Increased Expression and Activation of Stress-Activated Protein Kinases/c-Jun NH₂-Terminal Protein Kinases in Atherosclerotic Lesions Coincide with p53

Bernhard Metzler,* Yanhua Hu,[†]
Hermann Dietrich,[†] and Qingbo Xu[‡]

From the Division of Cardiology,* Department of Internal Medicine, and the Institute for General and Experimental Pathology,[†] University of Innsbruck Medical School; and the Institute for Biomedical Aging Research,[‡] Austrian Academy of Sciences, Innsbruck, Austria

Hyperlipidemia alters gene expression of arterial endothelial and smooth muscle cells (SMCs) and induces atherosclerotic lesions, in which cell proliferation and apoptosis co-exist. The signal transduction pathways that mediate these responses in the vessel wall *in vivo* have yet to be identified. Stress-activated protein kinases (SAPKs) or c-Jun NH₂-terminal protein kinases (JNKs) are thought to be crucial in transmitting transmembrane signals required for cell differentiation and apoptosis *in vitro*. In the present study, we investigated the localization and activity of SAPK/JNK in atherosclerotic lesions of cholesterol-fed rabbits. Immunofluorescence analysis revealed abundant and heterogeneous distribution of pan-SAPK/JNK and phosphorylated SAPK/JNK, which were mainly localized in cell nuclei of the lesional cap and basal regions. Double staining of the lesions demonstrated that a portion of α -actin⁺ SMCs and RAM11⁺ macrophages contained abundant phosphorylated SAPK/JNK proteins. SAPK/JNK protein levels in protein extracts from atherosclerotic lesions were two- to threefold higher than the vessels of chow-fed rabbits. SAPK/JNK activities were elevated three- to fivefold higher than the normal vessels. Interestingly, increased SAPK/JNK in lesions was co-localized or co-incided with high levels of transcription factor p53 as identified by double labeling and immunoprecipitation. Abundant pro-apoptotic protein BAX and BCL-X_s were also observed. Furthermore, low-density lipoprotein (LDL) and oxidized LDL stimulated SAPK/JNK activation in cultured SMCs in a time- and dose-dependent manner. LDL also induced SAPK/JNK activation in vascular SMCs derived from LDL-receptor-deficient Watanabe rabbits, indicating a LDL-receptor-independent process. Thus, SAPK/JNK persistently hyperexpressed and activated in lesions may

play a key role in mediating cell differentiation and apoptosis during the development of atherosclerosis via activation of transcription factor p53. (*Am J Pathol* 2000, 156:1875–1886)

Mammalian cells respond to extracellular stimuli by activating signal transduction pathways, which culminate in changes in gene expression. A critical component of signaling pathways is the activation of protein kinases that phosphorylate a host of cellular substrates, such as transcription factors controlling the induction of various genes. Stress-activated protein kinases (SAPKs) or c-Jun NH₂-terminal protein kinases (JNKs) are highly activated in the cardiovascular system in response to stresses,^{1–3} including acute hypertension,⁴ angioplasty,⁵ mechanical stress,^{6–8} ischemia/reperfusion,⁹ free radicals,¹⁰ heat shock,¹¹ and inflammatory cytokines¹² *in vivo* or *in vitro*. The pathways leading to SAPK/JNK activation have been extensively studied in cultured cells. Upstream activators of SAPKs/JNKs have been found involving SEK1/MKK4, MEKK1–3, and the small GTP-binding proteins Rac1 and Cdc42.^{13–15} JNK was named based on its ability to phosphorylate the c-Jun protein, leading to its enhanced transcriptional activity.^{16–18} Recently, they have also been shown to be capable of phosphorylating the transcription factors ATF₂, Elk1, and p53,^{19–23} which form heterodimers or homodimers binding to promoter regions present in numbers of genes that play a crucial role in mediating cell (de)differentiation and apoptosis.

The atherosclerotic lesion is defined by arterial intimal cell proliferation, lipid accumulation, and connective tissue deposition. The major cellular components of the human atherosclerotic plaques are smooth muscle cells (SMCs), which dominate the fibrous cap, and macrophages, which are the most abundant cell type in the

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Address reprint requests to Dr. Qingbo Xu, Institute for Biomedical Aging Research, Austrian Academy of Sciences, Rennweg 10, A-6020 Innsbruck, Austria. E-mail: qingbo.xu@oeaw.ac.at.

lipid-rich core region.^{24,25} Although the pathogenic mechanism of atherosclerosis is not fully understood, a high concentration of circulating cholesterol or low-density lipoproteins (LDL) is believed to be a major risk factor. LDL delivers cholesterol to vascular SMCs and macrophages, stimulates cell proliferation, and induces gene expression of platelet-derived growth factor (PDGF), PDGF receptors, *c-fos*, and *egr-1*.^{25,26} In the arterial wall. LDL or oxidized LDL stimulation can also evoke cell apoptosis,^{27–29} which has been observed in atherosclerotic lesions.^{30–33} In addition, p53 has been identified to be highly expressed in atherosclerotic lesions and to be involved in oxidized LDL-induced apoptosis.²⁷ However, the precise signal transduction pathways that link hypercholesterolemia or LDL stimulation to quantitative changes in gene expression and, to apoptosis in the pathogenesis of atherosclerosis, remain to be elucidated. In the present study, we examined SAPK/JNK expression, localization, and activation in atherosclerotic lesions of cholesterol-fed rabbits and provide the first evidence of SAPK/JNK overexpression and activation in lesions. Furthermore, we demonstrate that LDL and oxidized LDL stimulate SAPK/JNK activation in cultured SMCs independent of classic LDL receptors.

Materials and Methods

Rabbit Model for Atherosclerosis

New Zealand White male rabbits weighing between 1,800 and 2,200 g were obtained from Charles River (Kisslegg, Germany). All animals were selected for serum cholesterol levels under 100 mg/dl, individually housed in wire-bottomed cages at 22°C with a relative humidity of 55%. All received water *ad libitum* and were fed either a normal standard chow diet (T775; Tagger & Co., Graz, Austria) or a cholesterol-enriched diet (0.2% w/w) for 16 weeks, as described previously.^{34,35} Animals were sacrificed by heart puncture under ketamine (25 mg/kg) and xylazine (5 to 10 mg/kg) anesthesia. Serum was collected for cholesterol assays and LDL isolation. The aortas were carefully removed intact from the aortic arch to the iliac bifurcation, immediately put into cold phosphate-buffered saline (PBS) (4°C) and prepared for histological analysis, tissue culture, and protein extractions. The total surface area of aortas covered by lesions was evaluated. For conventional histology, tissue fragments were fixed in 4% buffered (pH 7.2) formaldehyde, embedded in paraffin, and sectioned for hematoxylin and eosin (H&E) staining. Serum cholesterol values were measured every 2 or 4 weeks using an enzymatic procedure (Sigma Chemical Co., St. Louis, MO).

Immunohistochemical and Immunofluorescence Double Staining

The procedure used for immunohistochemical staining was similar to that described elsewhere.^{34,35} Briefly, serial 4- μ m-thick frozen sections were overlaid with mouse monoclonal antibodies against α -actin (Sigma), macro-

phages (RAM11; DAKO, Copenhagen, Denmark), or T cells (L11/135; ATCC, Rockville, MD) and incubated with rabbit anti-mouse immunoglobulin (Ig) conjugated with peroxidase (DAKO) and developed for 20 minutes at room temperature using a substrate solution.

For immunofluorescence staining, a monoclonal antibody against SAPK/JNK1 (Transduction Lab., Lexington, KY) was added to the sections. After three washes with PBS, the sections were incubated with a rabbit anti-mouse Ig-tetramethylrhodamine B isothiocyanate (TRITC) conjugate (DAKO) for 30 minutes, or sections were incubated with a monoclonal antibody against phosphorylated SAPK/JNK conjugated with fluorescein isothiocyanate (FITC) (p-JNK; Santa Cruz Biochem., Santa Cruz, CA), mounted in glycerol/PBS, and examined in an epiillumination immunofluorescence microscope equipped with appropriate filter combinations for the three wavelength method (Leitz, Wetzlar, Germany).

For immunofluorescence double staining, frozen sections were incubated with monoclonal antibodies against α -actin Cy3 conjugate (Sigma) or RAM11 macrophages. After three washes with PBS, the sections were incubated with a rabbit anti-mouse Ig-TRITC conjugate (DAKO) for 30 minutes, rinsed, blocked with normal mouse serum (1:5), and stained with a mouse monoclonal antibody against phosphorylated-SAPK/JNK-FITC conjugate (p-JNK). For p53 and JNK1 double staining, a mouse monoclonal anti-p53 antibody and rabbit anti-JNK1 antibodies (Santa Cruz Biotech. Inc.) were used and visualized with swine anti-mouse Ig-FITC and goat anti-rabbit Ig-TRITC. For visualization of nuclei, sections were counterstained with the DNA stain Hoechst 33258 (1 μ g/ml PBS; Lambda Probes, Graz, Austria) for 1 minute.

Western Blot Analysis

The procedure used for protein extracts was similar to that described previously³⁶ with a slight modification. Briefly, the atherosclerotic-lesioned intima and media were dissected on ice from the remaining adventitia with tweezers and scissors. Tissues were homogenized in buffer A (20 mmol Hepes, pH 7.4, 50 mmol/L β -glycerophosphate, 2 mmol/L EGTA, 1 mmol/L dithiothreitol, 1 mmol/L Na_3VO_4 , 1% Triton X-100, 10% glycerol, 1 μ g/ml leupeptin, 100 μ mol/L phenylmethyl sulfonyl fluoride (PMSF), and 1 μ g/ml aprotinin), and proteins were extracted. Proteins (50 μ g/lane) were separated by electrophoresis through a 10% SDS-polyacrylamide gel and transferred to an immobilon-p transfer membrane. The membranes were processed with monoclonal antibodies against SAPK/JNK1, actin, p53, BAX, and BCL-X_{S/L} (Santa Cruz Biotech. Inc.), respectively, and specific antigen-antibody complexes were detected with the ECL Western Blot Detection Kit (Amersham Co., Little Chalfont, UK).

Immunoprecipitation and Kinase Assay

One-half milliliter of the supernatant containing 0.5 mg proteins was incubated with 10 μ l of rabbit anti-SAPK/JNK1 antibodies and 40 μ l of protein A-agarose suspen-

sion (Santa Cruz Biochem.). The immunocomplexes were precipitated by centrifugation and washed 2 times with buffer A, buffer B (500 mmol/L LiCl, 100 mmol/L Tris, 1 mmol/L dithiothreitol, 0.1% Triton X-100, pH 7.6), and buffer C (20 mmol/L Mops, 2 mmol/L EGTA, 10 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 0.1% Triton X-100, pH 7.2), respectively. The activities of SAPK/JNK in the immunocomplexes were measured as described previously.^{11,16} Briefly, immunocomplexes were incubated with 35 μ l of buffer C supplemented with glutathione S-transferase (GST)-c-Jun (5 μ g), γ -³²P-ATP (Amersham, Little Chalfont, UK), and MgCl₂ (50 mmol/L) for 20 minutes at 37°C with vortexing every 5 minutes. The GST-c-Jun fusion protein (plasmid provided by Dr. Woodgett, Toronto, Canada) was produced in *Escherichia coli* and isolated using glutathione Sepharose 4B RediPack Columns (Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer's protocol. To stop the reaction, 15 μ l of 4 $\times$ Laemmli buffer was added and the mixture was boiled for 5 minutes. Proteins in the kinase reaction were resolved by SDS-polyacrylamide gel electrophoresis (12% gel) and subjected to autoradiography.

For immunoprecipitation of SAPK/JNK1 and p53 Western blot analysis, 1 ml of the supernatant containing 2 mg of protein was incubated with 50 μ l of rabbit anti-SAPK/JNK1 antibodies and 100 μ l of protein A-agarose suspension. The immunocomplexes were precipitated as described for the kinase assay and resolved in SDS-polyacrylamide gel electrophoresis (15% gel).

Cell Cultures

Human and rabbit vascular SMCs were cultivated from arteries using the procedure described by Ross and Kariya³⁷ with a slight modification.³⁸ In short, specimens of human carotid arteries were obtained from the Department of Vascular Surgery, University Hospital of Innsbruck. Intima and inner layer of media were dissected from the arteries and cut into pieces, digested with collagenase and elastase, and cultured in Dulbecco's modified essential medium (DMEM; Life Technologies, Inc., Grand Island, NY). Thoracic aortas of chow- and cholesterol-fed rabbits were removed, and lesioned intima and normal media were carefully dissected from the vessel, cut into pieces (~1 mm³), and implanted onto a gelatin-coated (0.02%) plastic bottle (Becton Dickinson, Oxnard, CA). The bottle was incubated up-side-down at 37°C in a humidified atmosphere of 95% air/5% CO₂ for 3 hours, and then medium supplemented with 20% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) was slowly added. Cells were passaged by treatment with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) solution. Experiments were conducted on SMCs that had just achieved confluence. Similarly, aortic SMCs from Watanabe rabbits (La Roche, Basel, Switzerland) were cultivated and used for SAPK/JNK kinase assays.

LDL Isolation and Oxidation

EDTA plasma was pooled from normolipemic, fasting (12 to 14 hours) male and female donors, aged 20 to 30 years. Lipoproteins were prepared by differential centrifugation using solid KBr to adjust the density, as described previously.^{39,40} LDL were obtained in fraction between 1.020 to 1.050 g/ml. Concentrations of LDL were determined gravimetrically by aliquot weight after drying, and quantities of lipoproteins were expressed as total weights.^{39,40} LDL oxidation was performed by incubation of LDL (1 mg/ml PBS) with 10 μ mol/L CuCl₂ at 37°C for 18 hours.⁴¹ The extent of oxidation was assessed by measurement of thiobarbituric acid reactive substances (TBARS) (9.8 $\pm$ 1.3 nmol/mg).⁴²

Statistical Analysis

The Mann-Whitney *U* test was used for comparison between two groups. *P* < 0.05 was considered statistically significant.

Results

Atherosclerotic Lesions in Hypercholesterolemic Rabbits

Animals in the control group (chow diet) had blood cholesterol levels <100 mg/dl, whereas blood cholesterol levels in rabbits receiving the 0.2% cholesterol diet were significantly elevated and reached 500 mg/dl at 4 weeks, and ~700 mg/dl at 16 weeks. Rabbit aortas from both groups were examined morphologically and immunohistologically 16 weeks after chow or cholesterol feeding. Fifty percent to 80% of aortic intima was covered by atherosclerotic lesions, including fatty streaks and plaques in cholesterol-fed rabbits. Aortic lesions of rabbits fed with a cholesterol-enriched diet were characterized by cell proliferation, foam cell accumulation, and lipid deposition in the intima. Atherosclerotic lesions displayed great variations in cell type and distribution. The overlying endothelium was intact to variable degrees, and α -actin-positive SMCs appeared in various stages of lesions, most frequently in advanced lesions (Figure 1). Cells expressing the macrophage antigen identified by the RAM11 antibody were observed in all atherosclerotic lesions. T lymphocytes identified by monoclonal antibody L11/135 were also found in lesions (Figure 1).

SAPK/JNK Activation in Atherosclerotic Lesions

SAPK/JNK kinases are activated by dual phosphorylation of tyrosine and threonine residues in response to stress stimuli or mitogens.¹³⁻¹⁵ After activation, they translocate from cytoplasm into the nucleus in cultured cells. We performed an immunofluorescent analysis of atherosclerotic lesions using the antibody recognizing the pan-SAPK/JNK and phosphorylated SAPK/JNK, respectively. Intimal endothelium, media, and adventitia of normal aor-

Figure 1. Cells in atherosclerotic lesions. **A** and **B**: H&E-stained sections from animals receiving chow (**A**) or cholesterol-enriched diets (**B**) for 16 weeks. **C–F**: Cryostat sections from aortic segments of rabbits fed a 0.2% cholesterol diet for 16 weeks were labeled with normal mouse Ig as a negative control (**C**), or monoclonal antibodies against α -actin (**D**), macrophages (**E**; RAM11), or T lymphocytes (**F**; L11/135) and visualized with the peroxidase system. Note the presence of positive staining (dark) in lesions. **Arrowheads** indicate internal elastic lamina, and **arrows** point to examples of positive-stained cells. Original magnification, $\times 250$.

tas showed very weak SAPK/JNK staining (Figure 2, a and d), while the lesion-covered areas in intima from rabbits receiving a cholesterol-rich diet showed increased immunostaining intensity (Figure 2, c, e, and f). Nonspecific reactivity was minimal in the negative controls stained with a normal mouse Ig (Figure 2b). The pattern of pan-SAPL/JNK and phosphorylated SAPK/JNK staining in the lesioned aortas is shown in Figure 2, c, e, and f. Heterogeneity of pan-SAPK/JNK and phosphorylated SAPK/JNK staining became more evident in atherosclerotic plaques. Sites of increased pan-SAPK/JNK and phosphorylated SAPK/JNK were mainly within the cap and base regions of the atherosclerotic plaque (Figure 2, c, e, and f). Fatty streaks displayed elevated SAPK/JNK content in subendothelial regions.

Next, we performed immunofluorescence double staining using the antibody against phosphorylated-SAPK/JNK to identify the cells responsible for the elevated SAPK/JNK expression. Figure 3, a–d, shows data representing double immunostaining with antibodies against phosphorylated-SAPK/JNK (a and c; green), α -actin (b; red), and RAM11 macrophages (d; red). Non-specific staining was minimal in the corresponding neg-

ative controls stained with a normal mouse Ig. Typical double-positive cells are indicated by arrows, demonstrating a portion of SMCs and macrophages in lesions overexpressing SAPKs/JNKs.

Elevated Protein Levels and Activities of SAPKs/JNKs

Because of the increased intensity of immunostaining for lesions, it would be interesting to determine SAPK/JNK1 protein levels of atherosclerotic lesions. Protein extracts from tissues of normal intima/media, lesioned intima, and media were analyzed by Western blot analysis. Abundant SAPK/JNK1 proteins in atherosclerotic lesions were observed (Figure 4A). SAPK/JNK proteins in lesioned intima were significantly higher than intima/media of control animals and media of cholesterol-fed rabbits after normalization to the respect of actin contents (Figure 4C). To further demonstrate higher SAPK/JNK1 protein levels associated with increased SAPK/JNK1 activity, a kinase

Figure 2. SAPK/JNK staining in atherosclerotic lesions. Cryostat sections from aortic segments of rabbits receiving a standard-chow diet (**a** and **d**) or a 0.2% cholesterol-diet (**b**, **c**, **e**, and **f**) were labeled with normal mouse Ig as a negative control (**b**), or a monoclonal antibody against pan-SAPK/JNK (**a** and **c**) visualized with rabbit anti-mouse Ig-TRITC, or a monoclonal antibody against phosphorylated SAPK/JNK conjugated with FITC (**d**–**f**). Note the presence of positive staining in the cap (**c** and **e**) and base (**f**) regions of lesions. **Arrows** indicate the surface of intimal lesions, and **thick arrows** point to examples of positive-stained cells. Co, core region of the lesion. Original magnification, $\times 250$.

assay was performed using c-Jun as a substrate. Figure 4B shows the results of an experiment examining SAPK/JNK1 activities in the vessel wall, indicating elevated SAPK/JNK1 activities in atherosclerotic lesions. When immunocomplexes, obtained similarly as for the kinase assay, were subjected to Western blot analysis, the results shown in Figure 4B (lower panel) demonstrated equal efficacy of the immunoprecipitation from protein extracts of both normal vessels and atherosclerotic lesions and higher levels of SAPK/JNK proteins in the lesions. Figure 4C summarizes data from two independent experiments, implicating that SAPK/JNK activities were at low levels in control vessels and media of cholesterol-fed rabbits, but increased three- to fivefold in atherosclerotic lesions.

Expression of p53, BAX, and BCL-X_S

There is evidence that transcription factor p53 accumulates in human atherosclerotic tissues.^{43,44} Immunofluorescence staining confirmed high levels of p53 in the lesions induced by the cholesterol-diet in rabbits (Figure 5). Interestingly, the most p53⁺ cells showed an elevation in pan-SAPK/JNK1 proteins, ie, co-localized in atherosclerotic lesions (Figure 5, b and c). The distribution pattern of p53 was similar to pan-SAPK/JNK1. In addition, both p53 (Figure 5d) and SAPK/JNK1 (Figure 5e) proteins in cells of arterial walls from control rabbits were expressed at very low levels, if any. To further verify p53 levels in lesions, protein extracts were analyzed by West-

Figure 3. Immunofluorescence double labeling of phosphorylated SAPK/JNK and cell markers in atherosclerotic lesions. Cryostat sections from rabbit aortic tissue 16 weeks after administration of a cholesterol-rich diet were incubated with a mouse monoclonal antibody against phosphorylated SAPK/JNK conjugated with FITC (**a**) for 30 minutes at room temperature. After washing, sections were incubated with a monoclonal antibody against α -actin conjugated with Cy3 (**b**). **c** and **d**: Results of immunofluorescent double staining for phosphorylated SAPK/JNK and RAM11⁺ macrophages. **Arrow** indicates internal elastic lamina, and **thick arrows** point to an example of double-positive cells. Original magnification, $\times 250$.

ern blot using anti-p53 antibodies. Data shown in Figure 6 indicated that p53 protein content in the lesions was higher than proteins from the normal intima and media (Figure 6A). When SAPK/JNK1 was immunoprecipitated and analyzed with Western blot, a 53-kd band was identified by anti-p53 antibody (Figure 6B), indicating that a complex of SAPK/JNK1 and p53 is present in atherosclerotic lesions. These findings are concomitant with the

results obtained from *in vitro* studies using cultured cells.^{22,23}

BAX and BCL-X_S are considered to be pro-apoptotic proteins which are primarily mediated by transcription factor p53.^{44,45} Western blot analysis of protein extracts from the vessel wall revealed marked increases in both BAX and BCL-X_S proteins from atherosclerotic lesions, but not BCL-X_L (Figure 7).

Figure 4. Elevated SAPK/JNK proteins and activities in atherosclerotic lesions. Intima and media (I/M) from chow-fed rabbits and media (M) and atherosclerotic-lesioned intima (AS) from cholesterol-fed rabbits (16-week diet) were dissected on ice from the remaining adventitia with a tweezers and scissors. Tissues were frozen in liquid nitrogen and homogenized with a polytron homogenizer. Protein extracts (50 μ g/lane) were separated on 10% SDS-polyacrylamide gel, transferred onto membrane, and probed with the antibody against SAPK/JNK1 (**A**) and anti-actin antibodies after stripping (**A**, lower panel). Immunocomplexes were visualized by a Western blot detection kit. For the kinase assay, SAPK/JNK1 proteins were immunoprecipitated from the protein extractions at 0.5 mg for the kinase assay (**B**, upper panel) and 0.2 mg for Western blot (**B**, lower panel) and their kinase activity (**B**) measured based on phosphorylation of GST-c-Jun substrate. SAPK/JNK1 in immunoprecipitant (IP) was identified by Western blot analysis (blot). Each lane represents an individual animal. **C**: Graph of means $\pm$ SD obtained from five rabbits per group. *, Significant difference from the intima/media, $P < 0.05$.

LDL and Oxidized LDL-Activated SAPK/JNK in SMCs

LDL is a main lipoprotein carrying and transporting cholesterol in hypercholesterolemic rabbits induced by cholesterol feeding. LDL can be oxidized in the arterial wall. To test whether LDL and oxidized LDL can induce SAPK/JNK activation, vascular SMCs from humans and rabbits

were stimulated with human LDL and oxidized LDL and the kinase assay was performed. As shown in Figure 8, A and B, LDL and oxidized LDL (100 μ g/ml) treatment resulted in significantly increased SAPK/JNK activation in SMCs; kinetic analysis indicated that this response occurred as early as 10 minutes after treatment (Figure 8, A and B), with maximum levels (five- to eightfold greater than untreated control) achieved 20 minutes after treatment, almost returning to basal levels by 4 hours. To further establish the relationship between LDL or oxidized LDL treatment and SAPK/JNK activity, we performed a dose-response analysis of LDL- or oxidized LDL-induced SAPK/JNK activation. As shown in Figure 8, C and D, SAPK/JNK activities increased in a dose-dependent manner between 5 and 400 μ g/ml (LDL), and reached higher levels at concentrations between 100 to 400 μ g/ml. To verify the efficacy of immunoprecipitation and the phosphorylation of SAPK/JNK1 stimulated by LDL, the immunocomplexes, obtained similarly as for kinase assays, were subjected to electrophoreses and blotted with antibodies against phosphorylated SAPK/JNK (Figure 8E, upper panel) and pan-SAPK/JNK1 (Figure 8E, lower panel). The results indicate that oxidized LDL stimulated SAPK/JNK1 phosphorylation and did not alter protein levels (Figure 8E). Taken together, these findings demonstrate that LDL and oxidized LDL induced SAPK/JNK activation and phosphorylation, which were strongly potentiated by oxidized LDL compared to native LDL.

LDL can specifically bind to their receptors to deliver cholesterol to the cell, but whether the receptor binding initiates signaling pathways stimulated by LDL remains to be clarified. We performed several experiments to verify receptor involvement in LDL-induced SAPK/JNK activation. SMCs from normal or Watanabe rabbits lacking the classic LDL receptors were incubated with human LDL for 20 minutes, and kinase activities measured as described above. Figure 9A indicates that LDL stimulated SAPK/JNK activation in LDL receptor-deficient SMCs. No significant difference between the two types of SMCs was found. It has been established that heparin blocks LDL-receptor binding, but pretreatment of LDL with heparin did not inhibit LDL-stimulated SAPK/JNK activation (Figure 9B). When LDL binds to the receptors, the complexes are internalized and degraded, which can be inhibited by pretreatment of cells with chloroquine or NH_4Cl . To further exclude the involvement of LDL receptors, SMCs were pretreated with both reagents and stimulated with LDL, again with no apparent influence (Figure 9C), indicating LDL receptor-independent activation.

Discussion

Atherosclerosis is the leading cause of death among persons with a Western life style. Elevated plasma LDL levels are clearly major risk factors for cardiovascular disease, but the underlying mechanisms are not fully understood. LDL and oxidized LDL effectively stimulate SMCs and macrophages to express a large number of genes, such as TGF, FGF, PDGF, PDGF receptors, *c-fos*, and *egr-1*,^{25,26,46-48} which are important in the develop-

Figure 5. Immunofluorescence double labeling of p53 and pan-SAPK/JNK1 in atherosclerotic lesions. Cryostat sections from rabbit aortic tissues 16 weeks after administration of a cholesterol-rich diet (**a–c**) or standard-chow diet (**d** and **e**) were incubated with normal mouse serum (**a**), a mouse monoclonal antibody against p53 (**b** and **d**), and visualized with a swine anti-mouse Ig-FITC (**a**, **b**, and **d**). After washing and blocking with normal mouse serum (1:5), sections **b** and **d** were labeled with rabbit anti-pan-SAPK/JNK1 antibodies and visualized with goat anti-rabbit Ig conjugated with TRITC (**c** and **e**). **Open arrow** indicates internal elastic lamina and **filled arrows** point to examples of double-positive cells. Original magnification, $\times 250$.

ment of atherosclerotic lesions. In the present study, we provide the first evidence of increased SAPK/JNK activities in intimal lesions of cholesterol-fed rabbits, which coincide with high levels of transcription factor p53, pro-apoptotic protein BAX, and BCL-X_S. These observations could be important for understanding the mechanisms of signal transduction pathways between hypercholesterolemia and altered gene expression and/or apoptosis of SMCs and macrophages during atherogenesis.

It is well known that LDL specifically binds to apoB/E receptors to deliver cholesterol to SMCs,⁴⁹ but LDL-initiated signal transduction pathways leading to SMC gene expression are not fully elucidated. Recently, several groups reported that LDL and oxidized LDL activate extracellular signal-regulated kinases in cultured SMCs and macrophages.^{46,50–53} In the present study, we demonstrated, for the first time, that LDL and oxidized LDL

markedly stimulate SAPK/JNK activation, and do not influence SAPK/JNK protein levels in cultured SMCs. LDL-induced SAPK/JNK activation was observed in both normal and Watanabe rabbit SMCs, which are LDL-receptor deficient, and this induction was not influenced by heparin, an inhibitor of LDL-receptor binding, indicating a process independent of classic LDL receptors.

As described above, oxidized LDL accumulation in the vascular wall is a characteristic feature of atherosclerosis.^{54,55} Oxidized LDL can be taken up into monocytes/macrophages to form foam cells via scavenger receptors, eg, CD36.⁵⁶ CD36 has been demonstrated to be abundantly expressed in lipid-laden macrophages around the core region of atherosclerotic lesions.⁵⁷ Oxidized LDL-CD36 interaction leads to increased expression of CD11b, CD18, cytokines such as IL-1 and TNF, and PDGF.⁵⁸ Although the mechanisms that transduce

Figure 6. Elevated p53 proteins coincided with SAPK/JNK1. **A:** Western blot analysis. Intima and media (I/M) from chow-fed rabbits and media (M) and atherosclerotic-lesioned intima (AS) from cholesterol-fed rabbits (16-week diet) were dissected on ice from the remaining adventitia with a tweezer and scissors. Tissues were frozen in liquid nitrogen and homogenized with a polytron homogenizer. Protein extracts (50 μ g/lane) were separated on 15% SDS-polyacrylamide gel, transferred onto membrane, and probed with the antibody against p53 and anti-actin antibodies after stripping (**A, lower panel**). Immunocomplexes were visualized by a Western blot detection kit. Each lane represents an individual animal. **B:** Immunoprecipitation (IP) and Western blot analysis. SAPK/JNK1 proteins were immunoprecipitated from the protein extractions (2 mg). The immunoprecipitants were separated on 15% SDS-polyacrylamide gel, transferred onto membrane, and probed with the antibody against p53 and SAPK/JNK1 after stripping. Immunocomplexes were visualized by a Western blot detection kit. Each lane represents proteins derived from three animals.

signals from oxidized LDL-CD36 binding to the nucleus are not well defined, previous studies have suggested that CD36 mediates nuclear factor κ B activation in response to oxidized LDL.⁵⁹ We found that LDL and oxidized LDL strongly stimulate SAPK/JNK activation, independent of classic LDL receptors, implicating the involvement of scavenger receptors. Therefore, CD36 activation may be involved in oxidized LDL-activated SAPK/JNK signal pathways.

In addition to LDL, SAPK/JNK can be activated by cytokines, mechanical force, oxidative stress, and growth factors present in high concentrations in atherosclerotic lesions or involved in the pathogenesis of atherosclerosis.^{1–12} These factors may be partially responsible for elevated SAPK/JNK activities in lesions observed here. After activation, SAPK/JNK kinases can phosphorylate or activate several transcription factors, including c-Jun, ATF₂, Elk, and p53.^{16–23} ATF₂ can dimerize not only with c-Jun, but also with itself and some other members of the activation transcription factor family, including ATF₃, CREB, and nuclear factor κ B. Elk-1, together with serum response factor, controls transcription from the serum response element. These transcription factors regulate

Figure 7. Elevated protein levels of BAX and BCL-X_S in atherosclerotic lesions. Protein extracts from intima and media (I/M) from chow-fed rabbits and atherosclerotic-lesioned intima (AS) and media (M) from cholesterol-fed rabbits (16-week diet) were prepared as described in the legend to Figure 4. Western blot analysis was performed with antibodies against BAX (**A**) or BCL-X_{S/L} (**B**) and reprobbed with anti-actin antibodies (**lower panel**) after stripping. Immunocomplexes were visualized by a Western blot detection kit. Each lane represents an individual animal. Data are from two independent experiments.

gene expression, including matrix metalloproteinases, adhesion molecule E-selectin, NO synthase, IL-8, and proliferating cell nuclear antigen.^{1–3,60} These genes have been demonstrated to play a key role in cell growth and cellular homeostasis in the development of atherosclerosis. Thus, our findings of SAPK/JNK activation in atherosclerotic lesions could be important to understanding the mechanism controlling expression of these genes during atherogenesis.

Apoptosis of SMCs and macrophages has recently been demonstrated in atherosclerotic lesions of humans as well as animal models.^{30–33,61–63} Oxidized LDL induces apoptosis of endothelial cells, SMCs, and macrophages depending on p53.^{27–29} Interestingly, isolated SMCs from human atherosclerotic plaques were shown to have a higher propensity for both spontaneous and induced apoptosis compared with SMCs from normal vessels.³¹ On the other hand, activation of MEKK1 (a SAPK/JNK upstream kinase) SAPK/JNK pathways is implicated in the initiation of apoptosis in other cell types in response to stress stimuli.^{64,65} In our study, we demonstrated coincidence of p53, pro-apoptotic protein, BAX, and BCL-X_S with selective activation of SAPK/JNK in

Figure 8. LDL-activated and oxidized LDL-activated SAPK/JNK in SMCs. Vascular SMCs were dissociated from human carotid arteries by collagenase and cultivated in DMEM medium. LDL were isolated from human EDTA-plasma by density ultracentrifugation and oxidized by incubation with Cu^{+2} . Subconfluent cells were serum-starved for 3 days and incubated with LDL (**A**) or oxidized LDL (**B**) (100 µg/ml) at 37°C for the indicated times or for 20 minutes in the presence of LDL (**C**) or oxidized LDL (**D**) in the indicated concentrations. For kinase assay, SAPK/JNK1 proteins were immunoprecipitated from the protein extracts (0.5 mg) and their kinase activities measured based on phosphorylation of GST-c-Jun substrate. For Western blot analysis (**E**), immunoprecipitated SAPK/JNK1 from 0.1-mg protein extracts was probed with phosphorylated-SAPK/JNK and SAPK/JNK1 after stripping. Data are from two independent experiments. Ctl, negative control; S, fetal calf serum treatment without LDL addition.

tissues of atherosclerotic lesions, indicating the involvement of SAPK/JNK in mediating apoptosis *in vivo* in response to hypercholesterolemia. It is conceivable that some cells in the lesions with higher SAPK/JNK activities and p53 proteins are subjected to apoptosis, during

Figure 9. LDL-receptor-independent SAPK/JNK activation. Arterial SMCs from normal or LDL receptor-deficient (Watanabe) rabbits (**A**) or humans (**B** and **C**) were cultivated in DMEM medium supplemented with 20% FCS. SMCs were serum-starved for 3 days, and preincubated with chloroquine (Chl) or NH_4Cl (**C**) for 30 minutes. LDL were directly added (**A** and **C**) or preincubated with heparin (2 mg/ml; **B**) for 40 minutes before addition to SMCs. SAPK/JNK1 proteins were immunoprecipitated from the protein extractions and their kinase activities measured based on phosphorylation of GST-c-Jun substrate. Data are from two independent experiments. -, negative control of Watanabe rabbit SMCs, which have similar levels of kinase activities as those of normal rabbit SMCs; S, fetal calf serum treatment without LDL addition.

which, SAPK/JNK-mediated signal transduction pathways, ie, SAPK/JNK-activated p53, could be important *in vivo*.

As described above, LDL and/or oxidized LDL stimulate SMC proliferation as well as apoptosis, and both cell proliferation and apoptosis co-exist in atherosclerotic lesions. We believe that LDL and oxidized LDL can initiate several signaling pathways leading to (de)differentiation, proliferation, or apoptosis, and that the balance between the positive and negative signals in vascular SMCs is critical for maintaining homeostasis of the arterial wall. Further understanding of the mechanisms regulating SAPK/JNK activities could lead to new strategies for the prevention or treatment of atherosclerosis.

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