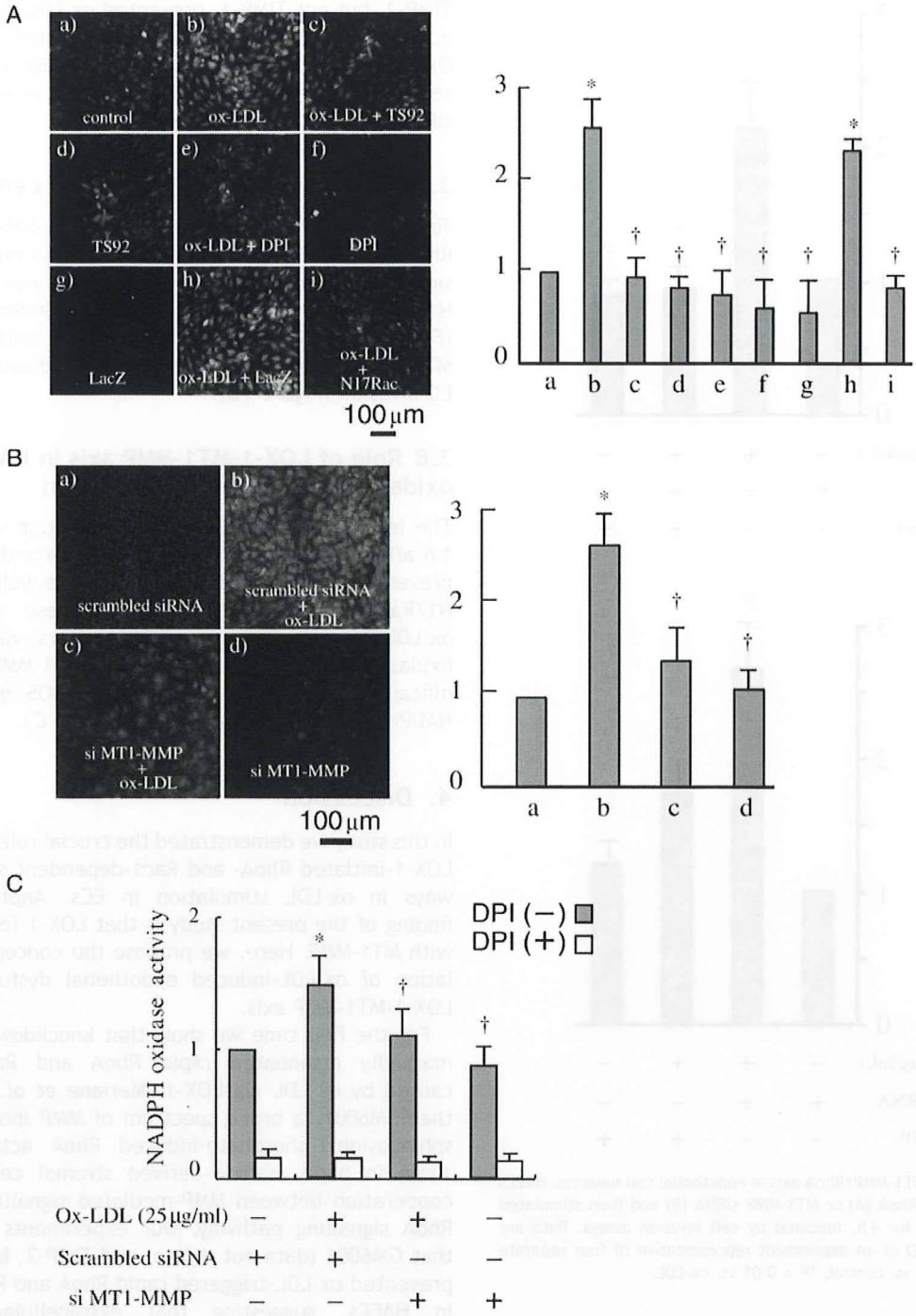

Figure 8 (*A*) HAECs were stimulated with 25 μg/mL ox-LDL for 60 min with or without pretreatment using 10 μg/mL TS92 for 1 h or 10 μmol/L DPI for 30 min. Cells were also infected with adenoviruses encoding N17Rac or *LacZ* (g, h, i). Then, intracellular ROS formation was assessed by H2DCF-DA oxidation-based fluorescence. Photomicrographs are from an experiment representative of six independent experiments. Bars represent mean ± SD of six independent experiments. (*B*, *C*) Effect of inhibition of MT1-MMP on ox-LDL-induced ROS generation and NADPH oxidase activity. (*B*) HAECs were stimulated with 25 μg/mL ox-LDL for 60 min with MT1-MMP siRNA or scrambled siRNA. Intracellular ROS formation was then assessed by H2DCF-DA oxidation-based fluorescence. Photomicrographs are from an experiment representative of six independent experiments. Bars are mean ± SD of quantitative analyses from six separate experiments. (*C*) Endothelial NADPH oxidase activity was measured with or without silencing of MT1-MMP 15 min after adding ox-LDL. Treatment of endothelial homogenates with DPI abolished the enzymatic activity of NAPDH oxidase. Data are the mean ± SD from five separate experiments. $^{*}P < 0.01$ vs. control; $^{\dagger}P < 0.01$ vs. ox-LDL.

Fluorescent immunostaining revealed that LOX-1 was colocalized with MT1-MMP to some degree in cultured HAECs. The notable finding of the present study by immunoprecipitation is that LOX-1 formed a complex with MT1-MMP in HAECs. To the best of our knowledge, the present study is the first to show the formation of a complex of LOX-1 with another molecule in ECs. It would be of importance to determine whether LOX-1 and MT1-MMP forms a complex *in vivo*. Our experiments suggested that LOX-1 also formed a complex with MT1-MMP in the aortae of Watanabe heritable hyperlipidaemic rabbits (data not shown). The issue should be extensively investigated in terms of the physiopathology

of vascular remodelling. We also found that ox-LDL did not change the levels of a complex of LOX-1 and MT1-MMP, although ox-LDL increased the protein expression of LOX-1 and MT1-MMP after 18 h of incubation (data not shown). We further attempted to determine whether MT1-MMP modulates LOX-1 in cultured HAECs. Our data indicated that silencing of MT1-MMP did not alter the uptake of DiI-labelled ox-LDL (data not shown). This would suggest that there is no change in LOX-1 function because of MT1-MMP knockdown at least inasfar as ox-LDL uptake is concerned. These findings of the present study might open new avenues in the understanding of signal transduction and physiopathology of ox-LDL-LOX-1-dependent endothelial dysfunction.

MT1-MMP reportedly plays a role in angiotensin-mediated vascular remodelling.[30] It has also been shown that MT1-MMP is involved in NO-induced endothelial migration and tube formation.[31] Various agonists related to vascular remodelling such as angiotensin II, endothelin, and thrombin regulate the cascade of Rac1/NADPH oxidase/ROS generation, which modulates nitric oxide synthesis including eNOS protein expression.[32] Here, we show that the silencing of MT1-MMP inhibited the RhoA-dependent eNOS protein downregulation and EC invasion, and Rac1-mediated NADPH oxidase activity and ROS generation. Thus, it would be of great interest to investigate the role of MT1-MMP in the agonist-triggered NADPH oxidase-dependent ROS generation and NO synthesis other than ox-LDL stimulation.

Recent studies of bone marrow transplantation of MT1-MMP$^{-/-}$ mice have demonstrated that macrophage-derived MT1-MMP plays a role in the pathogenesis of plaque stability.[33,34] Since LOX-1 is also expressed by plaque macrophages,[35] it will be of interest to investigate the involvement of the LOX-1-MT1-MMP axis in macrophages.

In summary, the present study provides new insights into the regulation by the LOX-1-MT1-MMP axis of ox-LDL-mediated RhoA and Rac1 activation and their downstream signalling pathways in ECs, and suggests that this axis may be a promising target for treating endothelial dysfunction in coronary artery disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Induction of Bovine Articular Chondrocyte Senescence With Oxidized Low-Density Lipoprotein Through Lectin-like Oxidized Low-Density Lipoprotein Receptor 1

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Objective. **Findings of recent in vivo and in vitro studies suggest that oxidized low-density lipoprotein (ox-LDL) plays a role in the degeneration of cartilage. The purpose of this study was to determine whether ox-LDL induces chondrocyte senescence through binding to lectin-like ox-LDL receptor 1 (LOX-1).**

Methods. **The effects of ox-LDL on senescence of cultured bovine articular chondrocytes (BACs) were investigated by observing senescence-associated (SA) β-galactosidase (β-gal) activity, cell proliferation activity, and telomerase activity. Telomerase activity was measured after adding LY294002 (a specific inhibitor of phosphatidylinositol 3-kinase [PI3K]) or after adding insulin-like growth factor 1 (IGF-1; an activator of PI3K) plus ox-LDL to the culture medium to elucidate the involvement of the PI3K/Akt pathway. Immunoblot analysis was used to investigate whether ox-LDL affects the phosphorylation of Akt. To ascertain whether these effects were attributable to ox-LDL binding to LOX-1, BACs were preincubated with TS-20, an anti-bovine LOX-1 blocking antibody.**

Results. **The activity of SA β-gal was increased and the incorporation of bromodeoxyuridine into BACs was decreased by ox-LDL in a dose-dependent manner. The telomerase activity of BACs was suppressed by the addition of ox-LDL in a time- and dose-dependent manner. LY294002 suppressed the telomerase activity of BACs, and IGF-1 reversed the ox-LDL–induced suppression of telomerase activity. In addition, ox-LDL rapidly decreased the amount of phosphorylated Akt in BACs. Pretreatment of cultured BACs with TS-20 recovered these effects.**

Conclusion. **These data show that ox-LDL binding to LOX-1 induces stress-induced premature senescence of chondrocytes and results in suppression of telomerase activity by inactivating the PI3K/Akt pathway. Oxidized LDL may play an important role in the pathogenesis of osteoarthritis by inducing chondrocyte senescence.**

Oxidized low-density lipoprotein (ox-LDL) was recently recognized to be one of the most important molecules that cause atherosclerosis (1). A novel receptor for ox-LDL, designated lectin-like ox-LDL receptor 1 (LOX-1), was recently cloned from cultured bovine aortic endothelial cells (2). The uptake of ox-LDL through this receptor, which is expressed on the vascular endothelium, is critically involved in endothelial activation and dysfunction in atherogenesis (3). The involvement of lipid peroxidation in the cartilage degeneration associated with both aging and the pathogenesis of osteoarthritis (OA) has been suggested by in vivo (4,5) and in vitro (6,7) studies. Some epidemiologic studies have suggested that OA and atherosclerosis share a common epidemiologic background in terms of the involvement of lipid peroxidation (8,9).

Interestingly, Nakagawa et al (10) showed the expression of LOX-1 and the presence of ox-LDL in chondrocytes from rats with zymosan-induced arthritis (ZIA); in addition, treatment with anti–LOX-1 blocking antibody was shown to suppress articular cartilage degeneration in ZIA, suggesting that ox-LDL binding to

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LOX-1 is involved in cartilage degeneration. Their in vitro study using cultured rat articular chondrocytes showed that LOX-1 expression is detectable under basal culture conditions and that ox-LDL reduces rat chondrocyte viability through LOX-1, which induces nonapoptotic cell death (11). We previously demonstrated that ox-LDL binding to LOX-1 in cultured bovine articular chondrocytes (BACs) increases the production of intracellular reactive oxygen species, resulting in the activation of NF-κB and the enhancement of monocyte chemoattractant protein 1 production, suggesting that ox-LDL exerts effects similar to those of interleukin-1β on the degradation of articular cartilage (12,13).

These observations suggest that the ox-LDL/LOX-1 system plays a role in both endothelial and chondrocyte dysfunction. More recently, we and other investigators (14,15) have reported the presence of ox-LDL and LOX-1 expression in articular cartilage from patients with rheumatoid arthritis and OA, respectively. In addition, ox-LDL was shown to penetrate the cartilage matrix and to associate with LOX-1, increasing the production of matrix metalloproteinase 3 by cultured explants of human articular cartilage (14). It was further demonstrated that the presence of ox-LDL and the expression of LOX-1 in chondrocytes correlates with grades of OA degeneration in cartilage (15). Simopoulou et al (16) also showed that ox-LDL is detectable in synovial fluid from OA joints and that LOX-1 messenger RNA (mRNA) and protein are expressed in chondrocytes derived from OA cartilage. This growing evidence suggests that the binding of ox-LDL to LOX-1 may modulate the cartilage degeneration seen in OA patients.

Epidemiologic studies have shown that age is the chief risk factor for atherosclerotic diseases (17) as well as for OA (18,19). Both the endothelial cells in atherosclerotic lesions (20,21) and the chondrocytes in OA cartilage (22,23) show attributes of cell senescence, and cell senescence and aging of the tissue are strongly correlated in both diseases. The purpose of the present study was to investigate whether ox-LDL induces cell senescence of cultured BACs. We first investigated whether ox-LDL affects senescence-associated (SA) β-galactosidase (β-gal) activity and cell proliferative ability. Next, we investigated the effects of ox-LDL on the telomerase activity of cultured BACs and on the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which may be involved in the regulation of the telomerase activity of chondrocytes. We also used a blocking antibody against LOX-1 to investigate whether ox-LDL–induced cell senescence occurs through the binding to LOX-1.

MATERIALS AND METHODS

Reagents. Nonspecific mouse IgG was purchased from Equitech-Bio (Kerrville, TX). LY294002 and recombinant human insulin-like growth factor (IGF-1) were purchased from Sigma (St. Louis, MO). Anti-Akt rabbit IgG, anti–phosphorylated-Akt (anti-pAkt; Ser^{473}) rabbit IgG, and anti-rabbit IgG horseradish peroxidase–linked antibodies were purchased from Cell Signaling Technology (Beverly, MA). The methods we used to prepare the native LDL and ox-LDL have been described previously (2,11).

Primary culture of BACs. Chondrocytes were isolated from articular cartilage of 10-month-old cows by enzymatic digestion with 2 mg/ml of collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 12 hours at 37°C. After filtration, cells were seeded in culture plates and cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 200 units/ml of penicillin, 40 μg/ml of streptomycin, and 10% fetal bovine serum. Cells were cultured at 37°C in a humidified and hypoxic atmosphere (5% O_2 and CO_2) to avoid acceleration of cell senescence as a result of oxidative stress (24,25).

RNA extraction and real-time polymerase chain reaction (PCR) analysis. Cells were seeded at a density of 1×10^5 cells/well in 24-well plates and allowed to grow to 70% confluence and then to 100% confluence. All experiments were performed in triplicate for each lot, and 2 different lots were used for this analysis. Cell pellets were resuspended in 350 μl of RNeasy (RLT) lysis buffer from an RNeasy kit and homogenized through QIAshredder columns (all from Qiagen Valencia, CA). RNA was extracted using RNeasy columns according to the manufacturer's instructions. Extracted RNA was eluted in 30 μl of RNase-free water. Single-strand complementary DNA (cDNA) was prepared from total RNA using a random primer under standard conditions and with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The cDNA from each sample was diluted and used to quantify the expression of type II collagen, aggrecan, and β-actin.

Quantitative real-time PCR with total cDNA was performed using Perfect Real-Time SYBR Green II (Takara Bio, Shiga, Japan) and the following primer sets: for β-actin, 5′-GGTCATCACCATTGGCAATG-3′ (forward) and 5′-CCACAGGACTCCATGCCC-3′ (reverse); for type II collagen, 5′-TGGTATCGCCGGACCCAAG-3′ (forward) and 5′-CTCGTCCACCGTCCTTCCC-3′ (reverse); and for aggrecan, 5′-CACCTGTAAAAAGGGCACAGTG-3′ (forward) and 5′-GCATTGATCTCGTATCGGTCC-3′ (reverse). The primer sets were designed to span exons in order to distinguish cDNA from genomic DNA products. PCR amplifications were performed using the 7700 real-time PCR system (Applied Biosystems) under the following conditions: 95°C for 10 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. To quantify the relative expression of each gene, the C_t values were normalized against the endogenous reference (ΔC_t = C_t target − C_t β-actin) and were compared with a calibrator, using the $\Delta\Delta C_t$ method ($\Delta\Delta C_t$ = ΔC_t sample − ΔC_t

calibrator). As a calibrator, we used the average C_t value for untreated chondrocytes. All experiments included negative controls, which consisted of no cDNA for each primer pair.

SA β-gal activity assay. SA β-gal activity was detected by cytochemical staining of chondrocytes using a senescence cells histochemical staining kit (Sigma) according to the manufacturer's instructions. Cells were seeded at 1×10^6/well in a 35-mm plate and allowed to grow to 70% confluence. Chondrocytes were stimulated for 24 hours with the indicated concentrations of ox-LDL or with 100 μg/ml of native LDL. Some plates were preincubated for 30 minutes with 40 μg/ml of TS-20 (an anti–LOX-1 blocking antibody) or with 40 μg/ml of nonspecific mouse IgG and then stimulated for 24 hours with 100 μg/ml of ox-LDL. After stimulation, cells were washed with ice-cold phosphate buffered saline (PBS) and incubated with fixation buffer (1.5 ml/well) for 7 minutes at room temperature. After the fixation process, cells were washed 3 times with PBS and incubated for 12 hours at 37°C with staining mixture (1 mg/well). Blue-stained cells were considered SA β-gal–positive cells.

Assay for the incorporation of 5′-bromo-2′-deoxyuridine (BrdU). The proliferative ability of chondrocytes was evaluated by measuring the incorporation of BrdU into newly synthesized DNA. The incorporation of BrdU in cultured BACs at 70% and 100% confluence and in precultured chondrocytes assessed immediately after isolation were examined using BrdU Labeling and Detection Kit I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Cells were incubated for 60 minutes at 37°C with 5 ml/well of BrdU-linked medium. The BrdU-linked medium was removed and the cells were washed and fixed for 1 hour at −20°C of 70% ethanol (1 ml/well). After fixation, cells were incubated for 30 minutes at 37°C with 3 ml/well of anti-BrdU reaction mixture and then incubated for 30 minutes with fluorescein isothiocyanate (FITC)–linked anti-mouse immunoglobulin reaction mixture (3 ml/well).

Cells were washed and examined with a confocal laser microscope (LCM5 Pascal Laser Scanning Microscope; Carl Zeiss Instruments, Oberkochen, Germany). The incorporation of BrdU into BACs cultured with various agents was quantified using a cell proliferation enzyme-linked immunosorbent assay (ELISA; Roche Diagnostics) according to the manufacturer's instructions. Chondrocytes were seeded in a Falcon 96-multiwell plate at a density of 2×10^4/well in 100 μl of culture medium. After incubation, 10 μl of BrdU solution was added to each well, and the plate was incubated for 2 hours at 37°C. After incubation, the culture medium was removed, the cells were fixed, and DNA was denatured by the addition of 200 μl/well of FixDenat for 1 hour. The reagent with peroxidase-labeled anti-BrdU antibody was added to the cells, and the plate was incubated for 90 minutes. At the end of the reaction time, the substrate was added for 5 minutes, and the absorbance at 370 nm and 492 nm (control) was measured with a microplate reader (Model 680; Bio-Rad, Hercules, CA).

Telomerase activity assay. Telomerase activity per 2×10^4 cells was measured by a stretch PCR method using TeloChaser (Toyobo, Osaka, Japan), according to the manufacturer's instructions. The cells were washed with PBS, and the cell pellet was resuspended in the lysis solution that came with the kit and then centrifuged at 15,000 revolutions per minute for 20 minutes. The supernatant was subjected to telomerase reactions at 37°C for 30 minutes. The products were denatured at 95°C for 2 minutes 30 seconds, amplified by PCR (33 cycles at 95°C for 30 seconds, 68°C for 30 seconds, and 72°C for 45 seconds) using Platinum *Taq* polymerase (Invitrogen, Auckland, New Zealand), subjected to electrophoresis on a 10% polyacrylamide gel in Tris−borate−EDTA buffer for 60 minutes at 100V, stained with 0.5 μg/ml of ethidium bromide for 15 minutes, and visualized with a blot detection system (Printgraph AE-6932; Atto, Tokyo, Japan). The signal intensities were quantified using image analysis software (ImageJ version 1.37; NIH Image, National Institutes of Health, Bethesda, MD; online at: http://rsbweb.nih.gov/ij/).

Western blot analysis. Chondrocytes were washed with ice-cold PBS, lysed in lysis buffer (sample buffer solution, Wako Pure Chemical Industries), and boiled for 5 minutes. The solution obtained was subjected to immunoblotting. Cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the proteins were transferred onto a polyvinylidene fluoride membrane. After blocking, the membrane was incubated overnight at 4°C with the primary antibody, incubated with the secondary antibody for 1 hour, and then visualized with an ECL Plus Western blot detection system (Amersham Biosciences, Little Chalfont, UK). The signal intensities were measured using NIH ImageJ software, version 1.37, as above.

Statistical analysis. Results are presented as the mean ± SD. Analyses of variance, Scheffe's tests, and Student's unpaired *t*-tests were used for statistical assessments. *P* values less than 0.05 were considered statistically significant.

RESULTS

Expression of matrix mRNA in cultured BACs. First, we investigated the expression of matrix genes in cultured BACs using real-time PCR analysis. Real-time PCR showed that the primary cultured BACs constitutively expressed mRNA for aggrecan and type II collagen, indicating that the cells did not dedifferentiate prior to stimulation by the agents. The expression of aggrecan and type II collagen mRNA in 70% confluent BACs was 7.6 ± 1.7–fold higher (mean ± SD) and 8.5 ± 1.8–fold higher, respectively, than in precultured chondrocytes that were assessed immediately after isolation. The expression of aggrecan and type II collagen mRNA was significantly higher in 70% confluent BACs than in 100% confluent BACs (data not shown).

Effects of ox-LDL on SA β-gal activity. To assess the effects of ox-LDL on chondrocyte senescence, SA β-gal cytochemical staining of cultured BACs was performed after 24 hours' incubation with ox-LDL (0, 50, or 100 μg/ml) or native LDL (100 μg/ml). Treatment with ox-LDL increased the ratio of SA β-gal–positive (blue-stained) cells and the intensity of the staining in a dose-dependent manner (Figures 1A–C), whereas native LDL did not increase the staining intensity (Figure 1D).

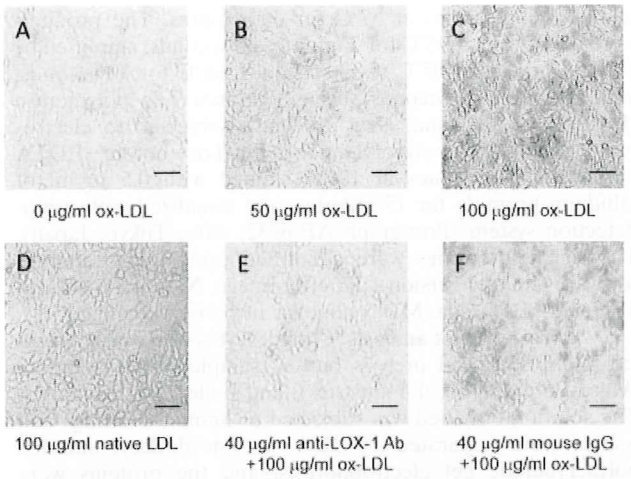

Figure 1. Effects of oxidized low-density lipoprotein (ox-LDL) on senescence-associated (SA) β-galactosidase (β-gal) activity in cultured bovine articular chondrocytes (BACs). SA β-gal activity in cultured BACs was investigated by cytochemical staining. Chondrocytes were incubated for 24 hours with 0 μg/ml of ox-LDL (**A**), 50 μg/ml of ox-LDL (**B**), 100 μg/ml of ox-LDL (**C**), or 100 μg/ml of native LDL (**D**). Some plates were pretreated for 30 minutes with 40 μg/ml of TS-20, an anti–lectin-like ox-LDL receptor 1 (anti–LOX-1) antibody (Ab) (**E**), or with 40 μg/ml of nonspecific mouse IgG (**F**) and then incubated with 100 μg/ml of ox-LDL. SA β-gal–positive cells are stained blue. Bars = 20 μm.

To examine the receptor specificity of the ox-LDL, we pretreated the BACs for 30 minutes with TS-20 (40 μg/ml), an anti–LOX-1 blocking antibody, before ox-LDL stimulation. The ox-LDL–induced increase in SA β-gal staining was significantly attenuated by pretreatment with TS-20 (Figure 1E) but not by pretreatment with 40 μg/ml of nonspecific mouse IgG (Figure 1F).

Effects of ox-LDL on the ability of cultured BACs to proliferate. The effects of ox-LDL on the proliferative ability of cultured BACs were investigated by measuring BrdU incorporation, as observed by fluorescence staining and as quantified by ELISA. Fluorescence images revealed that the preincubated chondrocytes tested immediately after isolation were BrdU negative and that more BrdU-positive BACs were observed in the 70% confluent BACs than in the 100% confluent BACs (data not shown). ELISA demonstrated that the mean BrdU incorporation was 6.3-fold higher in the 70% confluent BACs than in the preincubated chondrocytes. BrdU incorporation was significantly higher in the 70% confluent BACs than in the 100% confluent BACs (data not shown).

To examine whether ox-LDL affects the ability of cultured BACs at 70% confluence to proliferate, chondrocytes were incubated for 24 hours with ox-LDL or native LDL at various concentrations (0, 10, 50, or 100 μg/ml), and BrdU incorporation was quantified by ELISA. Chondrocytes preincubated with 40 μg/ml of TS-20 (anti–LOX-1 blocking antibody) or with 40 μg/ml of nonspecific mouse IgG for 30 minutes were also stimulated with the indicated doses of ox-LDL. Addition of ox-LDL suppressed BrdU incorporation into cultured BACs in a dose-dependent manner (Figure 2A), but native LDL did not (Figure 2B). Pretreatment with TS-20 recovered the ox-LDL–induced suppression of

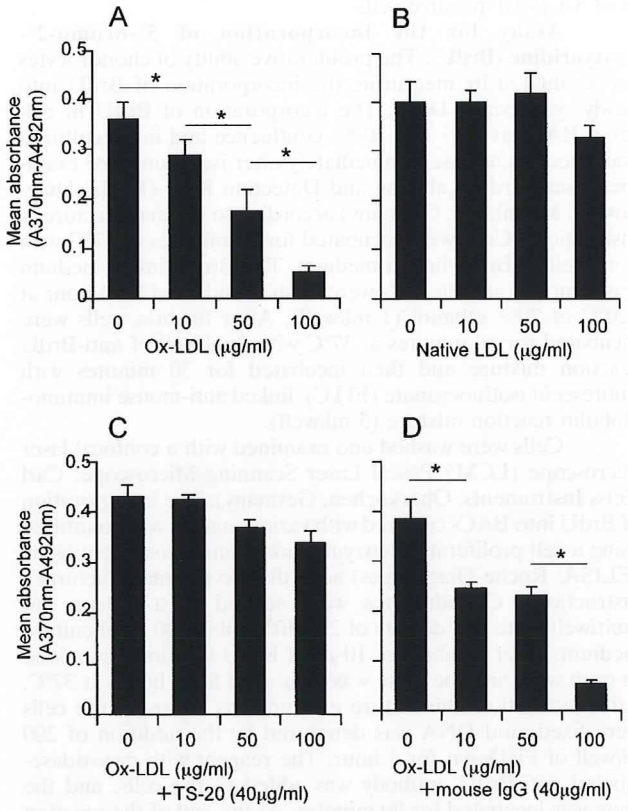

Figure 2. Effects of ox-LDL on the proliferative ability of cultured 70% confluent BACs. The proliferative ability of cultured BACs in 70% confluent cultures was quantified by bromodeoxyuridine incorporation using an enzyme-linked immunosorbent assay. Chondrocytes were incubated for 24 hours with the indicated concentrations of ox-LDL (**A**) or native LDL (**B**), or chondrocytes were pretreated for 30 minutes with 40 μg/ml of TS-20 (**C**) or with 40 μg/ml of nonspecific mouse IgG (**D**) and were then stimulated with the indicated concentrations of ox-LDL. Values are the mean and SD of 4 experiments per condition. * = $P < 0.01$. See Figure 1 for definitions.

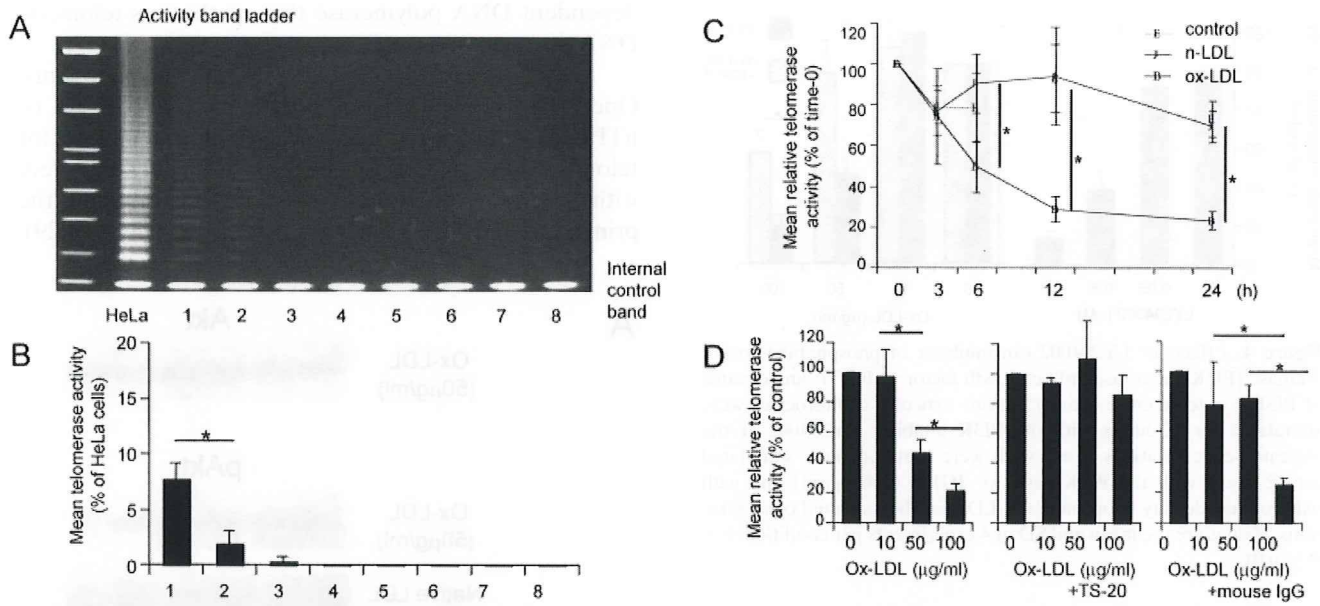

Figure 3. Telomerase activity in cultured BACs and time and dose effects of ox-LDL on telomerase activity. **A,** Telomerase activity was measured by a stretch polymerase chain reaction (PCR) method in positive control cells (HeLa), cultured BACs at 70% confluence (lane 1), cultured BACs at 100% confluence (lane 2), preincubated BACs (lane 3), heat-treated HeLa cells (lane 4), heat-treated BACs at 70% confluence (lane 5), heat-treated BACs at 100% confluence (lane 6), heat-treated, preincubated BACs (lane 7), and negative control (lane 8). Heat-treated samples were incubated at 85°C for 10 minutes. **B,** The relative telomerase activity was quantified with image analysis software, and the results for lanes 1–8 in **A** are shown. Compared with HeLa cells, the mean telomerase activity was 7.8% in 70% confluent BACs and 1.9% in 100% confluent BACs. **C,** BACs were incubated with culture medium (control), 50 μg/ml of native-LDL (n-LDL), or 50 μg/ml of ox-LDL, and telomerase activity was measured at the indicated times using a stretch PCR method and image analysis software. Thick vertical bars indicate the comparison groups with significant differences. **D,** BACs were incubated for 12 hours with the indicated concentrations of ox-LDL. BACs preincubated for 30 minutes with 40 μg/ml of anti–LOX-1 antibody (TS-20) or 40 μg/ml of nonspecific mouse IgG were also stimulated with various doses of ox-LDL. Values in **B–D** are the mean and SD of 4 experiments per condition. * = $P < 0.01$. See Figure 1 for other definitions.

BrdU incorporation (Figure 2C), but pretreatment with nonspecific mouse IgG did not (Figure 2D).

Telomerase activity of cultured BACs and effects of ox-LDL. Telomerase activity was lower in cultured BACs at 70% and 100% confluence than in HeLa cells but was detectable using a stretch PCR method (Figure 3A); relative to the activity in HeLa cells, the mean ± SD values were 7.8 ± 1.4% for 70% confluent cells and 1.9 ± 1.2% for 100% confluent cells (n = 4) (Figure 3B). Activity was barely detectable in the precultured chondrocytes (0.3 ± 0.6% of the values in the HeLa cells; n = 4) (Figure 3B).

We next investigated the effects of ox-LDL on the telomerase activity of cultured BACs at 70% confluence. BACs were incubated with 50 μg/ml of native LDL or ox-LDL for various times (0, 6, 12, or 24 hours). Incubation with ox-LDL, but not native LDL, suppressed telomerase activity in a time-dependent manner (Figure 3C). We further investigated the dose effects of ox-LDL on the telomerase activity of BACs. Chondrocytes preincubated for 30 minutes with 40 μg/ml of anti–LOX-1 blocking antibody (TS-20) or nonspecific mouse IgG were also stimulated with the indicated doses of ox-LDL. Telomerase activity was suppressed by ox-LDL in a dose-dependent manner. Pretreatment with TS-20 significantly reversed this suppression, but nonspecific mouse IgG had no effect (Figure 3D).

Effects of ox-LDL on the PI3K/Akt pathway. To clarify the mechanism of action of ox-LDL in the suppression of telomerase activity, we investigated the relationship between the PI3K/Akt pathway and the telomerase activity of BACs. BACs were stimulated for 12 hours with various concentrations of the PI3K inhibitor LY294002 (0, 0.05, 0.5, or 1 n*M*). Some wells were stimulated simultaneously with 100 ng/ml of IGF-1 (an activator of PI3K) and the indicated doses of ox-LDL for 12 hours. Incubation with LY294002 but without ox-LDL suppressed the telomerase activity of BACs in a

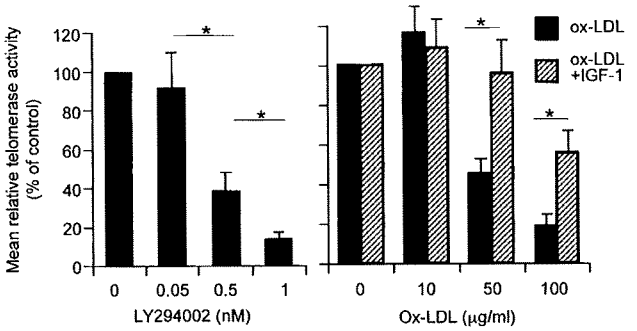

Figure 4. Effects of LY294002 (an inhibitor of phosphatidylinositol 3-kinase [PI3K]) and insulin-like growth factor 1 (IGF-1; an activator of PI3K) on telomerase activity. Bovine articular chondrocytes were stimulated for 12 hours with the PI3K inhibitor LY294002 at the indicated concentrations. Some wells were simultaneously stimulated for 12 hours with the PI3K activator IGF-1 (100 ng/ml) and with oxidized low-density lipoprotein (ox-LDL) at the indicated concentrations. Values are the mean and SD of 4 experiments per condition. * = $P < 0.01$.

dose-dependent manner. Addition of IGF-1 resulted in recovery of the suppressed telomerase activity that had been induced by ox-LDL (Figure 4).

Next, we used Western blot analysis to investigate whether ox-LDL affects the PI3K/Akt pathway in cultured BACs. Oxidized LDL at a concentration of 50 μg/ml rapidly decreased the amount of pAkt but had no effect on the amount of Akt. Native LDL did not affect the amount of pAkt. Pretreatment with 40 μg/ml of anti–LOX-1 blocking antibody (TS-20) significantly reversed the ox-LDL–induced decrease in pAkt levels, but nonspecific mouse IgG did not (Figure 5A). Treatment with LY294002 (0.5 n*M*) rapidly decreased the amount of pAkt in BACs, and 100 ng/ml of IGF-1 reversed the ox-LDL–induced decrease in pAkt levels (Figure 5B).

DISCUSSION

The telomere hypothesis is generally accepted as the explanation for the mechanism that underlies cell senescence (26,27). Structures of telomeres, the terminal, guanine-rich sequences of chromosomes (TTAGGG repeats in humans and other vertebrates), work to stabilize the chromosome during replication by protecting the chromosome end against exonucleases. Telomere length decreases with replication, and when this has decreased to a critical length, the cell is signaled to stop dividing and to enter cellular senescence (replicative senescence) (26,27). Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA sequences.

Telomerase consists of 2 essential components. One is the functional RNA component (called hTR, or hTERC, in humans), which serves as a template for telomeric DNA synthesis. The other is a catalytic protein with reverse transcriptase activity (hTERT) and the primary determinant for the enzyme activity (28,29).

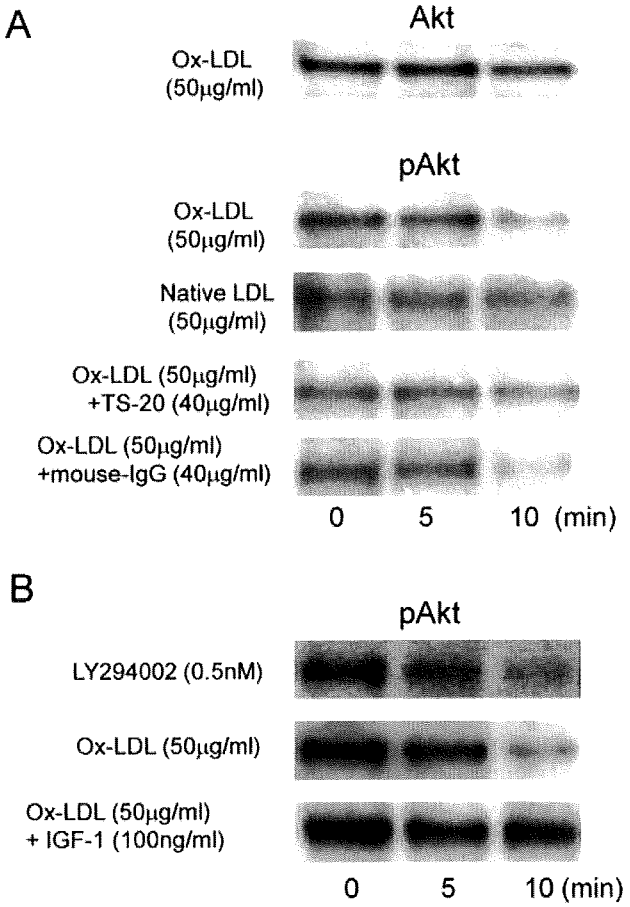

Figure 5. Effects of oxidized low-density lipoprotein (ox-LDL), LY294002 (an inhibitor of phosphatidylinositol 3-kinase [PI3K]), and insulin-like growth factor 1 (IGF-1; an activator of PI3K) on the amounts of pAkt in bovine articular chondrocytes (BACs). **A,** The amounts of Akt and pAkt were determined by Western blotting at the indicated times after stimulation with 50 μg/ml of ox-LDL or native LDL. BACs pretreated with 40 μg/ml of TS-20 (an anti–lectin-like ox-LDL receptor 1 antibody) or nonspecific mouse-IgG were also stimulated with 50 μg/ml of ox-LDL. **B,** The amount of pAkt was determined by Western blotting at the indicated times after stimulation with 0.5 n*M* LY294002 or 50 μg/ml of ox-LDL. Some wells were also stimulated simultaneously with 100 ng/ml of IGF-1 and 50 μg/ml of ox-LDL. All experiments were performed 4 times, and the results were similar.

Although hTERT is generally repressed in normal somatic cells, telomerase activation in human vascular smooth muscle cells protects telomere shortening with replication (30). Because vascular cell senescence occurs in human atherosclerotic lesions and is associated with telomere shortening, telomerase activity seems to be important in guarding against cell senescence (21). Telomere shortening has been demonstrated in OA chondrocytes (23,31,32), and the lifespan of senescent chondrocytes retrieved from OA cartilage can be increased by the exogenous expression of telomerase (33), indicating an important relationship between chondrocyte senescence and telomerase activity.

In addition to replicative senescence, stress-induced premature cell senescence (SIPS) also occurs; in SIPS, cells without discernible attrition of telomeres show a growth arrest (34,35). Some stressors identified include DNA damage, oxidative stress, suboptimal culture conditions, and inhibition of PI3K. Proatherogenic and proinflammatory factors, such as ox-LDL, tumor necrosis factor α, and hydrogen peroxide, have been implicated in SIPS (34,35), and these can suppress telomerase activity by inactivating the PI3K/Akt pathway (36). Both types of senescence are associated with suppressed cell proliferation and impaired physiologic cell function. It is likely that both types of cell senescence, the telomere shortening–initiated and the stress-induced, may contribute jointly to the pathogenic process of chronic diseases in vivo (34).

Clusters or clones of proliferating chondrocytes surrounded by newly synthesized matrix molecules constitute one of the histologic hallmarks of the chondrocyte response in the early phase of OA (37–39). Anabolic growth factors previously trapped in the matrix may be released in a process of matrix degradation, which activates chondrocytes to proliferate and synthesize matrix macromolecules (40). These factors in the synovial fluid may have better access to chondrocytes because of fissuring or loosening of the collagen network or because of damage to the collagen matrix itself (37). These phenomena are thought to represent repair responses of damaged cartilage. The progressive degeneration of cartilage in the later phase of OA may be attributed to limited repair responses caused by cell senescence associated with reduced cell function and proliferative ability (22,40). Chondrocytes in OA cartilage show distinctive features of the senescent cell, including higher activity of SA β-gal, reduced matrix production and proliferative abilities, and telomere shortening, indicating a strong relationship between chondrocyte senescence and cartilage degeneration (22,23,32).

In the present study, we first investigated the properties of the cultured BACs. Real-time PCR results revealed greater expression of aggrecan and type II collagen mRNA in proliferating 70% confluent chondrocytes than in 100% confluent chondrocytes, which had stopped proliferating because of contact inhibition, and in preincubated chondrocytes assessed immediately after isolation. The proliferative ability, as evaluated by BrdU uptake, was also greater in the 70% confluent chondrocytes than in the 100% confluent chondrocytes and the chondrocytes assessed immediately after isolation. These data suggest that the 70% confluent chondrocytes stimulated by fetal bovine serum have a phenotype similar to that of activated and proliferating chondrocytes in the early phase of OA.

We next investigated whether ox-LDL induces cell senescence of cultured BACs. SA β-gal activity has been recognized to be an important biologic marker of cell senescence (41), and it is higher in cloned chondrocytes in OA cartilage (23,32). Oxidized LDL increased the number of SA β-gal–positive BACs in a dose-dependent manner, but native LDL had no effect. Oxidized LDL reduced the cell proliferative ability, as evaluated by BrdU incorporation, in a dose-dependent manner. Pretreatment with anti–LOX-1 blocking antibody (TS-20) cancelled these effects of ox-LDL on BACs. Similar results have been reported in studies of endothelial progenitor cells (42). The induction of cell senescence caused by ox-LDL occurred within 24 hours and did not need subculturing, indicating that ox-LDL can induce SIPS in chondrocytes.

The "telomere hypothesis" is generally accepted as the explanation for replicative cell senescence. Telomerase is activated in proliferating cells of tissues under repair, which prolongs the cellular replicative capacity and postpones cell senescence (43–45). The regulation of telomerase activity is thought to play an important role in tissue repair and regeneration. We first investigated whether the telomerase activity of BACs changes with the culture conditions that induced distinct proliferating activity in chondrocytes. Compared with the telomerase activity of HeLa cells, the precultured chondrocytes assessed immediately after isolation showed little telomerase activity, 100% confluent chondrocytes showed 2% activity, and 70% confluent chondrocytes showed 8% activity. These results indicate that cultured chondrocytes with a higher proliferating activity have a higher telomerase activity, although the activity in all chondrocytes was lower than in HeLa cells. This

suggests that the telomerase activity in BACs is up-regulated during cell expansion. This result is consistent with the findings of previous reports on the telomerase activity of cultured chondrocytes (46,47) and proliferating somatic cells (48,49). Taken together, these data indicate that the telomerase activity in proliferating and cloning chondrocytes in the early phase of OA is up-regulated and plays an important role in tissue repair by postponing cell senescence and maintaining cell function.

We next investigated the effects of ox-LDL on the telomerase activity of the 70% confluent BACs. Telomerase activity was suppressed in a time-dependent manner to ~20% of the preincubation level after 24 hours of culture and in a dose-dependent manner to 40% of the control level 12 hours after the addition of 50 μg/ml of ox-LDL. This suppressive effect on the telomerase activity was reversed by pretreatment with the LOX-1 blocking antibody TS-20, indicating that ox-LDL suppresses telomerase activity through its receptor LOX-1. Oxidized LDL probably impairs the tissue repair of degenerative cartilage in the early phase of OA, since suppression of telomerase activity in proliferating cells results in telomere shortening and instability, leading to cell senescence. The telomere length of chondrocytes is relatively short in OA cartilage, and decreasing telomere length correlates strongly with increasing expression of SA β-gal and decreasing mitotic activity (23,32).

We also investigated the intracellular signaling pathway by which ox-LDL alters telomerase activity. Telomerase activity is regulated by phosphorylation of the reverse transcriptase (hTERT), and protein kinase C or protein kinase B (Akt) plays a critical role in the phosphorylation of hTERT (50). In general, the PI3K/Akt pathway plays important roles in the progress of the cell cycle, cell proliferation, regulation of nuclear transcription factors, cell survival (51,52), and chondrocyte differentiation and apoptosis (53). Activation of this pathway increases the production of aggrecan (54), and inactivation of this pathway suppresses cell viability in articular chondrocytes (11). Interestingly, Breitschopf et al (36) reported that ox-LDL suppresses telomerase activity by inactivating the PI3K/Akt pathway in endothelial cells.

We found that ox-LDL and LY294002 (a specific inhibitor of PI3K) suppressed the telomerase activity in a dose-dependent manner and that IGF-1 (an activator of PI3K) recovered the ox-LDL–induced suppression of telomerase activity. In addition, ox-LDL reduced the amount of pAkt without changing the amount of Akt,

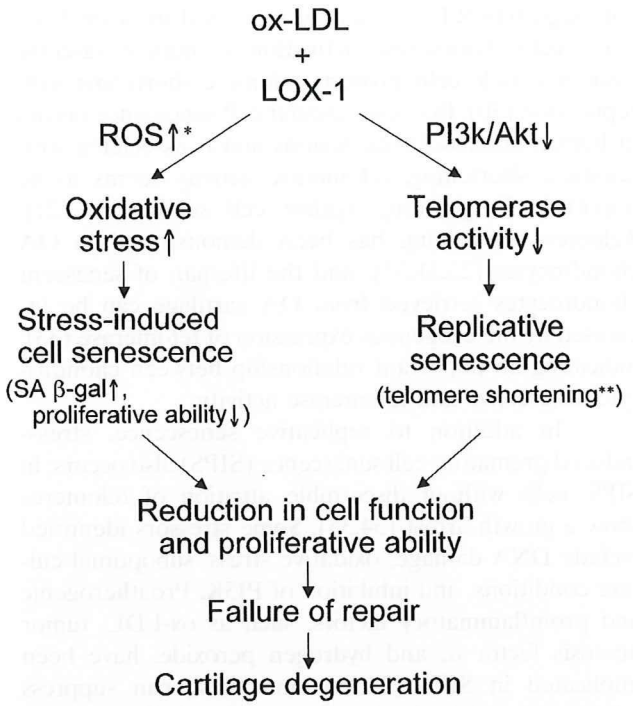

Figure 6. Flowchart summarizing the results of the present study and the assumptive relevance of the oxidized low-density lipoprotein (ox-LDL)/lectin-like ox-LDL receptor 1 (LOX-1) system to the pathogenesis of osteoarthritis. Production of reactive oxygen species (ROS) caused by ox-LDL binding to LOX-1 (*) was reported in our previous study (see ref. 12). Telomere shortening due to ox-LDL (**) is under investigation at our institution. PI3K = phosphatidylinositol 3-kinase; SA = senescence-associated; β-gal = β-galactosidase.

and pretreatment with the anti–LOX-1 blocking antibody recovered the ox-LDL–induced reduction in pAkt levels. Taken together, these results suggest that ox-LDL–induced suppression of telomerase activity can be attributed to inactivation of the PI3K/Akt pathway through binding to LOX-1 (Figure 6).

Our study has some important limitations because it was based on an in vitro model using cultured BACs. In this model, the activity of telomerase was only 8% of the HeLa activity, and then, we examined the effects of ox-LDL on this low telomerase activity. Furthermore, the telomerase activity in elderly people with OA may be even lower than the activity described in this study. The results of this study should be interpreted with caution. It is also unclear whether the ox-LDL–induced reduction in this low telomerase activity has any pathologic relevance in OA. However, considering the fact that chondrocytes proliferate in the early stage of OA and the fact that the telomere length of

chondrocytes is actually shortened in OA chondrocytes, telomerase activity seems to play some role in the maintenance of cell function and in the facilitation of tissue repair. We believe that long-lasting and chronic suppression of the telomerase activity in chondrocytes, even if it is initially at a low level, could result in an accumulated failure of tissue repair and a slow progression of cartilage degeneration.

An interesting question is whether proliferation of chondrocytes in the ox-LDL–added medium causes telomere shortening by suppressing telomerase activity, since previous studies have shown a correlation between telomere shortening and cell senescence (23,32). Another interesting question is whether ox-LDL activates the pathways that are linked mechanically to replicative senescence and SIPS, including the ataxia–telangiectasia mutated (ATM)/p53/p21/retinoblastoma (Rb) pathway and the p38/MAPK/p16/Rb pathway (34,35), respectively. We are especially interested in whether adding ox-LDL stabilizes p53, because a recent study showed that p53 destabilizes and permeabilizes lysosomes to shift β-gal from the lysosomes to the cytosol, which is recognized as cytosolic staining of SA β-gal (55).

In conclusion, our data show that ox-LDL binding to LOX-1 induces SIPS in chondrocytes and results in the suppression of telomerase activity through inactivation of the PI3K/Akt pathway. Oxidized LDL may play an important role in the pathogenesis of OA by inducing chondrocyte senescence.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Zushi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Zushi, Akagi, Sawamura.
Acquisition of data. Zushi, Akagi, Kishimoto, Teramura.
Analysis and interpretation of data. Zushi, Akagi, Hamanishi.

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Antiplatelet Therapy and Stent Thrombosis After Sirolimus-Eluting Stent Implantation

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Background—The influences of antiplatelet therapy discontinuation on the risk of stent thrombosis and long-term clinical outcomes after drug-eluting stent implantation have not yet been addressed adequately.

Methods and Results—In an observational study in Japan, 2-year outcomes were assessed in 10 778 patients undergoing sirolimus-eluting stent implantation. Data on status of antiplatelet therapy during follow-up were collected prospectively. Incidences of definite stent thrombosis were 0.34% at 30 days, 0.54% at 1 year, and 0.77% at 2 years. Thienopyridine use was maintained in 97%, 62%, and 50% of patients at 30 days, 1 year, and 2 years, respectively. Patients who discontinued both thienopyridine and aspirin had a significantly higher rate of stent thrombosis than those who continued both in the intervals of 31 to 180 days, 181 to 365 days, and 366 to 548 days after stent implantation (1.76% versus 0.1%, $P<0.001$; 0.72% versus 0.07%, $P=0.02$; and 2.1% versus 0.14%, $P=0.004$, respectively). When discontinuation of aspirin was taken into account, patients who discontinued thienopyridine only did not have an excess of stent thrombosis in any of the time intervals studied. Adjusted rates of death or myocardial infarction at 24 months were 4.1% for patients taking thienopyridine and 4.1% for patients not taking thienopyridine ($P=0.99$) in the 6-month landmark analysis.

Conclusions—Discontinuation of both thienopyridine and aspirin, but not discontinuation of thienopyridine therapy only, was associated with an increased risk of stent thrombosis. Landmark analysis did not suggest an apparent clinical benefit of thienopyridine use beyond 6 months after sirolimus-eluting stent implantation. (***Circulation.* 2009;119:987-995.**)

Key Words: aspirin ■ follow-up studies ■ stents ■ coronary disease ■ thrombosis

Concerns have been raised about the safety of drug-eluting stents (DES), and certain issues remain unresolved.[1,2] First, although premature discontinuation of antiplatelet therapy is reported to be the most powerful predictor of stent thrombosis (ST) and adverse cardiovascular outcomes,[3–5] the relative contribution of discontinuation of either aspirin or thienopyridine on ST rates has not been addressed adequately. Furthermore, the optimal duration of dual-antiplatelet therapy has not been well established, although dual-antiplatelet therapy beyond 1 year has become commonplace in clinical practice. To address these issues, a large-scale, multicenter registry of patients undergoing sirolimus-eluting stent (SES) implantation was designed with prospective data collection on the status of antiplatelet therapy during follow-up.

Clinical Perspective p 995

Methods

Study Population

The j-Cypher registry is a physician-initiated prospective, multicenter observational study in Japan enrolling consecutive patients undergoing SES implantation. The relevant review boards in all 37

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Table 1. Baseline Characteristics

Patient Characteristics	
No. of patients	10 778
Age, y	68.3±10.2
Age ≥80 y, n (%)	1362 (13)
Male, n (%)	8123 (75)
Body mass index	23.9±3.4
Hypertension, n (%)	8069 (75)
Diabetes mellitus, n (%)	4400 (41)
Taking insulin	996 (9.2)
Current smoking, n (%)	2119 (20)
eGFR <30 mL · min^{-1} · 1.73 m^{-2}, n (%)	
Without hemodialysis	522 (5.1)
With hemodialysis	594 (5.5)
ACS, n (%)	2308 (21)
STEMI	733 (6.8)
Non-STEMI	220 (2.0)
Unstable angina	1355 (13)
Prior MI, n (%)	3024 (28)
Prior stroke, n (%)	1007 (9.3)
Peripheral vascular disease, n (%)	1276 (12)
Prior heart failure, n (%)	1460 (14)
Prior PCI, n (%)	5179 (48)
Prior CABG, n (%)	787 (7.3)
Multivessel disease, n (%)	5392 (50)
Target of unprotected LMCA, n (%)	419 (3.9)
Ejection fraction, %	58.1±13.4
No. of vessels treated	1.22±0.47
Multivessel stenting, n (%)	2089 (19)
No. of lesions treated	1.37±0.66
Total No. of stents	1.75±1.04
Total length of stents, mm	38.9±25.6
Lesion and procedural characteristics	
No. of lesions	14 811
Lesion location, n (%)	
LAD	6138 (42)
LCx	3130 (21)
RCA	4913 (33)
LMCA	499 (3.4)
Saphenous vein graft	109 (0.7)
In-stent restenosis, n (%)	1895 (13)
Chronic total occlusion, n (%)	1348 (9.1)
Severe calcification, n (%)	1311 (8.9)
Bifurcation lesion, n (%)	2857 (19)
Side-branch stenting, n (%)	479 (3.2)
Lesion length ≥30 mm, n (%)	2146 (15)
Preprocedural reference diameter <2.5 mm, n (%)	4196 (29)
Use of intravascular ultrasound, n (%)	6681 (45)
Direct stenting, n (%)	3416 (23)
After dilation, n (%)	6491 (44)
Maximum inflation pressure, atm	17.9±3.3
No. of stents used	1.29±0.57
Length of stents used, mm	28.6±15.3
Minimal stent size, mm	2.89±0.37

eGFR indicates estimated glomerular filtration rate; STEMI, ST-elevation MI; non-STEMI, non–ST-elevation MI; LMCA, left main coronary artery; LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery; and RCA, right coronary artery.

Continuous variables are expressed as mean±SD.

Table 2. Clinical Event Rates Through 2 Years

	Cumulative Event Rate, %		
	30 d	1 y	2 y
Death	0.7	3.7	7.2
Cardiac death	0.6	2.2	3.7
Sudden death	0.02	0.7	1.4
MI	0.3	0.9	1.5
Related to ST	0.2	0.4	0.7
Stroke	0.4	1.8	3.1
ST			
Definite	0.34	0.54	0.77
Definite/probable	0.46	0.68	0.91
Definite/probable/possible	0.46	1.38	2.48
Target-lesion revascularization	0.5	6.9	10.2
CABG surgery	0.1	0.9	1.5
Any coronary revascularization	2.5	19.2	25.9

participating centers (online-only Data Supplement, Appendix I) approved the study protocol. Written informed consent was obtained from all patients.

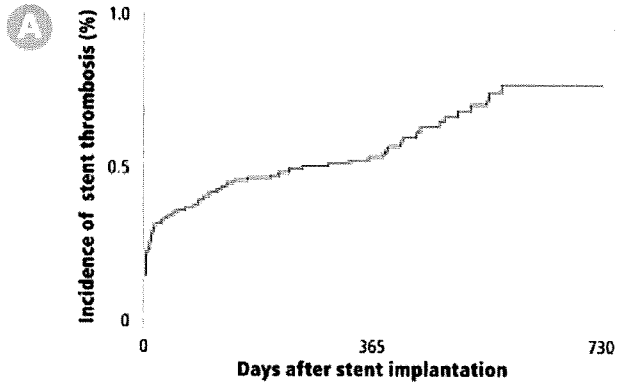
In an attempt to evaluate penetration of SES and to secure enrollment of truly consecutive patients, all patients undergoing percutaneous coronary intervention (PCI) in each center during the study interval were recorded on the PCI screening list by the technical staff in the catheterization laboratories. When SES implantation was undertaken, each patient was invited to participate in the j-Cypher registry. Although data entry was basically left to the individual sites, the clinical research coordinators (online-only Data Supplement, Appendix II) in the data management center (Kyoto University Hospital, Department of Cardiology) supported data entry when necessary. Obvious inconsistencies were resolved by inquiries to the site investigators and/or by audits against the original data sources. Follow-up data were obtained from hospital charts or by contacting patients and/or referring physicians at 30 days, 6 months, and 1 year and yearly thereafter. When death, myocardial infarction (MI), and ST were reported, the events were adjudicated with use of the original source documents by a clinical events committee (online-only Data Supplement, Appendix II).

Between August 2004 and November 2006, 15 155 patients were enrolled in the registry from among 29 555 consecutive patients recorded on the PCI screening list. SES penetration varied markedly across centers, with a median penetration rate of 53% (range 16% to 92%). After the exclusion of 2331 patients who were registered repeatedly because of PCI for restenosis or new lesions, 12 824 patients were enrolled in the registry for the first time. Of 19 675 target lesions, 17 050 were treated exclusively with SES. Treatment for the remaining 2625 lesions included bare-metal stent (BMS; 1259 lesions), combination of SES and other stent types (495 lesions), other DES (60 lesions), nonstent PCI (672 lesions), and failed procedure (139 lesions). Ultimately, 10 778 patients (84%) treated with SES exclusively constituted the study population for the present analysis.

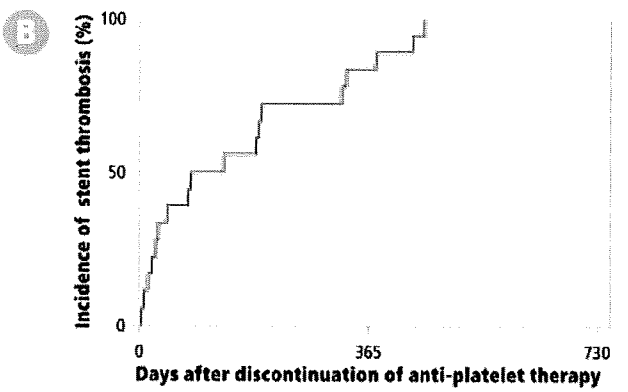
Complete 1-year follow-up (median of 491 days; interquartile range 387 to 730 days) was achieved in 96% of patients. At 1-year follow-up, information was collected from the hospital charts (75% of cases) or by contacting patients (25% of cases). Additional information was obtained from the referring physicians in 6% of cases.

Definitions

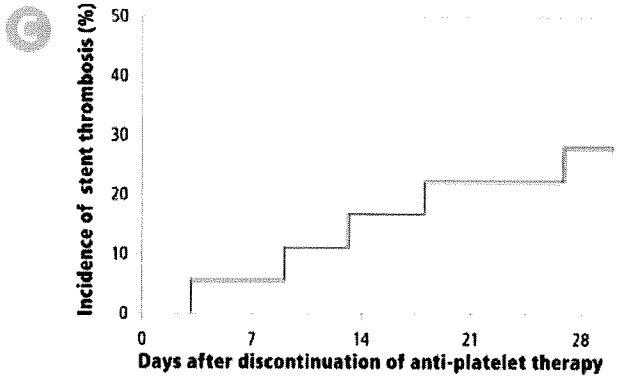
Coronary angiographic parameters were assessed in each participating center either by visual assessment or by quantitative angio-



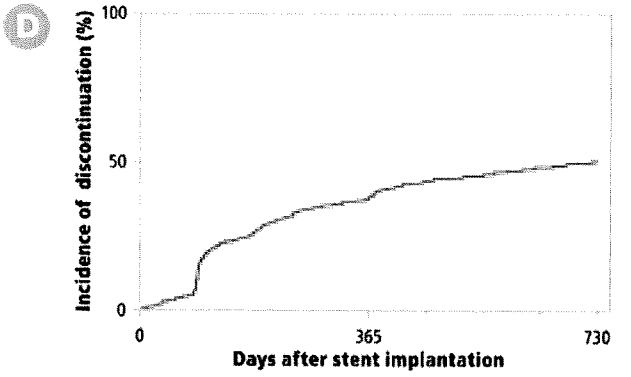
Days after stent implantation	30	365	730
Cumulative incidence	0.34%	0.54%	0.77%
Number of events	36	57	71
Number of patients at risk 10778	10612	9856	3608



Days after discontinuation	28	365	730
Cumulative incidence	28%	83%	100%
Number of events	5	15	180
Number of patients at risk 18	13	3	0



Days after discontinuation	7	14	21	28
Cumulative incidence	6%	17%	22%	28%
Number of events	1	3	4	5
Number of patients at risk 18	17	15	14	13



Days after stent implantation	120	365	730
Cumulative incidence	21%	38%	50%
Number of patients at risk 10778	8115	5998	1700

Figure 1. A, Kaplan–Meier curve showing the patient-based cumulative incidence of definite ST. B, Timing of ST after antiplatelet therapy discontinuation. C, Timing of ST within 28 days after antiplatelet therapy discontinuation. D, Kaplan–Meier curve showing the cumulative incidence of persistent thienopyridine discontinuation over time.

graphic measurement. Bifurcation lesion was defined as that involving a side branch of ≥2.2 mm in diameter.

Death was regarded as cardiac in origin unless obvious noncardiac causes could be identified. Any death during the index hospitalization was regarded as cardiac death. Sudden death was defined as unexplained death in previously stable patients. MI was adjudicated according to the definition in the Arterial Revascularization Therapy Study.[6] Within 1 week of the index procedure, only Q-wave MI was adjudicated as MI.

ST was defined according to the Academic Research Consortium definition.[7] Not only sudden death but also those deaths without enough information to exclude sudden death were regarded as possible ST. Unless otherwise noted, definite ST assessed on an individual patient basis was used as the end point for ST, because this was the end point used in a recent large-scale registry for DES.[8]

Antiplatelet Therapy

The recommended antiplatelet regimen was aspirin (≥81 mg daily) indefinitely and thienopyridine (200 mg of ticlopidine or 75 mg of clopidogrel daily) for at least 3 months. Duration of antiplatelet therapy was left to the discretion of each attending physician.

Dates of discontinuation of aspirin and thienopyridine were reported separately on the follow-up forms. When discontinuation was intended to be temporary, the dates the medications were restarted were also reported. When the attending physician intended to discontinue medications permanently, dates related to the restarting of medications after discontinuation were not systematically reported. Persistent discontinuation was defined as withdrawal that lasted at least 2 months.

ST incidences were evaluated according to the status of aspirin therapy and thienopyridine therapy. Analyses were made by time intervals after index PCI (ie, within 30 days, 31 to 180 days, 181 to 365 days, 366 to 548 days, and 549 to 730 days) in accordance with a previous report.[4] Those patients in whom occurrence of ST could be evaluated throughout the given intervals of interest were eligible for the analysis. Patients with known discontinuation of therapy for any duration until the end of the given intervals were assigned to the discontinuation group of patients without ST. In patients with ST, only discontinuation before the onset of ST was evaluated. Patients with acute ST and those with ST during the prior intervals were excluded from the analysis.

The influence of prolonged dual-antiplatelet therapy on clinical outcome was assessed with the so-called landmark analysis reported previously, which is a form of survival analysis that classifies patients on the basis of some nonoutcome event that occurs during follow-up (eg, discontinuation of thienopyridine at 6 months).[9] Eligible patients were those patients who continued taking aspirin and were free from death, MI, stroke, or ST at the 6-month landmark point.

Statistical Analysis

Categorical variables were compared with the χ^2 test. Continuous variables are expressed as mean±SD unless otherwise indicated. Continuous variables were compared with the Student t test or Wilcoxon rank sum test based on the distribution. Cumulative incidence was estimated by the Kaplan–Meier method, and differences were assessed with the log-rank test.

A Cox proportional hazard model was used to identify independent risk factors of ST. We used the variables listed in supplemental Table I as potential independent variables. The continuous variables were dichotomized by clinically meaningful reference values or median values. To determine the independent risk factors, we first selected variables with P values <0.05 in the univariable Cox models and for which proportional hazard assumptions were acceptable on the plots of log (time) versus log [−log (survival)] stratified by the variable. We then included them simultaneously in the multivariable models. Patients with missing values for any selected variable were excluded from the multivariable analysis. The robustness of independent risk factors for ST that were identified by the full model without selection of variables was confirmed by both forward and backward selection procedures.

Landmark analysis was conducted as described previously.[9] We computed the propensity score using logistic regression, with the dependent variable being continued thienopyridine use at 6 months and with the 23 independent variables listed in supplemental Table I. Next, we computed the adjusted survival curves of groups with and without thienopyridine use at the 6-month landmark using the Cox proportional hazard model in conjunction with methods described by Ghali et al,[10] adjusting for the propensity score and the above-mentioned 23 covariates.

All analyses were conducted by a physician (Takeshi Kimura) and an independent statistician (Takeshi Morimoto) with the use of SAS software version 9.1 (SAS Institute Inc, Cary, NC) and S-Plus version 7.0 (Insightful Corp, Seattle, Wash). All reported P values are 2-sided.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Baseline Characteristics

It was common for patients in the present study to have high-risk features, such as age ≥80 years, diabetes mellitus, renal failure, or unprotected left main disease (Table 1). Complex lesions such as chronic total occlusion, long lesions, and small vessels were also common. However, only 21% of patients presented with acute coronary syndrome (ACS). Procedures were characterized by use of high inflation pressure and a high rate of intravascular ultrasound guidance.

Clinical Outcome

Cumulative incidences of death, cardiac death, and sudden death at 2 years were 7.2%, 3.7%, and 1.4%, respectively (Table 2). Incidence of MI was 1.5% at 2 years. MI related to ST constituted 45% of all MIs, or 0.7% at 2 years. Incidence of target-lesion revascularization was 10.2% at 2 years.

Incidence, Clinical Sequelae, and Predictors of ST

Cumulative incidences of ST at 2 years were 0.77% for definite ST, 0.91% for definite or probable ST, and 2.48% for all ST (Table 2). Incidences of definite ST were 0.34% (95% confidence interval [CI] 0.23% to 0.45%) at 30 days, 0.54% (95% CI 0.4% to 0.68%) at 1 year, and 0.77% (95% CI 0.58% to 0.96%) at 2 years (Figure 1A). The slope of the linear portion of the cumulative incidence curve of ST between 30 days and 2 years was 0.2% per year. Clinical sequelae within 30 days of ST were MI in 85% to 95% of cases and death in 11% to 38% of cases, depending on ST timing (Table 3).

Table 3. Clinical Sequelae of ST and Antiplatelet Therapy at the Time of ST

	Early ST (≤30 d) (n=36)	Late ST (31–365 d) (n=21)	Very Late ST (366–730 d) (n=14)
Interim TVR, %	0	9.5	14
Clinical sequelae within 30 days of ST, %			
Death	11	38	18
MI	85	95	91
Q-wave infarction	56	70	83
Non–Q-wave infarction	29	25	8
Emergency CABG	11	0	0
Antiplatelet therapy at time of ST, %			
On dual antiplatelet therapy	86	57	36
On aspirin alone	8.3	14	43
On thienopyridine alone	0	4.8	0
Off both	2.8	24	21
Unknown	2.8	0	0
Discontinuation of thienopyridine, n	1	8	9
Median interval (IQR) between thienopyridine discontinuation and ST, d	9	29 (14–174)	196 (82–404)

TVR indicates target-vessel revascularization; IQR, interquartile range.

Univariable predictors for ST are shown in supplemental Table I. Multivariable analysis identified ACS (hazard ratio [HR] 2.53, 95% CI 1.3 to 4.92, P=0.006) and heart failure (HR 2.33, 95% CI 1.12 to 4.84, P=0.02) as independent predictors of early ST. Independent predictors of late or very late ST included hemodialysis (HR 6.86, 95% CI 3.05 to 15.45, P<0.001), end-stage renal disease (estimated glomerular filtration rate <30 mL · min^{-1} · 1.73 m^{-2}) without hemodialysis (HR 5.33, 95% CI 2.0 to 14.15, P<0.001), side-branch stenting (HR 3.5, 95% CI 1.36 to 9.03, P=0.01), and smoking (HR 2.36, 95% CI 1.17 to 4.76, P=0.02).

Discontinuation of Antiplatelet Therapy and ST

During the index hospitalization, aspirin and thienopyridine were administered in 98.9% and 99.5% (ticlopidine 96.9% and clopidogrel 2.6%) of patients, respectively. Cilostazol was administered in 3.2% of patients at the time of hospital discharge.

The status of antiplatelet therapy immediately before the onset of ST was known for the vast majority of patients with ST, except for 1 patient who presented with cardiogenic shock. The majority of patients (86%) with early ST were taking dual-antiplatelet therapy at the time of ST. The prevalence of dual therapy was 57% for late ST and 36% for very late ST, respectively (Table 3). Among 18 patients who

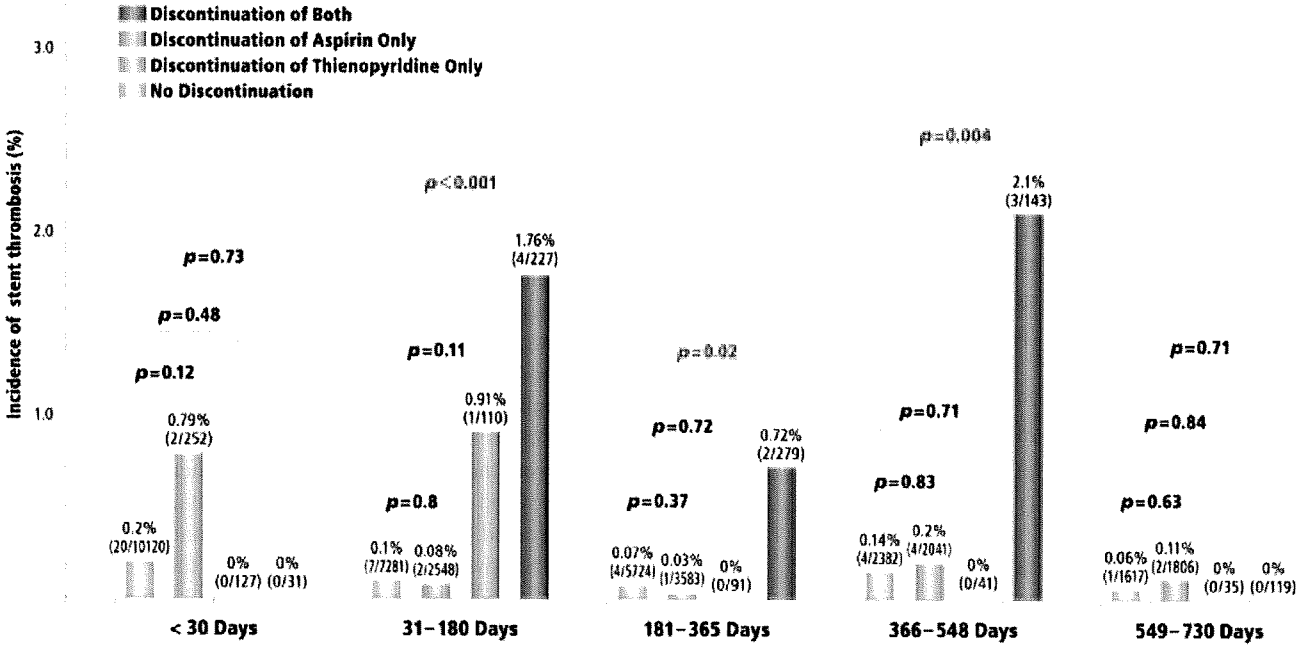


Figure 2. Relationship between thienopyridine and/or aspirin discontinuation and ST by time interval after stent implantation.

had ST after any antiplatelet therapy discontinuation, the majority of ST events occurred >1 week after discontinuation (Figure 1B and 1C).

Thienopyridine use was maintained in 97%, 62%, and 50% of patients at 30 days, 1 year, and 2 years, respectively. A steep rise was found at ≈3 months in the cumulative incidence curve of persistent discontinuation of thienopyridine (Figure 1D); however, a corresponding steep rise was not observed in the cumulative ST incidence curve (Figure 1A).

With regard to the relation between aspirin and/or thienopyridine discontinuation and ST, patients who discontinued both aspirin and thienopyridine had a significantly higher ST rate than those who continued both agents in the intervals of 31 to 180 days, 181 to 365 days, and 366 to 548 days after stent implantation (1.76% versus 0.1%, $P<0.001$; 0.72% versus 0.07%, $P=0.02$; and 2.1% versus 0.14%, $P=0.004$, respectively; Figure 2). When discontinuation of aspirin was considered, discontinuation of thienopyridine therapy only was not associated with increased ST risk in any of the time intervals.

Landmark Analysis Based on Thienopyridine Use

At 6 months, thienopyridine use was maintained in 7247 (73%) of 9875 patients eligible for the landmark analysis. Patients taking thienopyridine had significantly more complex characteristics, although some of these statistically significant differences might not be clinically relevant (Table 4). After adjustment for differences in baseline characteristics, the rates of death, MI, death or MI, and a combined end point of cardiac death, MI, or stroke at 24 months were not different between the 2 groups with or without thienopyridine therapy in the 6-month landmark analysis (Figure 3; Table 5).

The influence of ACS on the 6-month landmark analysis was evaluated. The cumulative rate of death or MI was significantly higher in patients with ACS than in those without ACS (Figure 4A); however, rates of death or MI beyond 6 months were similar in both groups (Figure 4B). Adjusted rates of death or MI at 24 months in the 2 groups either taking or not taking thienopyridine therapy at 6 months were similar in patients with or without ACS (Figure 4C and 4D).

Discussion

The main findings of the present study are that discontinuation of both aspirin and thienopyridine, but not discontinuation of thienopyridine therapy only, is associated with an increased ST risk and that no apparent clinical benefit is received from thienopyridine use beyond 6 months after SES implantation. More than 2 years ago, concerns were raised about DES safety.[1,2] Although more recent reports from registries and meta-analyses of randomized, controlled trials provided data supporting the relative safety of DES compared with BMS,[11,12] a cohort study conducted in Bern, Switzerland, and Rotterdam, Netherlands, demonstrated that definite ST continues to occur at the constant rate of 0.6% per year from 30 days to 3 years after DES implantation.[8] The present ongoing analysis using the same ST end point also showed that ST remained a continuous hazard up to 2 years after SES implantation, although the cumulative incidence of ST appeared to be considerably lower than that in the Bern and Rotterdam cohorts.[8]

We can suggest several potential reasons for the markedly lower rate of early ST in the present registry. First, there might be ethnic differences in the propensity for ST. We reported a 0.9% rate of early ST in 320 Japanese patients undergoing planned BMS implantation with an antithrom-

Table 4. Baseline and Procedural Characteristics in the 2 Groups Analyzed by Landmark Analysis at 6 Months

	Taking Thienopyridine (n=7247)	Not Taking Thienopyridine (n=2628)	*P*
Age, y	68.1±10.2	68.3±10.0	0.44
Male, %	76	75	0.12
Body mass index, kg/m^2	24.0±3.4	24.0±3.4	0.98
Hypertension, %	76	74	0.03
Diabetes mellitus, %	41	39	0.2
Current smoking, %	19	21	0.11
eGFR <30 mL · min^{-1} · 1.73 m^{-2}, %	9.7	8.0	0.009
ACS, %	20	22	0.03
Prior MI, %	28	28	0.62
Prior stroke, %	9.3	8.1	0.06
Peripheral vascular disease, %	11	12	0.54
Prior heart failure, %	13	10	<0.001
Prior PCI, %	50	44	<0.001
Prior CABG, %	7.3	7.0	0.58
Multivessel stenting, %	21	16	0.14
Target of unprotected LMCA, %	4.1	2.6	<0.001
Target of proximal LAD, %	49	49	0.51
Target of chronic total occlusion, %	12	9.7	0.005
Target of in-stent restenosis, %	17	15	0.046
Target lesion <2.5 mm in diameter, %	33	33	0.93
Intravascular ultrasound use, %	49	44	<0.001
Side-branch stenting, %	4.9	2.5	<0.001
Total length of stents, mm	39.8±26.5	36.0±22.5	<0.001

eGFR indicates estimated glomerular filtration rate; LMCA, left main coronary artery; and LAD, left anterior descending coronary artery.

Continuous variables were expressed as mean±SD.

botic regimen that included aspirin and warfarin.[13] This early ST rate appears to be markedly lower than the 2.7% in 550 US patients reported in the Stent Anticoagulation Restenosis Study using the same antithrombotic regimen and the same BMS.[14] Second, the incidence of ACS presentation, which is an established risk factor for early ST, was lower in the present study population than in the Bern and Rotterdam cohorts[9] (21% and 59%, respectively). Third, only 3% of patients in the present study discontinued thienopyridine within 30 days of SES implantation compared with the 14% discontinuation rate reported in the PREMIER registry (Prospective Registry Evaluating Myocardial Infarction: Events and Recovery).[5] We cannot provide a clear explanation for the lower rate of late and very late ST in the present study population compared with that in the Bern and Rotterdam cohorts,[8] because the mechanisms of late and very late ST have not yet been well clarified.

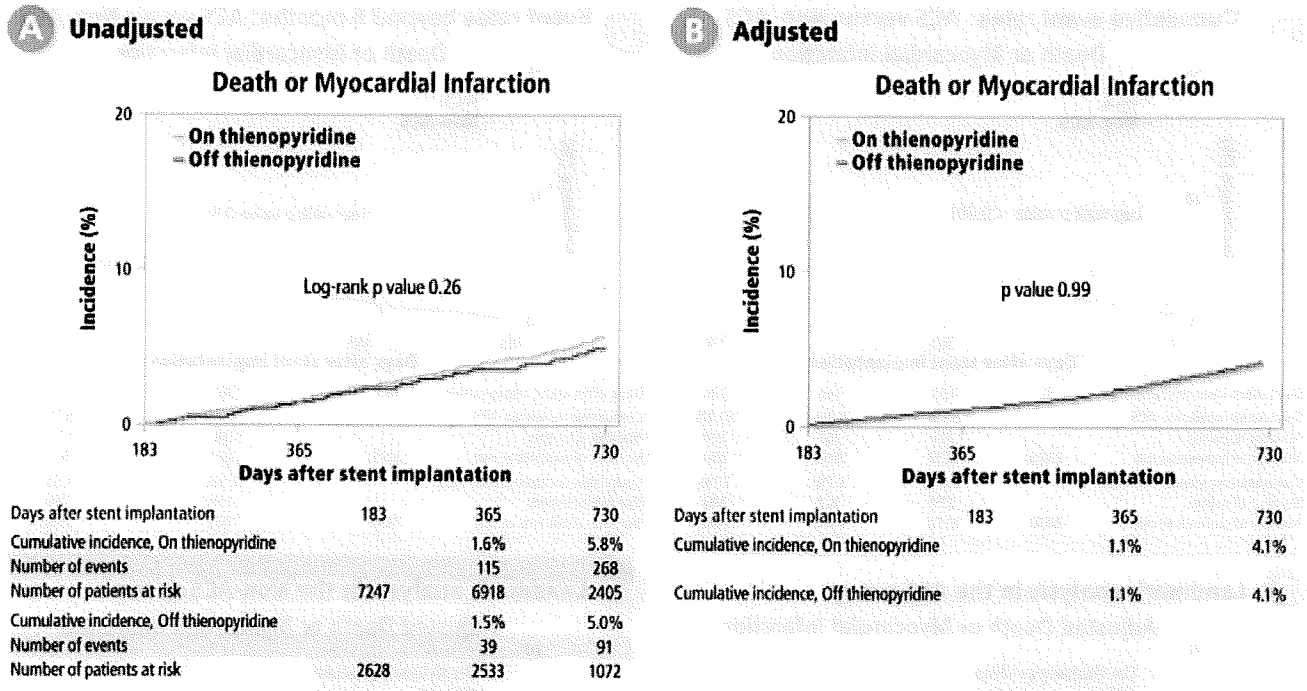
Although no randomized study has evaluated the role of dual-antiplatelet therapy in DES, the benefit of dual-antiplatelet therapy in preventing ST within 1 month after BMS implantation has been well established from randomized trials.[14,15] The TRITON-TIMI 38 trial (Trial to Assess Improvements in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis In Myocardial Infarction) also demonstrated that more intensive antiplatelet therapy with prasugrel was associated with a marked reduction in ST rate compared with standard antiplatelet therapy with clopidogrel.[16] Reduction of the ST rate in the prasugrel group was predominantly seen within 30 days after stent implantation, although the reduction in the rate of late ST was also of borderline significance. Therefore, the role of intensive antiplatelet therapy in reducing early ST appears to be firmly established.

However, the role of thienopyridine therapy in reducing late ST beyond 1 month after stent implantation has not been well addressed. Although premature discontinuation of antiplatelet therapy has been reported to be the most powerful predictor of ST and/or adverse outcome,[3–5] these previous reports did not discriminate the relative impact of discontinuation of either aspirin, thienopyridine, or both agents. The present analysis demonstrated that withdrawal of both thienopyridine and aspirin, but not of thienopyridine therapy alone, was associated with increased ST risk beyond 1 month after SES implantation. Aspirin withdrawal was reported to be responsible for admission with an ACS in 51 (4%) of 1236 patients, with a mean delay between aspirin cessation and hospitalization of 10±2 days.[17] It is noteworthy that late and very late ST at a mean of 16±7 months after BMS implantation was responsible for ACS in 10 (20%) of these 51 patients.

Furthermore, only one third of ST events after discontinuation of antiplatelet therapy (mostly thienopyridine) occurred within the first 28 days after discontinuation. This might lead to a discussion about whether or not a direct link in fact exists between discontinuation of antiplatelet therapy and ST, particularly very late ST.

Previous prospective studies demonstrated a clinical benefit of the prolonged use of thienopyridine for up to 1 year in patients undergoing PCI with BMS,[18,19] primarily in the setting of ACS. Extrapolation of these findings to DES might make it appear reasonable to advocate adherence to dual-antiplatelet therapy for 1 year after DES implantation; however, the present study results suggest that it is also reasonable to discontinue thienopyridine and adhere to aspirin monotherapy in situations in which continuation of dual-antiplatelet therapy appears to be otherwise clinically irrelevant. Furthermore, because the majority of ST events occurred >1 week after discontinuation of antiplatelet therapy, it appears important to make the duration of discontinuation as short as possible if discontinuation is unavoidable.

The optimal duration of dual-antiplatelet therapy has not been well established. A single-center observational study of 1216 DES patients and 2393 BMS patients reported that use of clopidogrel at 6 and 12 months was associated with a lower incidence of death or MI at 24 months in patients with DES but not in patients with BMS.[9] On the other hand, a similar



Days after stent implantation	183	365	730
Cumulative incidence, On thienopyridine		1.6%	5.8%
Number of events		115	268
Number of patients at risk	7247	6918	2405
Cumulative incidence, Off thienopyridine		1.5%	5.0%
Number of events		39	91
Number of patients at risk	2628	2533	1072

Days after stent implantation	183	365	730
Cumulative incidence, On thienopyridine		1.1%	4.1%
Cumulative incidence, Off thienopyridine		1.1%	4.1%

Figure 3. Unadjusted and adjusted cumulative incidences of death or MI using the 6-month landmark analysis.

analysis in 671 diabetic patients reported that use of clopidogrel at 6 months was associated with a significantly lower incidence of death or MI at 18 months in patients with BMS but not in those with DES.[20] Relatively small sample sizes in these subgroup analyses to evaluate hard clinical end points might be 1 of the reasons for the discrepancy. The present analysis of a larger number of patients by the same method demonstrated a similar long-term clinical outcome regardless of thienopyridine use at 6 months in SES patients. Given the increased risk of bleeding and the huge economic burden with prolonged dual-antiplatelet therapy,[18,19,21] the optimal duration of dual-antiplatelet therapy should be defined by prospective randomized trials evaluating the net clinical benefit after considering both ischemic events and bleeding complications.

The present study has several important limitations. First, baseline characteristics and procedural characteristics such as the high rate of intravascular ultrasound guidance in the present cohort might be markedly different from practices outside Japan. Also, ticlopidine was used as a thienopyridine antiplatelet agent in the vast majority of patients, in contrast to the use of clopidogrel in most other studies. These and other ethnic differences might make it difficult to apply the findings in the present study outside of Japan. Second, although data on antiplatelet therapy use were collected prospectively, no attempt was made to verify compliance with antiplatelet medications for patients in whom discontinuation was not reported; this might well have led to overestimation of compliance. In addition, we did not systematically evaluate the restarting of antiplatelet therapy after persistent discontinuation, which could have resulted in the potential underestimation of medication use. In fact, in the 6-month landmark analysis, 13% of patients who underwent repeated revascularization >6 months after the first procedure were likely to have restarted thienopyridine. The limitation of a landmark analysis is that it only examines specific points in time. A Cox proportional hazards model with a time-dependent covariate (thienopyridine discontinuation) might be able to examine the continuous risk of thienopyridine discontinuation. Furthermore, when follow-up information was obtained by contact with patients, dates of discontinuation of aspirin and thienopyridine were based on retrospective recall by the patients or relatives, which suggests a potential for recall bias. Third, unmeasured confounders related to thienopyridine discontinuation might be present because of the observational study design. Fourth, the number of patients at risk at 2-year follow-up was limited. Therefore, the results of the present study might be valid only during the first year after SES implantation. Finally, bleeding complications were not evaluated, which made it impossible to evaluate the net clinical efficacy of dual-antiplatelet therapy.

Table 5. Unadjusted and Adjusted 24-Month Outcomes Based on 6-Month Thienopyridine Use

	No. at Risk at 6 Months	No. of Events				No. at Risk at 24 Months	Unadjusted Event Rates, %				Adjusted Event Rates, %			
		Death	MI	Death or MI	Cardiac Death, MI, or Stroke		Death (*P*=0.22)	MI (*P*=0.81)	Death or MI (*P*=0.26)	Cardiac Death, MI, or Stroke (*P*=0.6)	Death (P=0.9)	MI (*P*=0.42)	Death or MI (*P*=0.99)	Cardiac Death, MI, or Stroke (*P*=0.79)
On thienopyridine	7247	238	41	268	228	2424	5.2	0.9	5.8	4.9	3.4	0.6	4.1	4.0
Off thienopyridine	2628	79	17	91	82	1078	4.4	0.8	5.0	4.3	3.4	0.8	4.1	4.1