

Lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) expression in human articular chondrocytes

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Abstract

Objective

To investigate the involvement of oxidized low density lipoprotein (ox-LDL) and the expression of its receptor lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) in osteoarthritis, by determining the ox-LDL in synovial fluid and the expression of LOX-1 mRNA and protein in osteoarthritic as well as normal cartilage. In addition, the effect of ox-LDL on chondrocyte viability and the effect of ascorbic acid (a well-known anti-oxidant) on LOX-1 expression were studied.

Methods

Fifteen patients were included in the study. Osteoarthritic articular cartilage was obtained from two distinct locations in the knee (n = 10) and hip (n = 5), specifically from weight-bearing and non-weight-bearing areas of the same joints. Five individuals were used as controls. mRNA and protein expression were studied by RT-PCR and immunofluorescence, respectively. Ox-LDL was measured in the synovial fluid and in paired serum samples from the patients using the ELISA method.

Results

Ox-LDL was detected in the synovial fluid and its receptor LOX-1 was detected in cartilage from both weight-bearing and non-weight-bearing areas, whereas no LOX-1 expression was found in normal cartilage. Ox-LDL reduced chondrocyte viability in cell cultures, while the addition of ascorbic acid to osteoarthritic chondrocytes resulted in a decrease in LOX-1 mRNA expression.

Conclusion

The detection of LOX-1 mRNA and protein expression in osteoarthritic cartilage drawn from both weight-bearing and non-weight-bearing regions of the same patients suggest that LOX-1 may be involved in the progression and pathogenesis of osteoarthritis.

Key words

LOX-1 expression, osteoarthritis, cartilage, chondrocytes.

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Introduction

Osteoarthritis (OA) is a common age-related joint disorder. As in other age-related degenerative diseases (for example, those of the cardiovascular system), altered lipid metabolism has been implicated as a critical player in disease development (1, 2). It has been shown that the lipid content in the superficial layer of normal articular cartilage increases with age (3, 4). Lipid deposition, seen early in the OA process and before any histological changes, is considered to reflect lipid involvement in the pathogenesis of OA (4). Furthermore, recent population studies have provided support for the hypothesis that serum cholesterol is a systemic risk factor for OA and an association has been observed between high serum cholesterol levels and radiologic OA (5-7). Lipid peroxidation has been linked to the structural destabilization of cartilage matrix, while lipid peroxidation inhibitors such as ascorbic acid and vitamin E have been shown to exert a protective role in extracellular matrix degradation (8-10).

Oxidized low density lipoprotein (ox-LDL), which is known to be accumulated under conditions of oxidative stress, is a molecule with many biological functions; it causes lipid accumulation, elicits pro-inflammatory changes including apoptosis, and also transforms macrophages and other cells to foam cells (11). Ox-LDL has been detected in the synovium and synovial fluid of patients with rheumatoid arthritis (RA) and recently it was demonstrated that it affects chondrocyte viability and proteoglycan synthesis synergistically with the cyclic tensile stretch load (12-14).

Lectin-like oxidized LDL receptor 1 (LOX-1) is a type II membrane protein belonging to the C-type lectin family of molecules, which can act as a cell-surface endocytosis receptor for ox-LDL (15). LOX-1 expression and ox-LDL accumulation have been detected in arthritic joints in a rat zymosan-induced arthritis model (16). In cultured rat chondrocytes, it was shown that treatment with ox-LDL and IL-1 β upregulated LOX-1 expression (17). Also, in bovine articular chondrocyte cultures, ox-LDL binding to LOX-1 increased

the production of intracellular reactive oxygen species (ROS) and activated NF- κ B, suggesting that ox-LDL has similar effects to IL-1 β on cartilage degradation (18). LOX-1 expression has been detected in human RA cartilage, whereas in the same study normal and OA cartilage did not present any immunoreactive staining, indicating that LOX-1 is not expressed in OA cartilage (19). However, a subsequent study by the same group produced conflicting results, as both OA and normal cartilage exhibited LOX-1 expression (20).

Although LOX-1 has been shown to be implicated in RA, its presence in OA and normal cartilage remains controversial and its role in the pathophysiology of OA remains unknown. In the present study we investigated LOX-1 mRNA and protein expression in osteoarthritic cartilage derived from both weight-bearing and non-weight-bearing areas of the same joint, as well as in normal articular cartilage. Furthermore, in order to assess the role of LOX-1 in osteoarthritis, we studied the effect of ascorbic acid, an anti-oxidant nutrient, on LOX-1 mRNA expression in osteoarthritic as well as in normal chondrocytes.

Materials and methods

Cartilage samples

Articular cartilage samples were obtained from the femoral head, femoral condyles and tibial plateaus of patients with primary OA who were undergoing hip and knee replacement surgery at the Orthopaedics Department of the University Hospital of Larissa. A total of 15 patients were included in the study (3 male/12 female; mean age 71.5 ± 5.51 , range 63-80; mean body mass index (BMI) 30.29 ± 3.81 , range 25.9 - 41.0). Two specimens were taken from every patient. Each sample was categorized according to its gross morphology, as either severely damaged (advanced OA, having being taken from the main defective area of maximal load) or mildly damaged (minimal OA, based on samples taken from areas with no obvious surface defects).

The macroscopic findings were validated by histological studies performed on 5 μ m serial sections of cartilage

Competing interests: none declared.

samples and graded using the Mankin scoring system (21, 22). Specimens with mild OA had a Mankin score of 1-4, while specimens with advanced OA had a Mankin score of 10-14. Normal cartilage was obtained from 5 individuals (2 male, 3 female; mean age 36.6 ± 12.6 , range 25-58; mean BMI 25.04 ± 0.83 , range 23.88-25.97) with a Mankin score of 0, who were undergoing fracture repair surgery and had no history of joint disease.

Radiographs were obtained before surgery and were graded according to the Kellgren-Lawrence system (23, 24). Controls had a zero (0) K/L score, while all patients had a K/L score > 2 . The radiographs were assessed by two independent observers who were blinded to all data regarding the subjects.

Patients with RA, or some other autoimmune disease, or chondrodysplasias, infection-induced OA and post-traumatic OA, were excluded from the study.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Local Ethical Committee of the University Hospital of Larissa.

Synovial fluid (SF) samples

SF samples were obtained from all 15 patients. The samples were collected in EDTA tubes, placed immediately on ice and subsequently centrifuged at 3000g for 30 min. The supernatant was separated and stored at -80°C until analysis, but never for longer than 3 weeks.

Ox-LDL measurements in SF and serum

In the quantitative determination of ox-LDL *in vitro*, synovial fluid and paired serum samples were analyzed for ox-LDL using a specific enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions. Dilution and spiking experiments were performed to validate the use of the commercially available ELISA kit for SF samples. Ox-LDL concentrations were determined in duplicate. According to the manufacturers, the detection limit for the ox-LDL assay was ≤ 0.3 U/l. The intra- and inter-assay coefficients of variation were 5.87% and 6.13%, respectively.

Total RNA isolation

Fresh cartilage was dissected within one hour after surgery and the total cellular RNA was extracted using Trizol reagent (Invitrogen, Life Technologies, Paisley, UK). The RNA was further purified using an RNA extraction kit (RNeasy Mini-kit, Qiagen, Hilden, Germany) according to the manufacturers' instructions. The preservation of 28S and 18S rRNA species was used to assess RNA integrity. All the samples included in the study had prominent 28S and 18S rRNA components. The yield was quantified spectrophotometrically.

RT-PCR of LOX-1 mRNA

1 μg of RNA was reverse transcribed to complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase (M-MLV-RT, Invitrogen, Life Technologies, Paisley, UK), as well as random primers (Gibco, BRL-UK). As an internal control, the cDNA was subjected to PCR analysis for the retinoic acid receptor alpha gene (RAR α). PCR primers were selected to amplify a 193-bp fragment for LOX-1 (forward 5'-TTACTCTCCATGGTGGTGCC-3'; reverse 5'-AGCTTCTTCTGCTTGTGCC-3') and a 250-bp fragment for RAR α (forward 5'-GGTGCCTCCCTACGCCTTCT-3'; reverse 5'-GGCGCTGACCCCATAGTGGT-3'). The thermal cycling conditions used for LOX-1 amplification were 35 cycles at 94°C for 45 sec, 58°C for 45 sec and 72°C for 50 sec. For RAR α , 40 cycles at 94°C for 1min, 53°C for 1min and 72°C for 1min were used. The amplified samples were visualized on 3% agarose gel and stained with ethidium bromide. A 100-bp DNA ladder (Gibco, BRL-UK) was employed as the molecular weight standard. Each LOX-1 mRNA band was normalized with a band of relative internal reference RAR α mRNA. The relative intensities of the bands of interest were analyzed by means of ethidium bromide-stained agarose gels scanned on Image Master[®]VDS (Pharmacia Biotech, Uppsala, Sweden) using the LISCAP and were expressed as a ratio to the RAR α mRNA band.

Detection of LOX-1 protein with immunofluorescence

To determine whether the ox-LDL receptor LOX-1 is expressed in human chondrocytes, we employed immunofluorescence microscopy and a rabbit antibody raised against amino acids 1-140 mapping near the N-terminus of the receptor (Santa Cruz Biotechnology, Inc). As secondary reagents, donkey anti-rabbit IgG conjugated with FITC and UltraCruz[™] Mounting Medium (Santa Cruz Biotechnology, Inc) were used. Briefly, osteoarthritic and normal chondrocytes from primary cultures were fixed in 2% (v/v) paraformaldehyde. After blocking with normal serum, the cells were incubated overnight with the primary antibody (dilution 1:100) in a moisture chamber. The slides were washed with Tris-buffer saline (TBS) and incubated with FITC-conjugated secondary antibody (1:150) at room temperature for 2h. Cells were viewed through a Zeiss axoplan fluorescence microscope. Images were captured using a digital cool camera and processed with Adobe Photoshop (Adobe Systems Inc, Mountain View, CA).

Primary cultures of human articular chondrocytes

Articular cartilage was transported from the surgical room in HBSS medium and dissected immediately. Explants were washed twice with HBSS without Ca^{++} / Mg^{++} (Gibco, BRL, UK). Cartilage samples were subjected to sequential digestion with 1 mg/ml pronase (Roche) for 90 min and 1 mg/ml collagenase P (Roche) for 3 hours at 37°C . The resulting cell suspension was recovered by centrifugation for 10 min. Chondrocytes were counted and checked for viability using trypan blue staining. More than 95% of the cells were viable after isolation. Chondrocytes were then seeded in 6-well plates with Dulbecco's Modified Eagles Medium/ Ham's F-12 (DMEM/F-12) (GIBCO BRL, UK) plus 5% foetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin and were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Effect of ascorbic acid on chondrocytes

After reaching confluence, cultured nor-

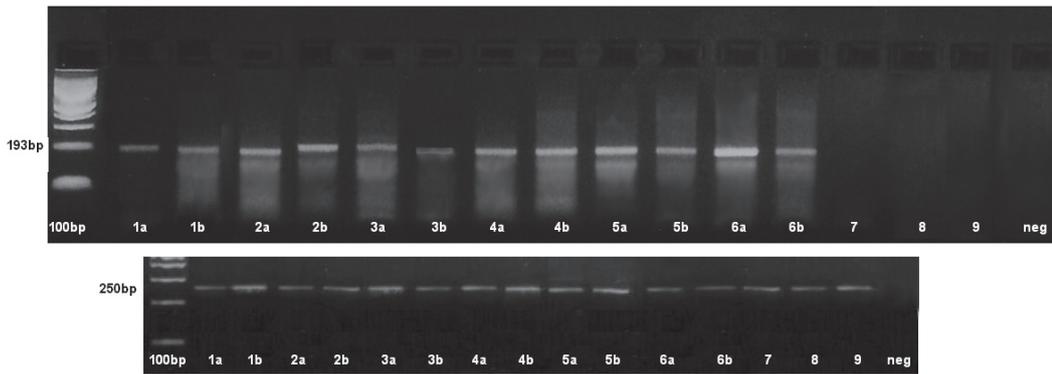


Fig. 1. Gel showing LOX-1 (193bp) and RARa (250bp) mRNA expression in the osteoarthritic cartilage from weight-bearing (a) and non-weight-bearing (b) regions (lanes 1-6) in 6 patients, and cartilage from 3 normal controls (lanes 7-9).

mal and osteoarthritic chondrocytes were first grown in serum-deprived medium (DMEM/F-12 without FBS) for 24 hours and subsequently with increasing concentrations of ascorbic acid (0-100 μ M) (Sigma, St. Louis, USA) for up to 20 days. RNA was then extracted from cultured cells and mRNA expression of LOX-1 was studied by RT-PCR.

Viability assay

Chondrocyte viability was assessed by means of an MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (R&D Systems, Minneapolis, USA) that allowed the quantification of viable cells. Human articular chondrocytes were seeded in 96-well plates and incubated in the presence of increasing concentrations of ox-LDL (10 to 40 μ g/ml) (Intracel, USA) for 24, 48 and 72 hours. The tetrazolium compound MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was then added to the wells and the cells were incubated for 2 hours. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals. At the end of the incubation period, detergent reagent was added to each well solubilizing the crystals. Absorbance of the converted dye was measured using a spectrophotometer at a wavelength of 570 nm with a reference wavelength of 650 nm. The number of viable cells per well in the presence of ox-LDL was compared with non-treated control cells. Wells containing only medium served as blanks.

Statistical analysis

Statistical analysis was conducted using SPSS software (version 11.0).

Differences between women and men were analyzed using the non-parametric Mann-Whitney U test. Correlation coefficients were calculated by Pearson rank correlation (r) and Spearman rank correlation where applicable. Comparisons of the levels between matched pairs of SF and serum samples were made by the paired sample t- test. A p value less than 0.05 was considered significant for differences and correlations.

Results

Detection of ox-LDL in the SF

SF samples obtained from OA patients were analyzed for ox-LDL. There was no significant difference between male and female patients with respect to age or BMI.

Ox-LDL was detected in each of the SF tested, with concentrations ranging from 12.3U/l to 75.85 U/l (mean 34.88 \pm 18.45 U/l), and were similar to those measured in the serum. Our study revealed that there was no correlation in ox-LDL values between the two body fluid compartments. Gender-specific or age-specific differences in ox-LDL levels were not detected. A significant correlation was observed between BMI and ox-LDL levels in the SF (Pearson rank correlation r = 0.881, p < 0.01).

LOX-1 mRNA and protein expression

Human adult articular chondrocytes derived from osteoarthritic cartilage expressed LOX-1 mRNA, while normal chondrocytes showed no such expression (Fig. 1). No significant differential

Table I. Quantification of the bands visualized in the gel. The ratio LOX-1/RARa mRNA represents the relative expression of LOX-1 mRNA in cartilage samples from weight-bearing and non-weight-bearing regions in 6 patients, as well as three normal cartilage samples.

	Sample	LOX-1 mRNA	RARa mRNA	LOX-1/RARa
Patient 1	Weight-bearing	39,831.00	22,817.00	1.745672
	Non-weight-bearing	55,229.00	44,776.00	1.233451
Patient 2	Weight-bearing	83,293.00	27,308.00	3.050132
	Non-weight-bearing	83,162.00	34,248.00	2.428229
Patient 3	Weight-bearing	40,794.00	37,709.00	1.081811
	Non-weight-bearing	36,824.00	27,700.00	1.329386
Patient 4	Weight-bearing	73,260.00	30,606.00	2.393648
	Non-weight-bearing	104,946.00	44,291.00	2.369466
Patient 5	Weight-bearing	131,883.00	38,322.00	3.441444
	Non-weight-bearing	88,885.00	47,463.00	1.872722
Patient 6	Weight-bearing	174,015.00	30,613.00	5.68435
	Non-weight-bearing	68,759.00	22,629.00	3.038535
Control 7		0		0
Control 8		0		0
Control 9		0		0

mRNA expression was observed between advanced and minimal osteoarthritic cartilage derived from the same patient (Table I). Our LOX-1 protein expression findings were in concordance with the mRNA studies. Microscopic immunofluorescence analysis demonstrated immunolocalization of LOX-1 in osteoarthritic chondrocytes (Fig. 2a). When normal cartilage was examined, no immunoreactive staining was found with anti-LOX-1 antibody (Fig. 2b). Non-specific control rabbit IgG yielded no positive staining in chondrocytes.

Effect of ox-LDL on cell viability

We investigated the effect of ox-LDL on chondrocyte viability. Increasing concentrations of ox-LDL were added to human chondrocytes and cell viability was assessed by the MTT assay after 24, 48 and 72 hours. We found that Ox-LDL reduced the viability of chondrocytes dramatically, decreasing the number of cells by one-half after 24h incubation with 20 $\mu\text{g/ml}$ (Fig. 3). The experiments were repeated 3 times with similar results.

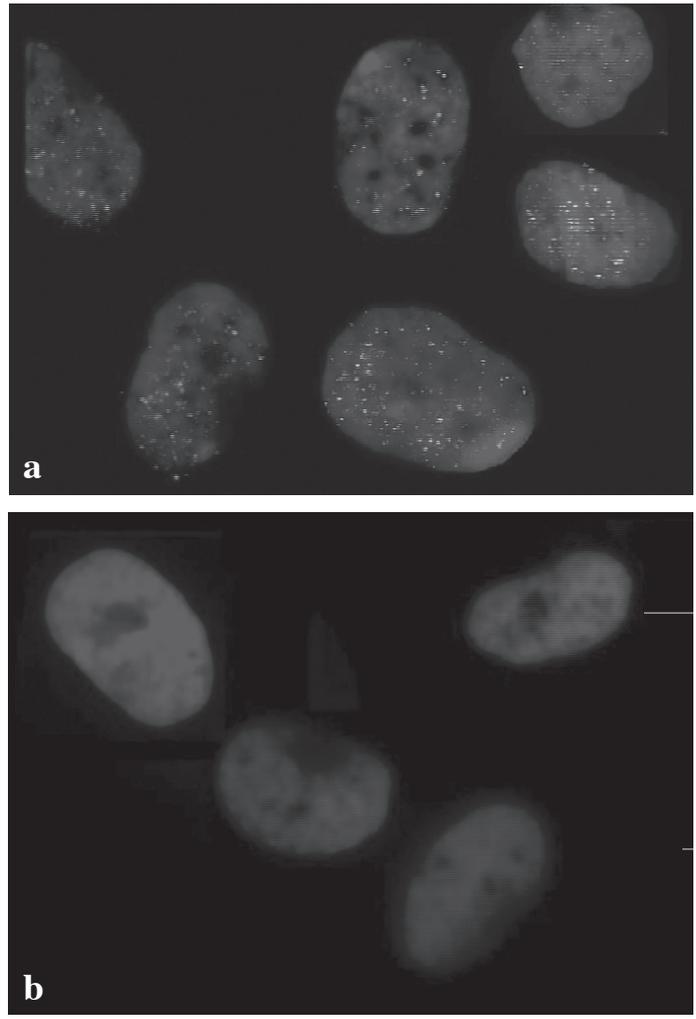
Effect of ascorbic acid on LOX-1 expression

LOX-1 mRNA expression was studied in cultured normal and osteoarthritic chondrocytes after the addition of ascorbic acid. Normal chondrocytes showed no LOX-1 mRNA expression with or without ascorbic acid. However, we did observe that in osteoarthritic chondrocytes LOX-1 mRNA expression decreased as the concentration of ascorbic acid increased (Fig. 4).

Discussion

LOX-1 has been initially identified as the major ox-LDL receptor in endothelial cells, related to the pathogenesis of atherosclerosis (15). Osteoarthritis and atherosclerosis are both extremely common age-related diseases. A high prevalence of cardiovascular risk factors has been described in OA (25), while cardiovascular mortality has been linked to the severity and extent of OA (26, 27). There are several explanations for this association, with decreased amounts of exercise and obes-

Fig. 2. Detection of LOX-1 protein by immunofluorescence. (a) Dots represent LOX-1 protein expressed on osteoarthritic chondrocytes. (b) Normal chondrocytes show no immunostaining.



ity being the most obvious, whereas a common genetic background, altered lipid metabolism, and microvascular atheromatous disease have been proposed as causative agents involved in the progression of OA (28).

In the present study, ox-LDL was detected in the synovial fluid of patients with osteoarthritis, and was significantly correlated with the patients' BMI. No correlation was observed between ox-LDL levels in the SF and paired serum samples. It has been suggested that lipid diffusion from synovial fluid occurs with aging and that the process of lipid diffusion can be detected in OA cartilage, depending on the severity of the disease (3, 4). Age-related changes in the lipid composition of cartilage could push the normally contained lipid peroxidation process into a state of uncontrolled oxidative stress, leading to the oxidation of cartilage collagen. This oxidation of collagen could cause

fragmentation, which would alter the material properties of collagen fibrils, making them more brittle and prone to mechanical fatigue failure, which in turn could initiate osteoarthritis (8). It is likely that LDL diffusing from serum into the synovial fluid could be oxidatively modified in the joint cavity. Excess weight as a factor in excess mechanical stress may explain our finding that the levels of ox-LDL in the SF correlated significantly with BMI.

Furthermore, we detected LOX-1 mRNA and protein expression in osteoarthritic chondrocytes. LOX-1 has been previously detected in RA cartilage (19), while concerning the LOX-1 mRNA expression in OA and normal cartilage there is conflicting data in the literature from the same research group. Kakinuma *et al.* failed to detect LOX-1 in osteoarthritic cartilage, while Akagi *et al.* detected LOX-1 in both normal and osteoarthritic cartilage

(19, 20). The discrepancy between the two studies was attributed to differences in the cartilage sampling sites (non-weight-bearing versus weight-bearing areas, respectively), as well as in the antibodies that were used for the immunohistochemistry.

In order to clarify this point, we used, for the first time, cartilage from both weight-bearing and non-weight-bearing regions in the same patients and performed histological studies to validate our macroscopic findings. We found that osteoarthritic chondrocytes showed LOX-1 mRNA and protein expression, whereas no such expression was detected in normal OA cartilage. It has been shown that LOX-1 expression is enhanced by oxidative stress, inflammatory mediators, shear stress and mechanical strain, as well as by its ligand, ox-LDL (14, 15, 29, 30). It is possible that mechanical stress may induce LOX-1 expression in chondrocytes in the weight-bearing regions of cartilage. Degraded OA cartilage matrix could provide easier access to ox-LDL and subsequent binding to LOX-1. The binding of ox-LDL to LOX-1 could oxidatively modify LDL to ox-LDL locally in the surroundings of chondrocytes, which in turn could upregulate LOX-1 expression in chondrocytes and contribute to further ROS generation (18). This vicious cycle of redox sensitive regulation may explain the presence of LOX-1 expression even in the non-weight-bearing regions of cartilage.

In the present study, in order to clarify whether LOX-1 is expressed or not in normal cartilage, we took into consideration that lipid deposition is seen early in the OA process, even before any histological changes, and we therefore used only specimens from young people (mean age 36.6 ± 12.6) for our controls, in contrast to the previous study which used samples from controls with a mean age of 80.3 ± 10.3 (20). We found that normal chondrocytes did not express LOX-1 mRNA or protein. Furthermore, as LOX-1 expression is enhanced in several pathologic settings, including hypertension, hyperlipidemia, diabetes and atherosclerosis (31), we were very careful to choose a control group without any of the above-mentioned factors

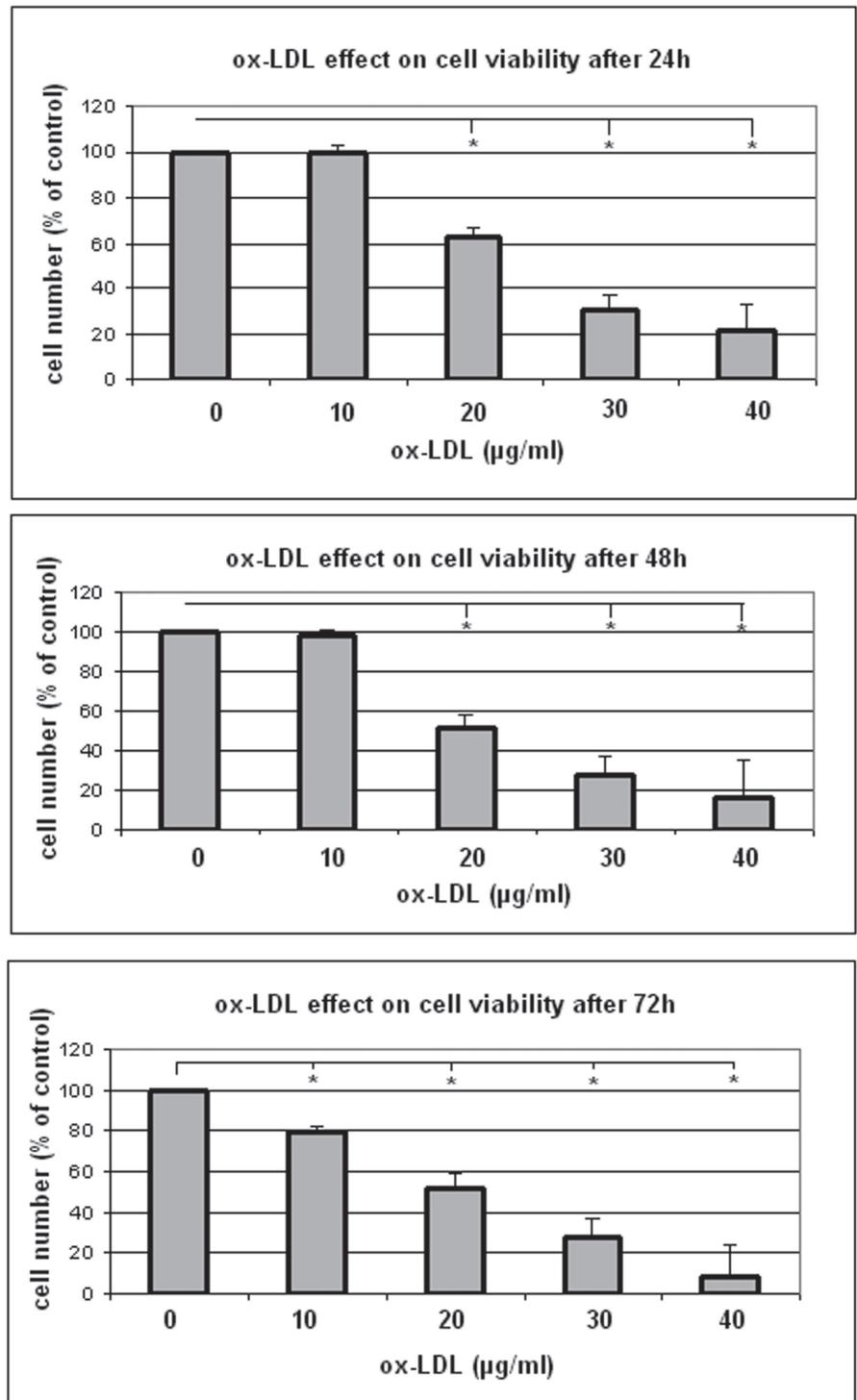


Fig. 3. Effect of increasing concentrations of ox-LDL (0-40ng/ml) on chondrocyte viability after 24, 48 and 72 hours. Cell numbers are expressed as a percentage of the controls (*i.e.*, cell cultures that had no ox-LDL). Data are shown as the mean \pm standard error of three independent experiments. (*) $p < 0.05$ was considered significant.

of co-morbidity, in contrast to the previous study, which used a control group that had all of these co-morbidity factors (20).

It has been shown *in vitro* that oxidized LDL exhibits, through binding to LOX-

1, a variety of biological properties involved in atherogenesis, and potentially in OA, including cytotoxicity (32). In addition, oxidized lipids promote calcification, partly by compromising cell viability and partly by the direct modula-

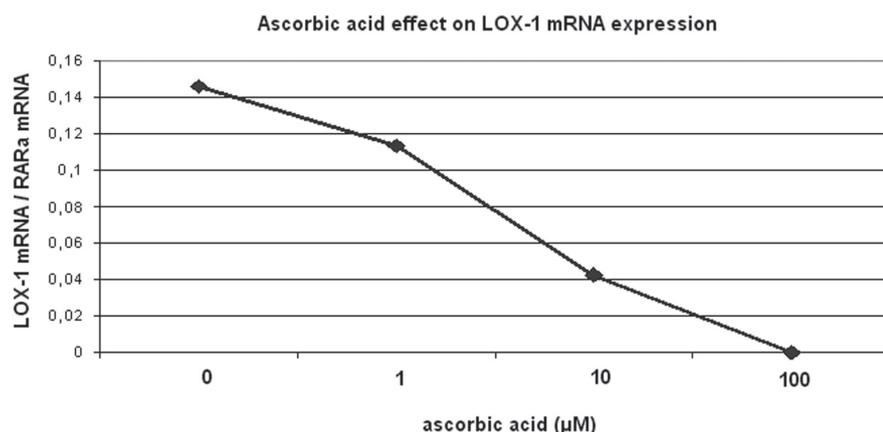


Fig. 4. Effect of increasing concentrations of ascorbic acid (0-100 µM) on relative LOX-1 mRNA expression (LOX-1 mRNA/RARa mRNA) in osteoarthritic chondrocytes. Data are shown as the mean relative expression of LOX-1 from three independent experiments.

tion of hydroxyapatite deposition in the matrix (33). We observed that ox-LDL at 10-40 µg/ml reduced chondrocyte viability. Previous studies have shown that ox-LDL induces the apoptosis of endothelial cells in association with NF-κB activation, and non-apoptotic cell death through the Akt pathway in cultured articular chondrocytes from rats (17). In addition, ox-LDL and cyclic tensile stretch load were found to synergistically induce LOX-1 expression in cultured bovine and human chondrocytes, resulting in decreased cell viability and proteoglycan synthesis, which have important implications for the progression of cartilage degeneration in osteoarthritis (14, 20). Our results show that ox-LDL has a cytotoxic effect on human chondrocytes proportional to the concentration of ox-LDL in the culture medium.

If oxidative modification of LDL plays a role in osteoarthritis, it can be assumed that its inhibition by an appropriate anti-oxidant would slow down disease progression. In human plasma, ascorbate is the only anti-oxidant that can completely protect lipids from the detectable peroxidative damage induced by aqueous peroxy radicals (34). Chondrocytes consume glucose as a primary substrate for ATP production in glycolysis and utilize glucosamine sulfate and other sulfated sugars (such as vitamin C) as structural components for extracellular matrix synthesis, and are dependent on hexose uptake and delivery to metabolic and biosynthetic

pools (35). We observed that the addition of ascorbic acid in osteoarthritic chondrocytes resulted in a decrease in LOX-1 mRNA expression, suggesting that this anti-oxidative nutrient has the ability to stop the cycle of ox-LDL binding to LOX-1. Ascorbic acid has been demonstrated to have specific effects in relation to the repair process in arthritic joints. More specifically, it has been shown to play a role in extracellular matrix production, as it is required for the synthesis of type II collagen, the most abundant protein in cartilage, (36), which moderately stimulates the synthesis of aggrecan (37), and its absence has been associated with the reduced mechanical resilience of collagen fibrils and increased turnover rates (38). Short-term studies in guinea pigs have suggested that ascorbic acid might be protective against osteoarthritis, and that a diet supplemented with vitamins (including vitamin C) and selenium diminishes the development of mechanically induced osteoarthritis in the knee joints of mice (39-41). The Framingham Knee Osteoarthritis Cohort Study showed that a high intake of anti-oxidant micronutrients, especially vitamin C, reduced the risk of cartilage loss and disease progression in OA patients (38). However, a prospective cohort study from Australia was unable to detect a significant effect of the main dietary anti-oxidant vitamins (taken in normal dietary amounts) on structural or symptomatic progression in subjects with OA of the knee over a period of 2

years, whereas they found an adverse effect of high dietary vitamin C intake on knee function (42).

In conclusion, we have demonstrated that ox-LDL present in the synovial fluid of patients with osteoarthritis is significantly correlated with the patients' BMI. In addition, no LOX-1 expression was found in normal chondrocytes, whereas LOX-1 mRNA and protein expression were detected in osteoarthritic cartilage derived from both the weight-bearing and non-weight-bearing regions of the same patients, suggesting the involvement of LOX-1 in the progression and pathogenesis of osteoarthritis.

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