OsteoArthritis and Cartilage (2004) **12,** 568–576

doi:10.1016/j.joca.2004.04.005

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Oxidized low-density lipoprotein (ox-LDL) binding to lectin-like ox-LDL receptor-1 (LOX-1) in cultured bovine articular chondrocytes increases production of intracellular reactive oxygen species (ROS) resulting in the activation of NF-κB

Shunji Nishimura M.D.†, Masao Akagi M.D.†*, Koji Yoshida M.D., Ph.D.‡, Sumio Hayakawa Ph.D.‡, Tatsuya Sawamura M.D., Ph.D.§, Hiroshi Munakata M.D., Ph.D.‡ and Chiaki Hamanishi M.D., Ph.D.†

† *Department of Orthpaedic Surgery, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama City, Osaka 589-8511, Japan*

‡ *Department of Biochemistry, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama City, Osaka 589-8511, Japan*

§ *Department of Bioscience, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita City, Osaka 565-8565, Japan*

Summary

Objective: To examine the effect of oxidized low-density lipoprotein (ox-LDL) on the intracellular production of reactive oxygen species (ROS) in bovine articular chondrocytes (BACs) and to investigate whether this increase occurs through binding to the receptor lectin-like ox-LDL receptor-1 (LOX-1). Furthermore, to ascertain whether the binding of ox-LDL to LOX-1 results in NF-κB activation.

Design: BACs were preincubated with 2′,7′-dichlorofluorescin diacetate (DCFH-DA), a dye that allows the monitoring of intracellular ROS production for DCF by spectrofluorometry. BACs were incubated with native LDL and ox-LDL (10, 50, and 100 μg/ml) for 5 min at 37°C and DCF formation was observed. BACs were also preincubated with anti-LOX-1 mAb (40 μg/ml) or ascorbic acid (10 μM). Nuclear extracts from BACs treated for the indicated periods with 50 μg/ml ox-LDL, and preincubated with anti-LOX-1 mAb or ascorbic acid, were prepared and analyzed by electrophoretic mobility shift assay (EMSA).

Results: ox-LDL induced a significant dose-dependent increase in ROS production after 5-min incubation with BACs ($P<0.001$). ROS formation was markedly reduced in BACs preincubated with anti-LOX-1 mAb and ascorbic acid ($P<0.001$). Activation in BACs of the transcription factor NF-κB was evident after 5-min incubation with ox-LDL and was attenuated by anti-LOX-1 mAb and ascorbic acid.

Conclusion: ox-LDL binding to LOX-1 in BACs increased the production of intracellular ROS and activated NF-κB. Reduction of NF-κB activation by ascorbic acid indicates that the activation, at least in part, is ROS-dependent. These observations support the hypothesis that hypercholesterolemia is one of several risk factors for arthritis, and that lipid peroxidation products such as ox-LDL are involved in cartilage matrix degradation.

Key words: Oxidized LDL, Cultured chondrocytes, ROS, NF-κB.

Introduction

Epidemiological and experimental studies have suggested the involvement of lipid peroxidation in the pathogenesis of articular cartilage degradation. Both hypercholesterolemia and hypertension have been reported to be associated with the risk of knee osteoarthritis (OA), independent of obesity[1]. Articular manifestations are frequent in familial hypercholesterolemia, which is characterized by a decreased removal of low-density lipoprotein (LDL), and treatment with a lipid-lowering diet decreases the incidence of joint involvement[2]. The Framingham Knee Osteoarthritis Cohort Study has demonstrated that a medium to high intake of antioxidants, such as ascorbic acid, β-carotene, and vitamin E, also reduces the risk of progressive knee OA[3]. In addition, it has been shown that the content of neutral lipids including cholesterol and triglycerides in the superficial layer of articular cartilage increases with aging[4,5]. *In vitro* studies have shown that lipid peroxidation by reactive oxygen species (ROS) generated by chondrocytes mediates cartilage matrix protein degradation. However, the studies also indicated that degradation of the extracellular matrix is inhibited by the lipid peroxidation inhibitors vitamins C and E[6,7], and *n*−3 fatty acids present in fish oils specifically modulate catabolic factors involved in articular cartilage degradation[8]. Furthermore, it has been reported that oxidized LDL (ox-LDL) is detected in synovial

* Address correspondence and reprint requests to: M. Akagi, M.D., Department of Orthopaedic Surgery, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama City, Osaka 589-8511, Japan. Tel: 81-72-366-0221; Fax: 81-72-366-0206; E-mail: makagi@med.kindai.ac.jp

Received 3 September 2003; revision accepted 5 April 2004.

fluid from rheumatoid arthritis (RA) patients[9,10]. In OA and RA joints, inflammation accelerates vascular porosity, which can facilitate the invasion of various inflammatory cells and the permeation of biological mediators into the synovial fluid, including ox-LDL that has been oxidatively modified extra-articularly. Inflammatory cells that have invaded the synovium and synovial fluid, activated endothelial cells in the inflamed synovium, and activated chondrocytes in degraded cartilage can all release ROS and increase intra-articular oxidative stress[11], which could lead to local oxidative modification of native LDL (n-LDL) to ox-LDL[12]. Collectively, these facts and speculations suggest that ox-LDL plays some role in the degradation of cartilage in OA and RA.

Recently, a novel receptor for ox-LDL, designated lectin-like ox-LDL receptor-1 (LOX-1) was cloned from cultured bovine aortic endothelial cells[13]. It has a type II membrane protein structure with a short N-terminal cytosolic domain and a long C-terminal extracellular domain, but is distinct from the type 1 and 2 scavenger receptors, CD36 and CD68. Although the potential roles of this receptor in atherogenesis are not yet fully understood, it has been suggested that ox-LDL uptake through this receptor expressed on the surface of vascular endothelium may be involved in endothelial activation or dysfunction in atherogenesis[13]. More recently, Nakagawa *et al.* reported LOX-1 expression in articular cartilage cells in rat zymosan-induced arthritis and the presence of ox-LDL in the articular cartilage[14]. Furthermore, they showed LOX-1 expression in cultured rat chondrocytes, which was detectable in basal culture conditions and enhanced by the treatment with ox-LDL and interleukin-1β. In addition, ox-LDL dose-dependently reduced chondrocyte viability, inducing non-apoptotic cell death[15]. These observations suggest that ox-LDL and its receptor LOX-1 may be significant regulating factors not only for endothelial dysfunction, but also for cartilage degradation.

In the current study, we investigated the effect of ox-LDL on the intracellular production of ROS in cultures of bovine articular chondrocytes (BACs), and whether the intracellular increase of ROS induced by ox-LDL is mediated specifically by binding to LOX-1. Furthermore, since NF-κB is well known as an oxidative stress-sensitive nuclear transcription factor and because intracellular ROS plays a major role in the translocation of NF-κB[16,17], this study also aimed to ascertain whether binding of ox-LDL to LOX-1 is associated with NF-κB activation in cultured BACs.

Materials and methods

PRIMARY BAC CULTURE

Articular cartilage slices were taken from the condyles of the metacarpophalangeal joints of freshly slaughtered calves aged about 10 months. Care was taken to exclude underlying bone marrow. Chondrocytes were obtained by sequential enzymatic dissociation at 37°C with 0.1% EDTA/phosphate buffered saline (PBS; pH 7.4) for 20 min, and 2 mg/ml collagenase for 10 h. After filtration through nylon mesh to remove debris, cells were seeded on culture plates and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD) 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 grown in the serum-free culture medium for 24 h.

PREPARATION OF N-LDL AND OX-LDL

Human LDL (density 1.019−1.063) was isolated from fresh plasma by sequential ultracentrifugation as described previously[13]. 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 charges on agarose gel electrophoresis were compared with n-LDL (relative electrophoretic mobility was 3.25).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION FOR LOX-1 MRNA

Total RNA (1 μg) extracted from BACs with Isogen (Nippon Gene, Tokyo, Japan) was reverse transcribed using a OneStep RT-PCR kit (QIAGEN Japan, Tokyo, Japan). Reverse-transcribed material (1.5 μl) was amplified with Taq DNA polymerase (Bex, Tokyo, Japan) using a primer pair specific to bovine LOX-1 (sense primer, 5′-GTGACTC-TAGGGGTCCTTTG-3′, antisense primer, 5′-TGGGCATC-CAAAGACAAGCA-3′). The PCR product was 415 bp in length. For PCR, 34 cycles were used at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. In the same experiments, bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with equal efficiency as a reference for quantification of LOX-1 mRNA. A primer pair for bovine GAPDH was used (sense primer, 5′-CCATCACCATCTTCGGAGC-3′, antisense primer, 5′-GGAAGGCCATGCCAGTGAGC-3′). The PCR product was 483 bp in length. For PCR, 34 cycles were used at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The RT-PCR-amplified samples were visualized on 1.5% agarose gels using ethidium bromide. Each LOX-1 mRNA band was normalized with a band of the relative internal reference GAPDH mRNA. The relative intensities of bands of interest were analyzed using Gel Doc 2000 (Bio-Rad Labs, Hercules, CA) and scan analysis software (Biosoft, Ferguson, MO), and expressed as a ratio to the GAPDH mRNA band.

DETECTION OF ROS PRODUCTION BY DCF FORMATION

The intracellular production of ROS was detected using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), as described previously[18,19] with modifications. DCFH-DA is a non-fluorescent probe that, upon diffusion into cells, is hydrolyzed by intracellular esterases to 2′,7′-dichlorodihydrofluorescin (DCFH2), which is trapped within the cells. In the presence of ROS, particularly peroxides, DCFH2 is oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescin (DCF). To observe intracellular ROS production through the oxidation of DCFH-DA, confluent BACs in 12-well plates were incubated in DMEM containing 10% FBS and 5 μM DCFH-DA (COSMO BIO, Tokyo, Japan) for 60 min at 37°C in a 5% CO_2 incubator. Increasing concentrations of n-LDL, ox-LDL, and IL-1β were then added to the medium for 5 min at 37°C. The intracellular ROS produced by BACs were observed by confocal microscopy (MRC 2400LSX imaging system, Bio-Rad Labs, Hercules, CA), and ROS production was monitored with a spectrofluorometer (CytoFluor-4000, PerSeptive Biosystems, Framingham, MA), as described previously[20]. Fluorescence intensities indicating ROS production by the chondrocytes were measured with an excitation wavelength of 560 nm and an emission wavelength of 580 nm.

WESTERN BLOTTING ANALYSIS FOR LOX-1 PROTEIN

BACs stimulated with ox-LDL for various times were lysed with CelLytic-M Mammalian Cell Lysis/Extraction Reagent (Sigma, St Louis, MO). The protein concentration was determined by the bicinchoninic acid (BCA) method. The samples from each experiment (30 μg per lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After incubation with 5% skim milk in 20 mM Tris-buffered saline/0.05% Tween 20, the membranes were incubated with primary monoclonal antibody to LOX-1, diluted 1:500, overnight at 4°C. Membranes were washed and then incubated for 1 h with secondary mouse monoclonal antibody diluted 1:10 000 (MBL, Nagoya, Japan). LOX-1 protein on the membrane was detected with the ECL system (Amersham, Piscataway, NJ), and relative intensities of protein bands were analyzed with a LAS-1000 luminoimage analyzer (FUJIFILM, Tokyo, Japan).

PREPARATION OF LOX-1 SMALL INTERFERING RNA CELLS

The selection of small interfering RNA (siRNA) duplexes from the target mRNA sequence was performed according to the method described by Elbashir *et al.*[21,22]. We searched for the sequence AA(N19)TT and chose those with approximately 50% G/C content. siRNA oligonucleotides directed to a 164-nucleotide coding sequence of the bovine LOX-1 mRNA were designed and manufactured by Dharmacon (Lafayette, CO) and were targeted as a 19-mer to the 5′-AAUUAUCCAGUCUCUGAUC-3′ part of the LOX-1 sequence. dsRNA sequences (sense 5′-UUAUCCCAGGUCUCUGAUCdTdT-3′ and antisense 5′-dTdTAAUAGGGUCCAGAGACUAG-3′) were synthesized. In one tube, 3 μl of 20 μM siRNA duplex was mixed with 50 μl of Opti-MEM (Gibco) and incubated for 10 min at room temperature. In another tube, 3 μl of Oligofectamine reagent (Invitrogen, Carlsbad, CA) was mixed with 12 μl of Opti-MEM and incubated under the same conditions. These solutions were then gently mixed by inversion and incubated for 25 min at room temperature. BACs were cultured in DMEM using 24-well plates (30−40% confluent). Each culture well was washed twice with Opti-MEM, and the mixture of the siRNA duplex and Oligofectamine reagent (total 68 μl) was added. After addition of 200 μl of Opti-MEM to each well, cultured BACs were incubated for 4 h, and the cells were further incubated for 4 days with 30% FBS, without antibiotics, at 37°C in a 5% CO_2 incubator. To test the response specificity, non-specific siRNA (Dharmacon) cells also were prepared using the same experimental conditions as mentioned above.

PREPARATION OF NUCLEAR AND CYTOSOLIC EXTRACTS

Nuclear and cytosolic extracts were isolated with a Nuclear/cytosol Fractionation kit (Biovision, Mountain View, CA). After the incubation period, BACs were collected by centrifugation at 600 *g* for 5 min at 4°C. Chondrocyte pellets were washed twice with ice-cold PBS, followed by the addition of 0.2 ml of Cytosol Extraction Buffer A and vigorous mixing for 5 s. Ice-cold Cytosol Extraction Buffer B (11 μl) was added to the solution. After vortex mixing, nuclei and cytosolic fractions were separated by centrifugation at 16 000 *g* for 5 min. The cytoplasmic extracts (supernatants) were stored at −80°C. Nuclear extraction buffer was added to the nuclei and cytosolic fractions (pellets), which were then mixed by vortex mixing on the highest setting for 15 s. Icing the mixture, a 15-s vortex was performed every 10 min for a total of 40 min. Nuclei were centrifuged at 16 000 *g* for 10 min. The nuclear extracts (supernatants) were stored at −80°C. The protein concentration was determined by the BCA method.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear transcription factor activity was studied using an EMSA Gel-Shift Kit (Panomics, Redwood City CA). Briefly, NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were used as a biotin-labeled probe, as described previously[23]. Nuclear extracts (5 μg) were equilibrated for 10 min in a binding buffer (2.0 μl 5×Binding Buffer, 1.0 μl Poly d (I-C), 5.0 μl distilled water). Then, 10 ng/μl of the labeled probe was added to the binding buffer, and incubated for 30 min at room temperature. To confirm the specificity of the reaction, a competition assay with unlabeled oligonucleotide was performed. Samples were separated on 6.0% polyacrylamide gels (1 ml 10×TBE, 4 ml 30% Bis-included acrylamide), 625 μl 80% glycerol, 14.375 ml dH_2O, 300 μl 10% APS, 20 μl TEMED, 20 ml total volume) and transferred to nylon membranes by electroblotting. After DNA complexes were fixed to the membranes using a UV cross-linker, blocking buffer and 1:1000 diluted streptavidin-HRP conjugate were added to the membrane for 15 min. After washing three times, NF-κB expression on the membrane was detected as a blot with a luminescent detection kit (Panomics, Redwood City, CA), and relative intensities of the bands were analyzed using the LAS-1000 luminoimage analyzer.

STATISTICAL ANALYSIS

Results are presented as the mean±SD. Analysis of variance (ANOVA), Scheffe's test and Student's *t* test were used for statistical assessments. A level of $P<0.05$ was considered statistically significant.

Results

INDUCTION OF LOX-1 EXPRESSION ON THE CULTURED BACS WITH OX-LDL

The level of LOX-1 gene expression was minimal at time 0. Six hours after addition of ox-LDL, LOX-1 mRNA expression reached a maximal level [about fivefold, Fig. 1(A, B)]. The level of LOX-1 protein at time 0 was also minimal. Treatment with ox-LDL caused a maximal increase in LOX-1 protein expression at 24 h [Fig. 1(C)].

OBSERVATION OF INTRACELLULAR ROS PRODUCTION BY CONFOCAL MICROSCOPY

We used confocal microscopy to observe whether BACs produce intracellular ROS following stimulation with IL-1β (5 ng/ml), n-LDL (50 μg/ml), and ox-LDL (10, 50 μg/ml). The distribution of luminescence resulting from DCF formation was observed to correspond to the cytoplasmic morphology of BACs (Fig. 2). The luminescence intensity resulting from ROS production following IL-1β and ox-LDL treatment (10 and 50 μg/ml) was significantly higher than that in cells stimulated with n-LDL. Ox-LDL-induced ROS production was dose-dependent.

MEASUREMENT OF THE AMOUNTS OF DCF FORMATION THROUGH INTRACELLULAR ROS PRODUCTION

We also investigated the amount of DCF formation with a spectrofluorometer. Addition of n-LDL (10, 50, or 100 μg/ml)

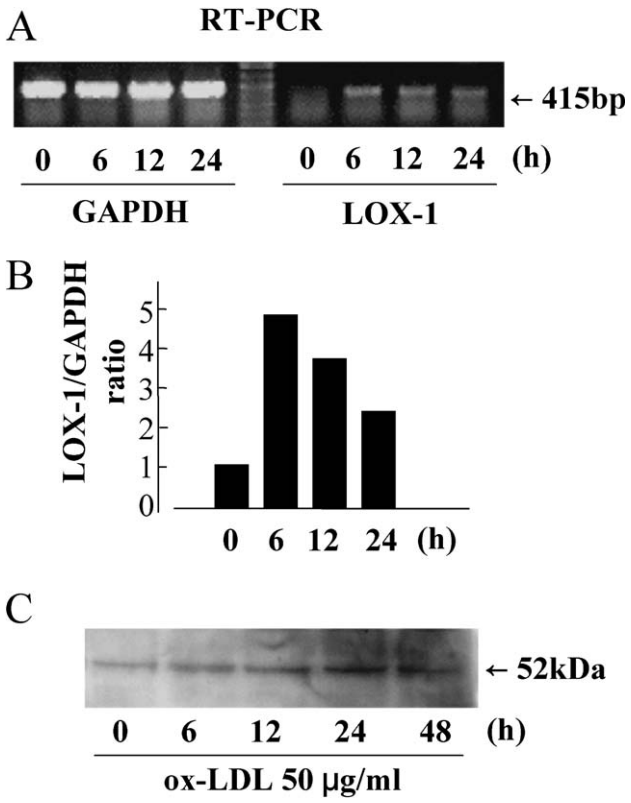

Fig. 1. Induction of LOX-1 expression in bovine articular chondrocytes (BACs) by ox-LDL. (A) After incubation with ox-LDL (50 μg/ml) for 0, 6, 12, or 24 h, LOX-1 mRNA expression in BACs was assessed by RT-PCR. The level of LOX-1 gene expression was minimal at time 0 and reached a maximum at 6 h. The expected size of the LOX-1 PCR product was 415 bp. (B) Relative intensity of LOX-1 and GAPDH bands was analyzed by scan analysis software and expressed as a ratio to a GAPDH mRNA band, which increased about fivefold at 6 h after the addition of ox-LDL. (C) After incubation with ox-LDL (50 μg/ml) for 0, 6, 12, 24, or 48 h, LOX-1 protein in BACs was assessed by western blotting. The level of LOX-1 protein expression at time 0 was minimal. Treatment with ox-LDL caused a maximal increase in LOX-1 protein expression at 24 h.

did not result in any change in DCF formation (mean fluorescent intensities (MFI): 18.1±1.3, 19.5±2.1, 16.6±2.2, respectively, control: 16.2±1.5, Fig. 3). By contrast, 10, 50, or 100 μg/ml ox-LDL dose-dependently increased the amounts of DCF formation (MFI: 49.2±3.0, 62.4±3.4, and 89.7±5.8, respectively, Fig. 3). We next performed time-course experiments of DCF formation in BACs that indicated that ROS production was evident 60 s after addition of ox-LDL (Fig. 4). The effect of an anti-LOX-1 mAb on DCF formation in BACs was then investigated. DCF formation was markedly reduced in BACs incubated with an anti-LOX-1 mAb (40 μg/ml JTX-20) on treatment with ox-LDL (ox-LDL 10, 50, and 100 μg/ml, MFI: 47.2±3.4, 60.3±3.8, and 91.2±5.3, respectively; ox-LDL 10, 50, and 100 μg/ml with 40 μg/ml JTX-20, MFI: 14.3±4.4, 25.5±4.1, and 29.9±5.5, respectively; ox-LDL 10, 50, and 100 μg/ml with comparable amounts of non-specific mouse IgG (Funakoshi, Tokyo, Japan), MFI: 42.4±4.9, 57.5±6.1, 86.6±5.8, Fig. 5). To confirm that the ROS produced by BACs resulted in the DCF formation, BACs preincubated with ascorbic acid, a known radical scavenger, were stimulated with ox-LDL. DCF formation was significantly reduced by the preincubation with ascorbic acid (ox-LDL 10, 50, and 100 μg/ml with ascorbic acid, MFI: 12.3±1.9, 29.9±6.1, and 34.3±4.8, respectively, Fig. 5).

INHIBITION OF ROS PRODUCTION IN LOX-1 SIRNA CELLS

In addition, we created LOX-1 siRNA cells to confirm that ROS is produced through interaction between LOX-1 and ox-LDL. First, we investigated the inhibitory rate of LOX-1 expression by LOX-1 siRNA. Basal expression of LOX-1 mRNA was observed by RT-PCR in control, LOX-1 siRNA, and non-specific siRNA cells [Fig. 6(A)]. The inhibitory ratio of LOX-1/GAPDH mRNA in control cells to that in LOX-1 siRNA and non-specific siRNA cells averaged 54.8±6.1% and 4.0±0.5%, respectively [$n = 5$, Fig. 6(B)]. We then investigated the inhibition of DCF formation in LOX-1 siRNA cells stimulated with ox-LDL. Intracellular ROS production stimulated by ox-LDL was moderately inhibited in the LOX-1 siRNA cells compared with control and non-specific siRNA cells (ox-LDL 10, 50, or 100 μg/ml, MFI: 43.6±5.0, 58.7±2.5, and 90.4±6.8 in the control cells, 38.4±3.2, 50.5±2.9, and 69.6±3.8 in the LOX-1 siRNA cells, respectively, and 44.1±4.2, 55.3±4.4, 87.6±6.1 in the non-specific siRNA cells, Fig. 7).

INHIBITION OF INTRACELLULAR ROS PRODUCTION BY DPI TREATMENT

DCF formation was markedly reduced in BACs preincubated with DPI (5 μM) when stimulated by 50 μg/ml ox-LDL (78±4.1% inhibition compared with control cells incubated with ox-LDL alone, Fig. 8).

NF-κB ACTIVATION BY OX-LDL STIMULATION

Finally, the effect of ox-LDL stimulation on NF-κB activation in BACs was studied. In order to ascertain a causal relationship between IL-1β and ox-LDL on NF-κB activation, a time-course study was performed. NF-κB activation was evident after a 5 min of incubation with both IL-1β and ox-LDL, and reached a peak at 60 min (Fig. 9). In addition, the effect of anti-LOX-1 mAb and ascorbic acid on ox-LDL-induced NF-κB activation was also investigated. The activation of NF-κB was attenuated in BACs incubated with anti-LOX-1 mAb and ascorbic acid (Fig. 10).

Discussion

In this study, by monitoring intracellular oxidation of the dye DCFH-DA, which is a process known to depend on the intracellular production of ROS[24–26], we have shown that ox-LDL increased the intracellular formation of ROS in cultured BACs, and that ox-LDL is a strong inducer of intracellular ROS production. The increased intracellular production of ROS was prevented by preincubating BACs with ascorbic acid, one of a number of antioxidants known to work as radical scavengers. These data support the conclusion that the incubation of ox-LDL with BACs is associated with an increased intracellular production of ROS and that the rise in DCF formation is specifically related to ROS formation. Our results agree with the conclusions of a series of papers showing that ox-LDL increased the production of ROS in different cells[24,26–28]. The increase in intracellular ROS formation induced by ox-LDL in BACs was very fast, the

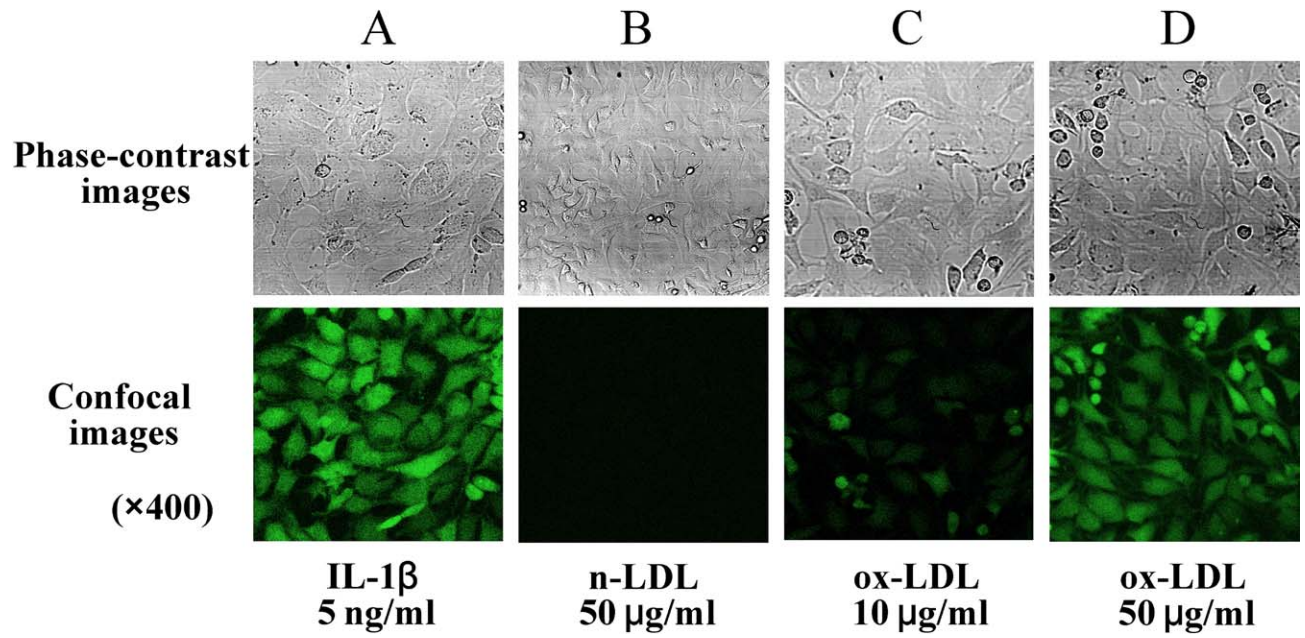

Fig. 2. Fluorometric micrographs of intracellular ROS production in BACs. DCF formation following ROS production was observed by confocal microscopy after treatment with (A) IL-1β (5 ng/ml), (B) n-LDL (10 μg/ml), (C) ox-LDL (10 μg/ml), and (D) ox-LDL (50 μg/ml) for 5 min in the presence of DCFH-DA. Upper: phase-contrast images, lower: confocal images (original magnification ×400).

signal from ROS production being clearly visible after 60 s. This suggests that intracellular ROS increases may be secondary to ox-LDL interacting with a specific receptor on BACs. The fact that DCF formation was markedly reduced in BACs preincubated with an anti-LOX-1 mAb indicates that ox-LDL binding to LOX-1 may play a role in intracellular ROS generation. This conclusion is further supported by the results we obtained with LOX-1 siRNA cells, where DCF formation stimulated by ox-LDL was also reduced significantly.

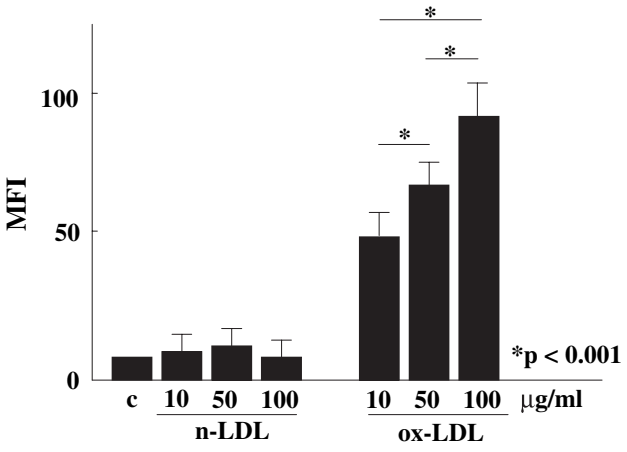

Fig. 3. Amounts of DCF formation in BACs treated with n-LDL or ox-LDL. BACs were preincubated with DCFH-DA for 60 min at 37°C. After incubation with n-LDL (10, 50, and 100 μg/ml) or ox-LDL (10, 50, and 100 μg/ml) for 5 min, DCF formation was measured using a spectrofluorometer with an excitation wavelength of 560 nm and an emission wavelength of 580 nm. Control (c): non-treated cells. Results are expressed as means ± SD ($n = 8$). There were statistically significant differences in mean fluorescence intensities between 10 and 50 μg/ml ox-LDL, 50 and 100 μg/ml ox-LDL, and 10 and 100 μg/ml ox-LDL (ANOVA and Scheffe's method as the post-hoc test, $P < 0.001$).

There are many enzymatic sources of ROS in almost all cell types. It has been hypothesized that the activity or expression of these enzymes can be regulated by cytokines and growth factors. Thus, receptor–ligand interaction is likely to trigger free radical signaling through activation of one or more of these enzymes[29]. In the current study, intracellular ROS production following binding of ox-LDL to LOX-1 in BACs was suppressed markedly by the addition of DPI, a well-known inhibitor of flavoproteins, strongly suggesting the involvement of NADPH oxidase activation in the ROS production. Unfortunately, we could not completely exclude the involvement of mitochondrial complex-1, which is also inhibited by DPI. Further studies are needed to confirm that ox-LDL binding to LOX-1 generates intracellular ROS through activation of NADPH oxidase.

It has been reported that ROS are required for transformation of n-LDL to ox-LDL[30]. The ROS produced by the binding of ox-LDL to LOX-1 could oxidatively modify n-LDL to ox-LDL locally in the surrounds of chondrocytes, which in turn could up-regulate LOX-1 expression in chondrocytes and contribute to further ROS generation. This phenomenon could be amplified in patients with high plasma concentrations of LDL, such as in hypercholesterolemia. Therefore, the results of the current study support the hypothesis that hypercholesterolemia is one risk factor of OA, and that lipid peroxidation products are involved in the degradation of articular cartilage.

Since ox-LDL is a large molecule of about 550 kDa, it is not likely that ox-LDL penetrates into the extracellular matrix of normal articular cartilage and binds to LOX-1 expressed on chondrocytes. However, degenerative OA and RA cartilages are known to release high molecular weight molecules, such as chondroitin sulfate, keratan sulfate,

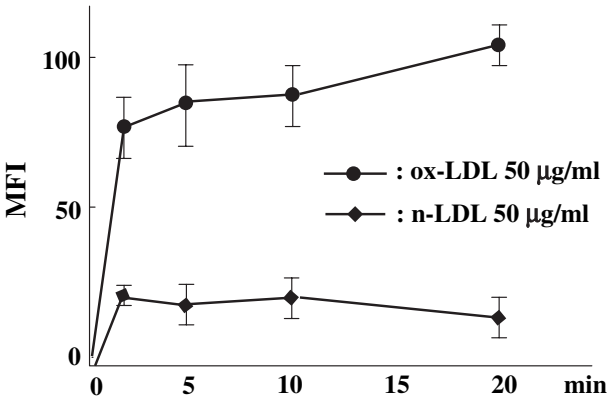

Fig. 4. Time course of DCF formation induced by ox-LDL in BACs. BACs were preincubated with DCFH-DA for 60 min at 37°C. Then, 50 μg/ml of n-LDL or ox-LDL were incubated with the bovine chondrocytes for the indicated times. DCF formation was measured using a spectrofluorometer with an excitation wavelength of 560 nm and an emission wavelength of 580 nm. Results are expressed as means ± SD ($n = 8$).

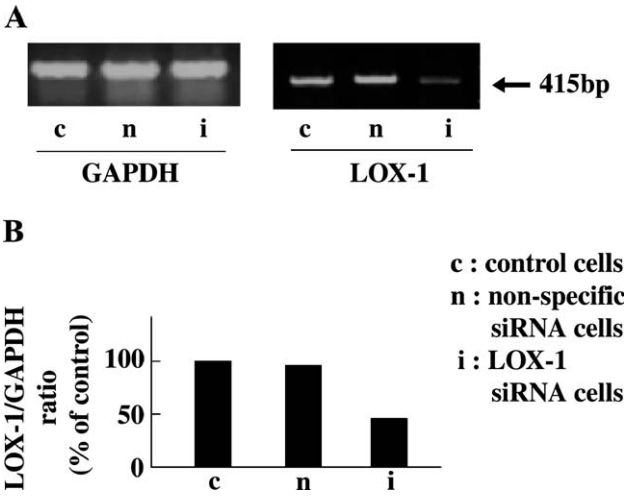

Fig. 6. Inhibition of LOX-1 expression in LOX-1 siRNA cells. (A) LOX-1 mRNA induction in control, non-specific siRNA, and LOX-1 siRNA cells was assessed by RT-PCR. (B) The ratios of LOX-1/GAPDH mRNA in control cells to that in LOX-1 and non-specific siRNA cells were analyzed by scan analysis software. Inhibition ratio of LOX-1 expression in LOX-1 siRNA cells ($n = 5$) and in non-specific siRNA cells averaged 54.8 ± 6.1% and 4.0 ± 0.5%, respectively. c: control cells, n: non-specific siRNA cells, i: LOX-1 siRNA cells.

and hyaluronic acid, from the inner layer, suggesting the possibility of permeation of ox-LDL into degenerative cartilage. Furthermore, in OA and RA, articular cartilage is subjected not only to deterioration of mechanical properties due to degradation of the matrix, but also to morphological changes. Fibrillation of articular cartilage, which is the earliest morphological change in OA, can make it easier for ox-LDL to gain access to chondrocytes. Anti-ox-LDL antibody-reactive substances have also been detected in articular cartilage from an animal arthritis model[14]. From this point of view, the role of ox-LDL may not primarily be involved in the degradation of articular cartilage. However, ox-LDL could play an important role in acceleration of cartilage degradation in chronic inflammatory joint diseases.

Finally, the results of the current study indicate that ox-LDL binding to LOX-1 induced NF-κB activation in BACs. Numerous studies have provided strong support for a proposal that intracellular ROS serve as common intracellular

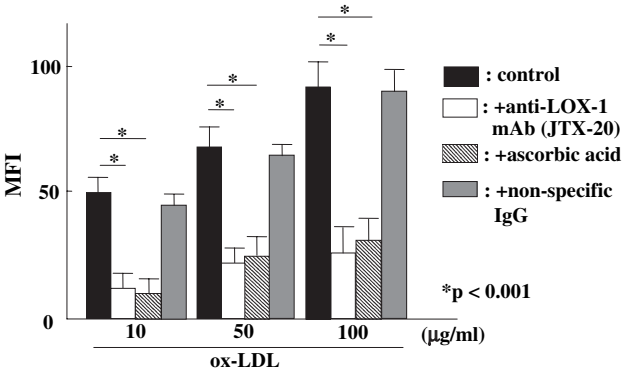

Fig. 5. Effect of preincubation of BACs with anti-LOX-1 mAb on ox-LDL-induced DCF formation. BACs were preincubated with DCFH-DA for 60 min at 37°C. BACs were also preincubated with anti-LOX-1 mAb (40 μg/ml), ascorbic acid (10 μM) and non-specific mouse IgG for 120 min at 37°C. Thereafter, ox-LDL (10, 50, and 100 μg/ml) was added for 5 min at 37°C. DCF formation was measured using a spectrofluorometer with an excitation wavelength of 560 nm and an emission wavelength of 580 nm. Results are expressed by means ± SD ($n = 8$). There were statistically significant differences in mean fluorescence intensities (MFI) between controls and anti-LOX-1 mAb groups of 10, 50, and 100 μg/ml ox-LDL (Student's unpaired t test, $P < 0.001$).

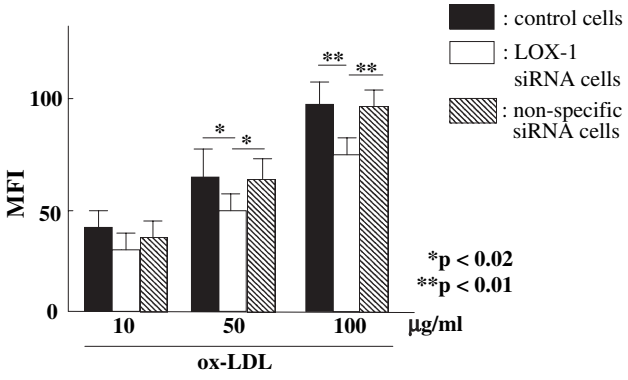

Fig. 7. DCF formation in control, LOX-1 siRNA, and non-specific siRNA cells. Control, LOX-1 siRNA and non-specific siRNA cells were preincubated with DCFH-DA for 60 min at 37°C and then incubated with ox-LDL (10, 50, and 100 μg/ml) for 5 min at 37°C. DCF formation was measured using a spectrofluorometer with an excitation wavelength of 560 nm and an emission wavelength of 580 nm. Results are expressed as means ± SD ($n = 5$). There were statistically significant differences in mean fluorescence intensities (MFI) between control and LOX-1 siRNA cells and between LOX-1 siRNA and non-specific siRNA cells incubated with 50 and 100 μg/ml ox-LDL (Student's unpaired t test, $P < 0.02$ for 50 μg/ml ox-LDL; and $P < 0.01$ for 100 μg/ml ox-LDL).

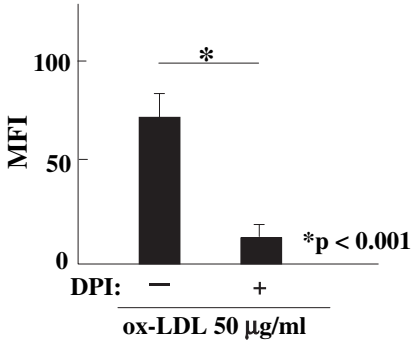

Fig. 8. Effect of preincubation of bovine chondrocytes with DPI on ox-LDL-induced DCF formation. BACs were preincubated with DPI (5 μM) for 60 min at 37°C. BACs were also preincubated with DCFH-DA for 60 min at 37°C. Thereafter, ox-LDL (50 μg/ml) was added for 5 min at 37°C. DCF formation was measured using a spectrofluorometer with an excitation wavelength of 560 nm and an emission wavelength of 580 nm. Results are expressed as means ± SD ($n = 8$). There was a statistically significant difference in mean fluorescence intensity between control cells and cells preincubated with DPI (Student's unpaired t test, $P < 0.001$).

downstream messengers of the various stimulus-specific pathways leading to NF-κB activation[31–33]. A kinetic coincidence of the two events, NF-κB activation and ROS production, in response to ox-LDL, indicates a causal relationship between the two events. In the current study, peak ROS production by BACs is within 60 s of the addition of ox-LDL. NF-κB activity did not strongly increase until 5 min after the addition of ox-LDL. These data show that NF-κB activation follows the production of ROS in ox-LDL-treated cells, which is consistent with the idea that ROS serve as a messenger of NF-κB activation. Furthermore, the fact that preincubation with both anti-LOX-1 mAb and ascorbic acid suppressed the ox-LDL-induced NF-κB activation suggests that the binding of ox-LDL to LOX-1 and the consequent formation of ROS may be the first event in the chain of reactions leading to NF-κB activation.

NF-κB transcription factor controls the expression of a number of proinflammatory molecules, including cytokines (TNF-α, IL-1β, IL-6), chemokines (IL-8, macrophage inflammatory protein-1β), enzymes (COX-2, inducible nitric oxide synthase, cPLA2, metalloproteinases), and adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1)[34]. In chronic inflammatory joint disease such as RA, the negative regulating loop with its inhibitor, IκB-α, is overwhelmed by a positive one involving NF-κB activation by TNF-α and IL-1β, and NF-κB-dependent expression of these two major proinflammatory cytokines. Indeed, RA is associated with persistent in situ NF-κB activity[35]. In the context of chronic arthritis, therefore, induction of NF-κB activity in chondrocytes would facilitate degradation of the extracellular matrix of cartilage, although it has been reported that NF-κB activity protects articular chondrocytes from apoptotic cell death caused by NO[36].

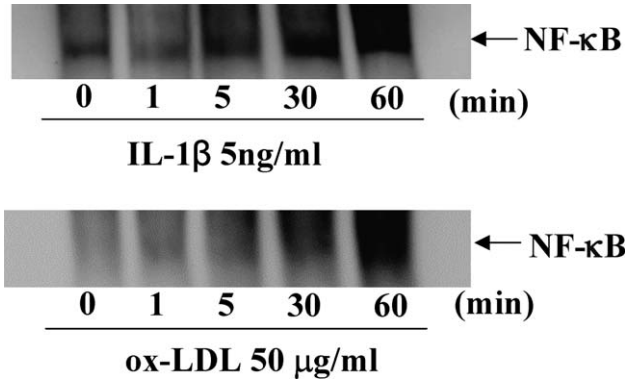

Fig. 9. Activation of NF-κB in BACs with IL-1β and ox-LDL. BACs were incubated for 0, 1, 5, 30, and 60 min with IL-1β (5 ng/ml) and ox-LDL (50 μg/ml). Activation of NF-κB was evident 5 min after incubation with ox-LDL and IL-1β, and reached a peak at 60 min.

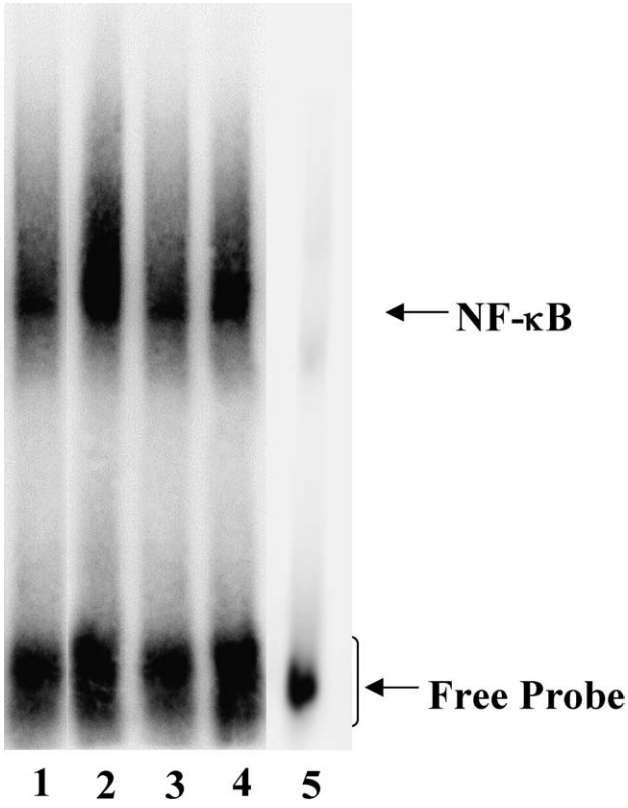

Fig. 10. Effect of anti-LOX-1 mAb and ascorbic acid on ox-LDL (50 μg/ml)-induced NF-κB activation. BACs were preincubated for 120 min with anti-LOX-1 mAb (40 μg/ml) and ascorbic acid (10 μM)), and were then stimulated with ox-LDL (50 μg/ml) for 60 min. (1) non-stimulated, (2) treated with ox-LDL (50 μg/ml) alone, (3) treated with ox-LDL (50 μg/ml) following anti-LOX-1 mAb (40 μg/ml) preincubation, (4) treated with ox-LDL (50 μg/ml) following ascorbic acid (10 μM) preincubation, (5) cold NF-κB probe. The positions of the specific NF-κB complexes are indicated.

In conclusion, ox-LDL binding to LOX-1 in bovine cultured chondrocytes increased production of intracellular ROS and activated NF-κB. Reduction of NF-κB activation by ascorbic acid indicates that the activation, at least in part, is ROS-dependent. These observations support the hypothesis that hypercholesterolemia is one of the risk factors of arthritis, and lipid peroxidation products such as ox-LDL are involved in cartilage matrix degradation in OA and RA.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Culture, Sports and Technology of

Japan, the Ministry of Health, Labour and Welfare of Japan. We thank Ohkubo M, and Masui A for their secretarial help in preparing and submitting the manuscript. We also thank Tokuyama T for technical assistance in the biochemical experiments.

References

1. Hart DJ, Doyle DV, Spector TD. Association between metabolic factors and knee osteoarthritis in women: the Chingford Study. J Rheumatol 1995;22:1118–23.
2. Carroll GJ, Bayliss CE. Treatment of the arthropathy of familial hypercholesterolaemia. Ann Rheum Dis 1983; 42:206–9.
3. McAlindon TE, Jacques P, Zhang Y, Hannan MT, Aliabadi P, Weissman B, *et al*. Do antioxidant micronutrients protect against the development and progression of knee osteoarthritis? Arthritis Rheum 1996; 39:648–56.
4. Lippiello L. The association of lipid abnormalities with tissue pathology in human osteoarthritic articular cartilage. Metabolism 1991;40:571–6.
5. Stockwell RA. Lipid in the matrix of ageing articular cartilage. Nature 1965;207:427–8.
6. Tiku ML, Shah R, Allison GT. Evidence linking chondrocyte lipid peroxidation to cartilage matrix protein degradation. Possible role in cartilage aging and the pathogenesis of osteoarthritis. J Biol Chem 2000;275:20069–76.
7. Tiku ML, Gupta S, Deshmukh DR. Aggrecan degradation in chondrocytes is mediated by reactive oxygen species and protected by antioxidants. Free Radic Res 1999;30:395–405.
8. Curtis CL, Hughes CE, Flannery CR, Little CB, Harwood JL, Caterson B. N-3 fatty acid specifically modulate catabolic factors involved in articular cartilage degradation. J Biol Chem 2000;275:721–4.
9. Dai L, Zhang Z, Winyard PG, Gaffney K, Jones H, Blake DR, *et al*. A modified form of low-density lipoprotein with increased electronegative charge is present in rheumatoid arthritis synovial fluid. Free Radic Biol Med 1997;22:705–10.
10. Dai L, Lamb DJ, Leake DS, Kus ML, Jones HW, Morris CJ, *et al*. Evidence for oxidised low density lipoprotein in synovial fluid from rheumatoid arthritis patients. Free Radic Res 2000;32:479–86.
11. Bostan M, Brasoveanu LI, Livescu A, Manda G, Neagu M, Iordachescu D. Effects of synovial fluid on the respiratory burst of granulocytes in rheumatoid arthritis. J Cell Mol Med 2001;5:188–94.
12. Steinberg D, Lewis A. Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. Circulation 1997;95:1062–71.
13. Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, *et al*. An endothelial receptor for oxidized low-density lipoprotein. Nature 1997;386: 73–7.
14. Nakagawa T, Akagi M, Hoshikawa H, Chen M, Yasuda T, Mukai S, *et al*. Lectin-like oxidized low-density lipoprotein receptor 1 mediates leukocyte infiltration and articular cartilage destruction in rat zymosan-induced arthritis. Arthritis Rheum 2002;46:2486–94.
15. Nakagawa T, Yasuda T, Hoshikawa H, Shimizu M, Kakinuma T, Chen M, *et al*. LOX-1 expressed in cultured rat chondrocytes mediates oxidized LDL-induced cell death—possible role of dephosphorylation of Akt. Biochem Biophys Res Commun 2002; 299:91–7.
16. Iademarco MF, McQuuillan JJ, Rosen GD, Dean DC. Characterization of the promoter for vascular cell adhesion molecule-1. J Biol Chem 1992;267:16323–9.
17. Neish AS, Williams AJ, Palmer HJ, Whitley MZ, Collins T. Functional analysis of the human vascular cell adhesion molecule-1 promoter. J Exp Med 1992; 176:1583–93.
18. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol 1983;130:1910–7.
19. LeBel CP, Ali SF, Mckee M, Bondy SC. Organometal-induced increases in oxygen reactive species: the potential of 2′, 7′-dichlorofluorescin diacetate as an index of neurotoxic damage. Toxicol Appl Pharmacol 1990;104:17–24.
20. Rosenkranz AR, Schmaldienst S, Stuhlmeier KM, Chen W, Knapp W, Zlabinger GJ. A microplate assay for the detection of oxidative products using 2′, 7′-dichlorofluorescin diacetate. J Immunol Methods 1992;156: 39–45.
21. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in mammalian cell culture. Nature 2001;411:494–8.
22. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Gene Dev 2001;15:188–200.
23. Rodgers JT, Patel P, Hennes JL, Bolognia SL, Mascotti DP. Use of biotin-labeled nucleic acids for protein purification and agarose-based chemiluminescent electromobility shift assays. Anal Biochem 2000;277: 254–9.
24. Zulueta JJ, Sawhney R, Yu FS, Cote CC, Hassoun PM. Intracellular generation of reactive oxygen species in endothelial cells exposed to anoxia-reoxygenation. Am J Physiol 1997;272:897–902.
25. Cominacini L, Garbin U, Pasini AF, Davoli A, Campagnola M, Pastorino AM, *et al*. Oxidized low density lipoprotein increases the production of intracellular reactive oxygen species in endothelial cells: inhibitory effect of lacidipine. J Hypertens 1998;16:1913–9.
26. Schmidt KN, Traenckner EB, Meier B, Baeuerle PA. Induction of oxidative stress by okadaic acid is required for activation of transcription factor NF-kappa B. J Biol Chem 1995;270:27136–42.
27. Tsao PS, Buitrago R, Chan JR, Cooke JP. Fluid flow inhibits endothelial adhesiveness. Nitric oxide and transcriptional regulation of VCAM-1. Circulation 1996;94:1682–9.
28. Maeba R, Maruyama A, Tarutani O, Ueta N, Shimasaki H. Oxidized low density lipoprotein induces the production of superoxide by neutrophils. FEBS Lett 1995;377:309–12.
29. Lander HM. An essential role for free radicals and derived species in signal transduction. FASEB J 1997;11:118–24.
30. Cathacart MK, McNally AK, Morel DW, Chisolm GM. Superoxide anion participation in human monocyte-mediated oxidation of low-density lipoprotein to a cytotoxin. J Immunol 1989;142:1963–9.
31. Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messenger

in the activation of the NF-kappa B transcription factor and HIV-1. EMBO J 1991;10:2247–58.
32. Schreck R, Meier B, Mannel DM, Droge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of NF-kappa B activation in intact cells. J Exp Med 1992;175:1181–94.
33. Weber C, Erl W, Pietsch A, Strobel M, Ziegler-Heitbrock HW, Weber PC. Antioxidants inhibit monocyte adhesion by suppressing NF-kappa B mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Arterioscler Thromb 1994;14:1665–73.
34. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999;18:6853–66.
35. Handel ML, McMorrow LB, Gravallese EM. Nuclear factor-kappa B in rheumatoid synovium. Localization of p50 and p65. Arthritis Rheum 1995;38: 1762–70.
36. Relic B, Bentires-Alj M, Ribbens C, Franchimont N, Guerne PA, Benoit V, *et al*. TNF-α protects human primary articular chondrocytes from nitric oxide-induced apoptosis via NF-kappa B. Lab Invest 2002; 82:1661–72.