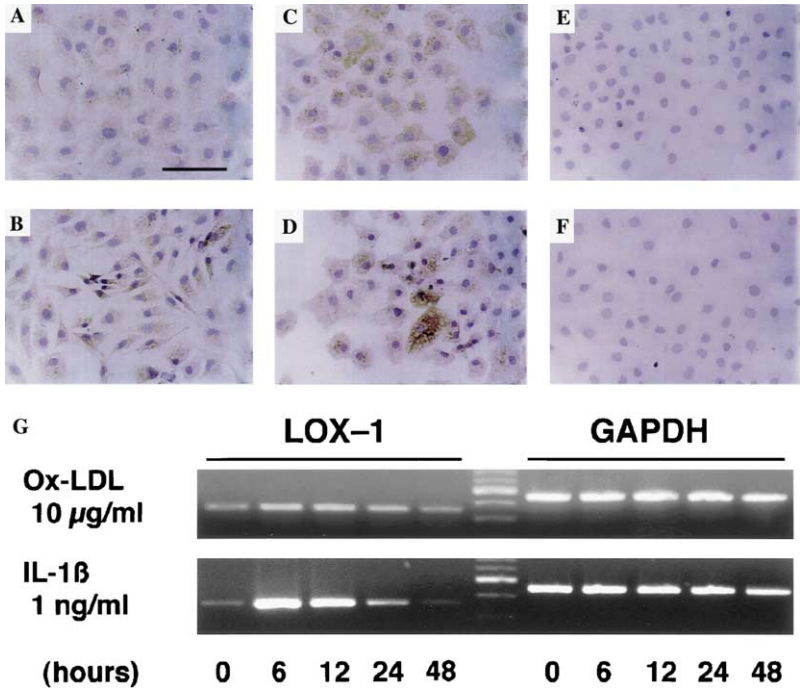

Fig. 1. Expression of LOX-1 protein and *LOX-1* mRNA in chondrocytes. (A–F) Immunocytochemistry of rat primary chondrocytes without treatment (control: panel A), with 1 ng/ml IL-1β (panel B), 10 μg/ml Ox-LDL (panel C), or 40 μg/ml Ox-LDL (panel D). Negative controls for B (panel E) and D (panel F) lacked primary monoclonal antibody. Original magnification 400×. Bar, 100 μm. (G) *LOX-1* mRNA induction in rat chondrocytes stimulated with Ox-LDL and IL-1β. After incubation with Ox-LDL (10 μg/ml) or IL-1β (1 ng/ml) for 0, 6, 12, 24, or 48 h, LOX-1 mRNA in rat chondrocytes was assessed by RT-PCR. Rat GAPDH was used as an internal positive control. The DNA marker represents a 100-bp DNA ladder.

cultures of rat chondrocytes (Fig. 1A). Treatment with 1 ng/ml IL-1β, a proinflammatory cytokine, resulted in increased LOX-1 expression in rat chondrocytes, together with morphological changes into fibroblastic shapes (Fig. 1B). Ox-LDL, a ligand for LOX-1, also enhanced LOX-1 expression in a dose-dependent manner, although morphological changes into fibroblastic shapes were not observed (Figs. 1C and D). When the primary antibody against LOX-1 was omitted, no positively stained cells were detected, suggesting the specificity of the immunostaining (Figs. 1E and F).

Effects of Ox-LDL and IL-1β on LOX-1 expression

LOX-1 mRNA expression was further confirmed by RT-PCR analyses with the specific amplification of the 361-bp fragment of rat LOX-1 cDNA. The level of LOX-1 gene expression at time 0 was detectable as the case of immunocytochemistry (Fig. 1A). Treatment with Ox-LDL and IL-1β caused a maximal increase in the LOX-1 gene expression at 6 h (Fig. 1G). In this experiment, we used 10 μg/ml Ox-LDL, because 40 μg/ml Ox-LDL significantly reduced cell viability at 20 h from our results as below (Fig. 3).

LOX-1 mediates Ox-LDL binding in chondrocytes

To confirm that expressed LOX-1 on chondrocytes mediates Ox-LDL binding, we used DiI-labeled Ox-LDL in combination with anti-LOX-1 antibody. After 24 h, DiI-labeled Ox-LDL was detected inside of chondrocytes (Fig. 2B). This binding was specific, displaced

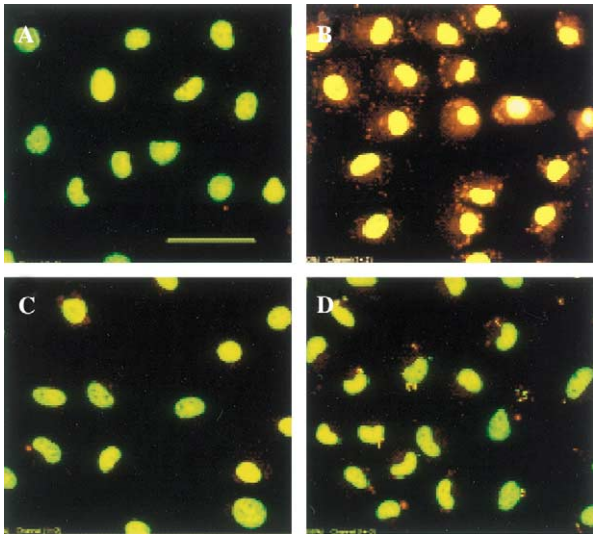

Fig. 2. LOX-1 involvement in Ox-LDL binding in chondrocytes. Cells were incubated for 24 h with none (A; control), DiI-labeled Ox-LDL alone (5 μg/ml) (B), and DiI-labeled Ox-LDL (5 μg/ml) plus excessive amount of non-labeled Ox-LDL (100 μg/ml) (C), DiI-labeled Ox-LDL plus anti-LOX-1 antibody (40 μg/ml) (D). Original magnification 800×. Bar, 50 μm.

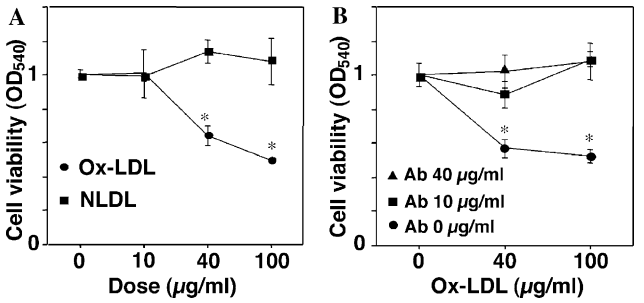

Fig. 3. Effects of Ox-LDL and LOX-1 on chondrocyte viability. (A) Cells were incubated with Ox-LDL and NLDL (10, 40, and 100 μg/ml) and assessed by MTT assay. (B) After 30 min preincubation with or without anti-LOX-1 antibody (10 and 40 μg/ml), cells were stimulated with Ox-LDL and then assessed by MTT assay. The values are given as mean $OD_{540} \pm$ SEM of replicates ($n = 4$). OD, optical density. The experiments were repeated three times with similar results. *$P < 0.01$ by t test.

by excess amount of unlabeled Ox-LDL (100 μg/ml) (Fig. 2C). Application of anti-LOX-1 antibody almost abolished the Ox-LDL uptake (Fig. 2D), suggesting that LOX-1 predominantly mediated the Ox-LDL binding by chondrocytes, and that other kinds of receptors for ox-LDL, if existed, are at negligible level in chondrocytes in this condition.

Effects of Ox-LDL on cell viability

We next investigated the events resulting from the binding of Ox-LDL to LOX-1. We added increasing levels (0–100 μg/ml) of either Ox-LDL or native LDL to rat primary chondrocytes and assessed cell viability by MTT assay. Ox-LDL significantly reduced chondrocyte viability at 40 and 100 μg/ml, whereas NLDL did not (Fig. 3A). From this result, we chose 40 μg/ml Ox-LDL for further analyses of the cell viability. Pretreatment with anti-LOX-1 antibody significantly reversed this

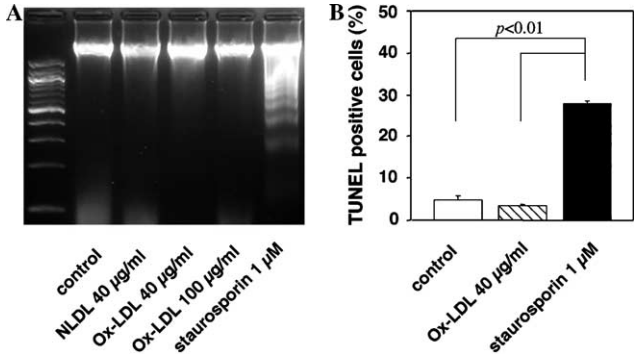

Fig. 4. DNA fragmentation assay (A) and in situ TUNEL assay (B). After 24 h of serum-starvation, chondrocytes were incubated with Ox-LDL (40 and 100 μg/ml), NLDL (40 μg/ml) or staurosporin (1 μM) for another 48 h. (B) The percentages of TUNEL positive cells were counted in each group and the values presented here are given as means ± SEM of each group ($n = 5$).

reduction in viability of chondrocytes (Fig. 3B), suggesting that LOX-1 mediated the cell-death induced by Ox-LDL. Then, we investigated whether the chondrocyte-death is carried out by the process of apoptosis, using DNA fragmentation analysis and in situ TUNEL staining. We found neither DNA fragmentation (Fig. 4A) nor significant numbers of TUNEL-positive cells, while we found the phenomena by the treatment with staurosporin (controls $4.89 \pm 0.96\%$; Ox-LDL, $3.55 \pm 0.37\%$; staurosporin, $27.77 \pm 0.86\%$: Fig. 4B). These results indicated that Ox-LDL induced non-apoptotic death in chondrocytes. Furthermore, NF-κB inhibitor, PDTC, had no effects on Ox-LDL-induced chondrocyte death (data not shown).

Effects of PI3 kinase/Akt pathway on Ox-LDL-induced cell death

To clarify the mechanism of the action of Ox-LDL in chondrocyte death, we investigated whether Ox-LDL has any effects on PI3 kinase/Akt pathway. Ox-LDL (40 μg/ml) rapidly decreased the amount of phosphorylated Akt (pAkt) (Figs. 5A and B) from the baseline activity, whereas NLDL (40 μg/ml) did not (Fig. 5C). Furthermore, anti-LOX-1 treatment significantly inhibited the reduction of pAkt by Ox-LDL (Figs. 5A and B).

Then, we further confirmed the importance of Akt activation in keeping chondrocyte viability. The treatment with PI3 kinase inhibitor, LY294002, inhibited pAkt activity in chondrocyte without Ox-LDL (Fig. 6A), accompanying the decrease in the cell viability in dose-dependent manner (Fig. 6B). In addition, IGF-I (100 ng/ml), a potent activator of PI3 kinase/Akt pathway, inhibited Ox-LDL-induced cell death (Fig. 6D). This concentration of IGF-I could also reverse the Ox-LDL-induced rapid reduction of pAkt (Fig. 6C). These results indicate that Ox-LDL-induced rapid PI3 kinase/Akt inactivation is of importance in facilitating chondrocyte death via LOX-1.

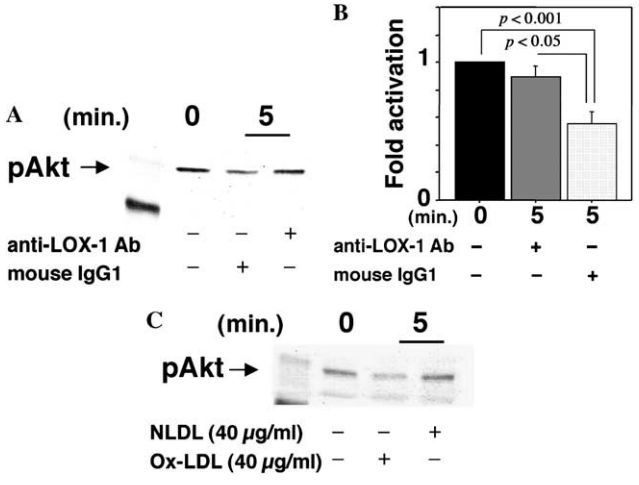

Fig. 5. (A) Immunoblot analysis of phospho-Akt in Ox-LDL-stimulated chondrocytes with or without anti-LOX-1 antibody. (B) Signal intensities of immunoblot analysis (panel A) at indicated time points of phospho-Akt measured using NIH image 1.62 software ($n = 4$ each). $^*P < 0.01$ by t test between anti-LOX-1 antibody- and normal mouse IgG_1-treated cells at the same concentration. (C) Immunoblot analysis of phospho-Akt in NLDL-stimulated chondrocytes.

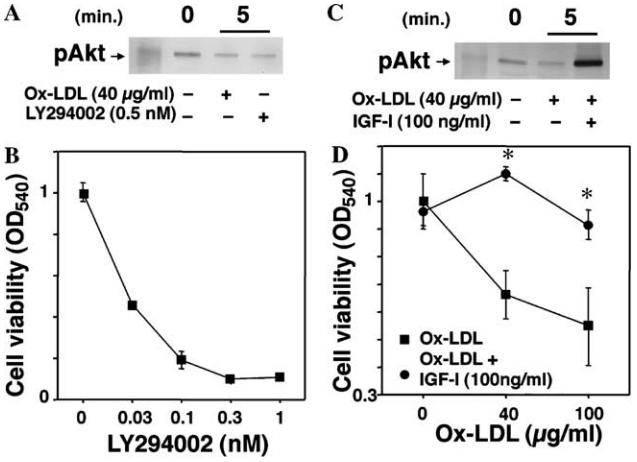

Fig. 6. Effect of PI3 kinase/Akt pathway on Ox-LDL-induced cell death. (A) Immunoblot analysis of pAkt activation by LY294002 (10 nM). (B) Effect of increasing concentrations of PI3 kinase/Akt inhibitor, LY294002 on chondrocyte viability. Pretreatment with PI3 kinase/Akt inhibitor, LY294002 (0–1 nM), increased cell death in a dose-dependent manner ($n = 5$ each). (C) Immunoblot analysis of pAkt activation by PI3 kinase/Akt activator, IGF-I (100 ng/ml), with Ox-LDL. (D) Pretreatment with IGF-I (100 ng/ml) inhibited Ox-LDL-induced cell death ($n = 6$ each). The data presented here are means of $OD_{540} \pm SEM$. $^*P < 0.05$ by t test between groups at the same concentrations.

Discussion

The involvement of the lipid peroxidation in the pathogenesis of arthritis has been suggested by epidemiological study and by animal experiments. Ox-LDL is one of the lipid peroxidation products that affect cellular phenotype. The effects of Ox-LDL are mediated by some classes of Ox-LDL receptors. LOX-1 is an oxidized LDL receptor cloned from vascular endothelial cells, which is expressed in non-phagocytes as well as macrophages, e.g., vascular smooth muscle cells and platelets [14,27–29]. Although researchers have focused on macrophages and endothelial cells for the target cells of Ox-LDL, here, we demonstrated that LOX-1 is expressed in rat cultured chondrocytes. The expression of LOX-1 is upregulated by both Ox-LDL, the ligand of LOX-1, and IL-1β, a proinflammatory cytokine that accumulates in osteoarthritic cartilage. Therefore, it is likely that the production of lipid peroxidation products, such as Ox-LDL, leads to the enhancement of LOX-1 expression in chondrocytes in vivo, modulating the cartilage degradation in individuals with OA. We

confirmed the expression of LOX-1 in human cartilage from OA patients (unpublished observation).

Although Ox-LDL is a large molecule of about 550 kDa, degenerative OA and RA cartilages are known to release high molecular weight molecules, such as chondroitin sulfate, keratan sulfate, and hyaluronic acids from the inner layer. Actually, we found that Ox-LDL could penetrate into OA cartilage and that anti-OxLDL antibody-reactive substances were accumulated in inflamed joint in vivo (unpublished observation). Furthermore, Ox-LDL could be detectable in degenerative cartilage such as OA cartilage (unpublished observation).

In the present study, LOX-1 stimulation resulted in chondrocyte death via a non-apoptotic pathway. Reduced numbers of chondrocytes, exemplified by empty lacunae, is one of the major characteristics of OA [30], and chondrocyte death is of particular interest in relation to reduced cell numbers [31]. These suggest the possible importance of LOX-1 in the pathogenesis of OA. We propose that Ox-LDL might be one of the important oxidation products that induce chondrocyte death.

It has been reported that exogenous reactive oxygen species (ROS) cause non-apoptotic cell death in chondrocytes [31]. The binding of Ox-LDL to LOX-1 could induce the generation of ROS in endothelial cells [18]. Therefore, LOX-1-mediated ROS generation might also be involved in the mechanisms of chondrocyte-death. However, an NF-κB inhibitor, PTDC, did not affect the Ox-LDL-induced cell death (data not shown), while NF-κB is well known as an oxidative stress-sensitive transcription factor. On the contrary, Ox-LDL binding via LOX-1 rapidly reduced pAkt. Furthermore, PI3 kinase inhibitor could induce cell death; and IGF-I, a PI3 kinase/Akt activator, inhibited Ox-LDL-induced cell death. These results suggest that PI3 kinase/Akt inactivation has an important role in the chondrocyte death.

Recently, Mochizuki et al. [32] have shown that PI3 kinse/Akt activation inhibited ceramide-induced, caspase-independent, non-apoptotic programmed cell death (type 2 physiological cell death) in glioma cell line and proposed that unidentified Akt substrate(s) may be involved in this event, although PI3 kinase/Akt is also reported to inhibit apoptosis by phosphorylating the Bcl-2 family member BAD and inhibiting the caspases [33–35]. From these points, Ox-LDL-induced non-apoptotic chondrocyte death may be mediated by pAkt reduction. Increase in lipid peroxide and decrease in IGF during ageing might be related to chondrocyte loss in OA through the decreased signal of Akt pathway.

In summary, Ox-LDL binding to LOX-1 initiates non-apoptotic chondrocyte death through rapid inactivation of PI3 kinase/Akt pathway. The decrease in cell numbers in osteoarthritic cartilage might be caused, at least in part, by the binding of Ox-LDL to LOX-1.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health, Labor and Welfare of Japan, the Organization for Pharmaceutical Safety and Research, and Mitsubishi Foundations. We thank Tamlyn Thomas for editing the manuscript.

References

[1] R.A. Stockwell, Lipid in the matrix of ageing articular cartilage, Nature 207 (1965) 427–428.
[2] L. Lippiello, T. Walsh, M. Fienhold, The association of lipid abnormalities with tissue pathology in human osteoarthritic articular cartilage, Metabolism 40 (1991) 571–576.
[3] M.L. Tiku, R. Shah, G.T. Allison, Evidence linking chondrocyte lipid peroxidation to cartilage matrix protein degradation. Possible role in cartilage ageing and the pathogenesis of osteoarthritis, J. Biol. Chem. 275 (2000) 20069–20076.
[4] C.L. Curtis, C.E. Hughes, C.R. Flannery, C.B. Little, J.L. Harwood, B. Caterson, $n-3$ fatty acids specifically modulate catabolic factors involved in articular cartilage degradation, J. Biol. Chem. 275 (2000) 721–724.
[5] T.E. McAlindon, P. Jacques, Y. Zhang, M.T. Hannan, P. Aliabadi, B. Weissman, D. Rush, D. Levy, D.T. Felson, Do antioxidant micronutrients protect against the development and progression of knee osteoarthritis?, Arthritis Rheum. 39 (1996) 648–656.
[6] R.A. Memon, I. Staprans, M. Noor, W.M. Holleran, Y. Uchida, A.H. Moser, K.R. Feingold, C. Grunfeld, Infection and inflammation induce LDL oxidation in vivo, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1536–1542.
[7] R. Ross, The pathogenesis of atherosclerosis: a perspective for the 1990s, Nature 362 (1993) 801–809.
[8] P. Tontonoz, L. Nagy, J.G. Alvarez, V.A. Thomazy, R.M. Evans, PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL, Cell 93 (1998) 241–252.
[9] D. Steinberg, Low density lipoprotein oxidation and its pathobiological significance, J. Biol. Chem. 272 (1997) 20963–20966.
[10] D.J. Hart, D.V. Doyle, T.D. Spector, Association between metabolic factors and knee osteoarthritis in women: the chingford study, J. Rheumatol. 22 (1995) 1118–1123.
[11] T. Kodama, P. Reddy, C. Kishimoto, M. Krieger, Purification and characterization of a bovine acetyl low density lipoprotein receptor, Proc. Natl. Acad. Sci. USA 85 (1988) 9238–9242.
[12] S.L. Acton, P.E. Scherer, H.F. Lodish, M. Krieger, Expression cloning of SR-BI, a CD36-related class B scavenger receptor, J. Biol. Chem. 269 (1994) 21003–21009.
[13] G. Endemann, L.W. Stanton, K.S. Madden, C.M. Bryant, R.T. White, A.A. Protter, CD36 is a receptor for oxidized low density lipoprotein, J. Biol. Chem. 268 (1993) 11811–11816.
[14] T. Sawamura, N. Kume, T. Aoyama, H. Moriwaki, H. Hoshikawa, Y. Aiba, T. Tanaka, S. Miwa, Y. Katsura, T. Kita, T. Masaki, An endothelial receptor for oxidized low-density lipoprotein, Nature 386 (1997) 73–77.
[15] T. Aoyama, M. Chen, H. Fujiwara, T. Masaki, T. Sawamura, LOX-1 mediates lysophosphatidylcholine-induced oxidized LDL uptake in smooth muscle cells, FEBS Lett. 467 (2000) 217–220.
[16] M. Chen, S. Narumiya, T. Masaki, T. Sawamura, Conserved C-terminal residues within the lectin-like domain of LOX-1 are essential for oxidized low-density-lipoprotein binding, Biochem. J. 355 (2001) 289–296.
[17] E. Iwai-Kanai, K. Hasegawa, T. Sawamura, M. Fujita, T. Yanazume, S. Toyokuni, S. Adachi, Y. Kihara, S. Sasayama, Activation of lectin-like oxidized low-density lipoprotein receptor-

1 induces apoptosis in cultured neonatal rat cardiac myocytes, Circulation 104 (2001) 2948–2954.
[18] L. Cominacini, A.F. Pasini, U. Garbin, A. Davoli, M.L. Tosetti, M. Campagnola, A. Rigoni, A.M. Pastorino, V. Lo Cascio, T. Sawamura, Oxidized low density lipoprotein ox-LDL binding to (ox-LDL) receptor-1 in endothelial cells induces the activation of NF-κB through an increased production of intracellular reactive oxygen species, J. Biol. Chem. 275 (2000) 12633–12638.
[19] L. Cominacini, A. Rigoni, A.F. Pasini, U. Garbin, A. Davoli, M. Campagnola, A.M. Pastorino, V. Lo Cascio, T. Sawamura, The binding of oxidized low density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide, J. Biol. Chem. 276 (2001) 13750–13755.
[20] M. Nagase, K. Ando, T. Nagase, S. Kaname, T. Sawamura, T. Fujita, Redox-sensitive regulation of lox-1 gene expression in vascular endothelium, Biochem. Biophys. Res. Commun. 281 (2001) 720–725.
[21] D. Li, J.L. Mehta, Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells, Circulation 101 (2000) 2889–2895.
[22] D. Li, J.L. Mehta, Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1116–1122.
[23] M. Kakutani, T. Masaki, T. Sawamura, A platelet-endothelium interaction mediated by lectin-like oxidized low- density lipoprotein receptor-1, Proc. Natl. Acad. Sci. USA 97 (2000) 360–364.
[24] M. Nagase, S. Kaname, T. Nagase, G. Wang, K. Ando, T. Sawamura, T. Fujita, Expression of LOX-1, an oxidized low-density lipoprotein receptor, in experimental hypertensive glomerulosclerosis, J. Am. Soc. Nephrol. 11 (2000) 1826–1836.
[25] A.M. Khatib, A. Lomri, F. Moldovan, H. Soliman, J. Fiet, D.R. Mitrovic, Endothelin 1 receptors, signal transduction and effects on DNA and proteoglycan synthesis in rat articular chondrocytes, Cytokine 10 (1998) 669–679.
[26] T. Hamada, N. Suda, T. Kuroda, Immunohistochemical localization of fibroblast growth factor receptors in the rat mandibular condylar cartilage and tibial cartilage, J. Bone Miner. Metab. 17 (1999) 274–282.
[27] H. Yoshida, N. Kondratenko, S. Green, D. Steinberg, O. Quehenberger, Identification of the lectin-like receptor for oxidized low-density lipoprotein in human macrophages and its potential role as a scavenger receptor, Biochem. J. 334 (1998) 9–13.
[28] H. Kataoka, N. Kume, S. Miyamoto, M. Minami, H. Moriwaki, T. Murase, T. Sawamura, T. Masaki, N. Hashimoto, T. Kita, Expression of lectinlike oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions, Circulation 99 (1999) 3110–3117.
[29] M. Kakutani, M. Ueda, T. Naruko, T. Masaki, T. Sawamura, Accumulation of LOX-1 ligand in plasma and atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits: identification by a novel enzyme immunoassay, Biochem. Biophys. Res. Commun. 282 (2001) 180–185.
[30] H.J. Mankin, H. Dorfman, L. Lippiello, A. Zarins, Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. II. Correlation of morphology with biochemical and metabolic data, J. Bone Joint Surg. Am. 53 (1971) 523–537.
[31] F.J. Blanco, R.L. Ochs, H. Schwarz, M. Lotz, Chondrocyte apoptosis induced by nitric oxide, Am. J. Pathol. 146 (1995) 75–85.
[32] T. Mochizuki, A. Asai, N. Saito, S. Tanaka, H. Katagiri, T. Asano, M. Nakane, A. Tamura, Y. Kuchino, C. Kitanaka, T. Kirino, Akt protein kinase inhibits non-apoptotic programmed cell death induced by ceramide, J. Biol. Chem. 277 (2002) 2790–2797.
[33] M.A. Lawlor, D.R. Alessi, PKB/Akt: a key mediator of cell proliferation, survival and insulin responses?, J. Cell Sci. 114 (2001) 2903–2910.
[34] J. Downward, How BAD phosphorylation is good for survival?, Nat. Cell Biol. 1 (1999) E33–35.
[35] S.R. Datta, A. Brunet, M.E. Greenberg, Cellular survival: a play in three Akts, Genes Dev. 13 (1999) 2905–2927.