Hyperexpression and Activation of Extracellular Signal–Regulated Kinases (ERK1/2) in Atherosclerotic Lesions of Cholesterol-Fed Rabbits

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Abstract—A hallmark of hyperlipidemia-induced atherosclerosis is altered gene expression that initiates cell proliferation and (de)differentiation in the intima of the arterial wall. The molecular signaling that mediates this process in vivo has yet to be identified. Extracellular signal–regulated kinases (ERKs) are thought to play a pivotal role in transmitting transmembrane signals required for cell proliferation in vitro. The present studies were designed to investigate the activity, abundance, and localization of ERK1/2 in atherosclerotic lesions of cholesterol-fed rabbits. Immunofluorescence analysis revealed abundant and heterogeneous distribution of ERK1/2, mainly localized in the cap and basal regions of atheromas. A population of ERK-enriched cells was identified as α-actin–positive smooth muscle cells (SMCs). ERK1 and 2 were heavily phosphorylated on tyrosyl residues and coexpressed with proliferating cell nuclear antigen in atherosclerotic lesions. ERK1/2 protein levels in protein extracts from atherosclerotic lesions were 2- to 3-fold higher than the vessels of chow-fed rabbits, and their activities were elevated 3- to 5-fold over those of the normal vessel. SMCs derived from atherosclerotic lesions had increased migratory/proliferative ability and higher ERK activity in response to LDL stimulation compared with cells from the normal vessel. Inhibition of ERK activation by PD98059, a specific inhibitor of mitogen-activated protein kinase kinases (MEK1/2), abrogated LDL-induced SMC proliferation in vitro. Taken together, our findings support the proposition that persistent activation and hyperexpression of ERK1/2 may be a critical element to initiate and perpetuate cell proliferation during the development of atherosclerosis.

Key Words: atherosclerosis ■ animal models ■ MAP kinases ■ ERK ■ signal transduction

Mitogen-activated protein (MAP) kinase–mediated signal transduction pathways contribute to cell growth and differentiation. The MAP kinase isoforms of 42 and 44 kDa, so-called extracellular signal–regulated kinases (ERK1/2), are expressed in most, if not all, mammalian cell types. ERK1 and 2 were initially identified as 2 protein kinases that became phosphorylated on tyrosine in response to growth factors. ERK-mediated signal pathways are a multistep phosphorylation cascade that transmits signals from the cell surface to cytosolic nuclear targets, which are responsible for the activation and phosphorylation of a number of other regulatory proteins, including p90rsk, cPLA2, and transcription factors needed for the expression of genes involved in cell proliferation. In addition, the activation of the cascade is also required for passing through certain checkpoints in the cell cycle, eg, G1/S and G2/M, in proliferating cells in vitro. Therefore, MAP kinase–mediated signal pathways play a key role in initiating cell proliferation and differentiation.

A high concentration of circulating cholesterol or LDLs is believed to be a major risk factor for atherosclerosis. The main pathophysiological role of LDL is to deliver cholesterol to vascular smooth muscle cells (SMCs) and macrophages, which form foam cells in the development of atherosclerosis. In addition to lipid transport, LDL can effectively stimulate SMC proliferation, a key event in the formation of atherosclerosis. There is evidence that LDL induces gene expression of platelet-derived growth factor (PDGF), PDGF receptors, c-fos, and egr-1, which are essential transcription factors for SMC proliferation. However, the precise signal transduction pathways that link to hypercholesterolemia and quantitative changes in gene expression in the pathogenesis of atherosclerotic lesions are largely unknown.

Most of our knowledge concerning the activation and function of ERK1/2 has come from studies on cultured cells; little is known about their activation in vivo and their relevance to atherogenesis in animal models. We examined ERK1/2 expression, localization, and activation in atherosclerotic lesions of cholesterol-fed rabbits and provide the first evidence of ERK overexpression and activation in lesions.

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Moreover, we demonstrate that the increased migratory/proliferative ability of SMCs derived from the lesions correlates with ERK1/2 activities, which are induced by LDL from chow- and cholesterol-fed rabbits.

Methods

Rabbit Model for Atherosclerosis

Twenty New Zealand White male rabbits weighing between 1800 and 2200 g were obtained from Charles River (Kisslegg, Germany). All animals were selected for serum cholesterol levels <100 mg/dL and were 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 (TT75; Tagger & Co) or a cholesterol-enriched diet (0.2% wt/wt) for 16 weeks, as described previously. Animals were killed 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 placed into cold PBS (4°C), and prepared for histological analysis, tissue culture, and protein extractions. For conventional histology, tissue fragments were fixed in 4% buffered (pH 7.2) formaldehyde, embedded in paraffin, and sectioned for hematoxylin-eosin staining.

Blood Cholesterol

Blood (1 to 2 mL) was taken from the central ear artery of rabbits that had been fasted for 16 hours. Serum total cholesterol values were measured every 2 or 4 weeks by an enzymatic procedure (Sigma). Briefly, 10 μL serum was added to 1 mL solution of cholesterol test kit and incubated for 18 minutes at room temperature followed by photometer measurement at 500-nm excitation wavelength (Dynatech Laboratories Inc).

Immunohistochemical and Immunofluorescence Double Staining

The procedure used for immunohistochemical staining was similar to that described elsewhere. Briefly, serial 4-μm-thick frozen sections were overlaid with mouse monoclonal antibodies against α-actin (Sigma), macrophages (RAM11; Dako), or CD3+ T cells (L11/135; ATCC; catalogue No. TIB188); incubated with rabbit anti-mouse Ig conjugated with peroxidase (Dako); and developed for 20 minutes at room temperature with a substrate solution. For immunofluorescent staining, a mouse monoclonal antibody against ERK1/2 (Transduction) was added to the sections. After 30 minutes, the supernatant was collected, and protein concentration was measured with Bio-Rad protein assay reagent.

Western Blot Analysis

Protein extracts (50 μg/lane) prepared from the arterial tissues described above were separated by electrophoresis through a 10% SDS-polyacrylamide gel and transferred to an Immobilon-P transfer membrane. The membranes were processed with the monoclonal antibody against ERK1/2 or phosphorlated ERK1/2 (Santa Cruz Biotechnology). Specific antigen-antibody complexes were then detected with the ECL Western Blot Detection Kit (Amersham). The blots were stripped for 3 minutes at 70°C in the buffer containing 60 mmol/L Tris, 2% SDS, and 100 mmol/L 2-mercaptoethanol; labeled with a monoclonal antibody against β-actin (Sigma); and developed as described above. Graphs of blots were obtained in the linear range of detection and were quantified and normalized to the level of actin by scanning laser densitometry (Power-look II, UMAX Data System Inc) of graphs.

Kinase Assay

Supernatant (0.5 mL) containing 0.5 mg proteins was incubated with 10 μL of goat anti-ERK2 antibodies (Santa Cruz Biotechnology) for 2 hours at 4°C with rotation. Subsequently, 40 μL of protein G–agarose suspension (Santa Cruz Biotechnology) was added, and rotation continued for 1 hour at 4°C. The immunocomplexes were precipitated by centrifugation and washed 2 times with buffers A, B (500 mmol/L LiCl, 100 mmol/L Tris, 1 mL/mmol DTT, 0.1% Triton X-100; pH 7.6), and C (20 mL/mmol MOPS, 2 mL/mmol EGTA, 10 mL/mmol MgCl2, 1 mL/mmol DTT, 0.1% Triton X-100; pH 7.2), respectively. ERK2 activities in the immunocomplexes were measured as described previously.

Briefly, immunocomplexes were incubated with 35 μL of buffer C supplemented with myelin basic protein (MBP; 6 μg; Upstate Biotechnology), [γ-32P]ATP (5 μCi), and MgCl2 (50 mL/mL) for 20 minutes at 37°C, with vortexing every 5 minutes. To stop the reaction, 15 μL of 4X Laemmli buffer was added, and the mixture was boiled for 5 minutes. Proteins in the kinase reaction were resolved by SDS-PAGE (15% gel) and subjected to autoradiography.

Cell Culture and Proliferation Assays

Rabbit vascular SMCs were cultivated from their aortas by a modification of the procedure described by Ross and Kariya. In short, thoracic aortas of chow- and cholesterol-fed rabbits were removed and washed with RPMI 1640 medium (Gibco). Intima with lesions and normal media were carefully dissected from the vessel, cut into pieces (~1 mm²), and explanted onto a 0.02% gelatin-coated plastic bottle (Falcon). The bottle was incubated upside-down at 37°C in a humidified atmosphere of 95% air/5% CO2 for 3 hours, resulting in firm attachment of the explanted tissues, and then medium supplemented with 20% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL) was slowly added. The outgrowths of SMCs from the explants were counted at days 5, 10, and 15 under the microscope. The percentage of the outgrowth was determined by counting positive tissue explants with cell growth over all explanted tissue segments (50 to 100 pieces per bottle). Cells were passaged by treatment with 0.05% trypsin/0.02% EDTA solution. Experiments were conducted on SMCs between passages 5 and 10 that had just achieved confluence. The purity of SMCs was routinely confirmed by immunostaining with antibodies against α-actin.

For proliferation assays, SMCs (1×10⁴) cultured in 96-well plates in medium containing 10% FCS at 37°C for 24 hours were serum-starved for 2 days. SMCs were treated with PD 98059 (Calbiochem) for 30 minutes, and then LDL (100 μg/mL) in 2% serum was added and incubated at 37°C for 24 hours. [3H]Thymidine was added 6 hours before cell harvest. Radiation activities were measured.

LDL Isolation

EDTA plasma was collected from normocholesterolemic and hypercholesterolemic rabbits fasted overnight. Lipoproteins were prepared by differential centrifugation with solid KBr to adjust the density as described previously. LDLs were obtained in the fraction between 1.020 and 1.050 g/mL. The sample was dialyzed against 30 minutes, the supernatant was collected, and protein concentration was measured with Bio-Rad protein assay reagent.

Hu et al ERK Activation in Atherosclerosis 19

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150 mmol/L NaCl with 0.1 mmol/L EDTA, sterilized through a 0.2-μm Millipore membrane, and stored at 4°C up to 3 weeks. No oxidation of LDL was observed at least 3 weeks after LDL isolation, as determined by measurement of malondialdehyde by the thiobarbituric acid method. Endotoxin contents of freshly isolated LDL and LDL after 3 weeks of storage at 4°C were both below the detection limit (<1 ng/mL, Endotoxin Kit, Sigma). Concentrations of LDL were determined gravimetrically by aliquot weight after drying, and quantities of lipoproteins were expressed as total weights.\(^{22,23}\)

**Statistical Analysis**

ANOVA was performed for multiple comparisons. The Mann-Whitney U test was used for comparison between 2 groups. A value of \(P<0.05\) was considered statistically significant.

**Results**

**Atherosclerotic Lesions in Hypercholesterolemic Rabbits**

Blood cholesterol levels in rabbits receiving a 0.2% cholesterol diet were significantly elevated and reached 350 mg/dL at 2 weeks and >600 mg/dL at 16 weeks. Animals in the control group (chow diet) had blood cholesterol levels <100 mg/dL. (Figure 1). To verify atherosclerotic lesions in cholesterol-fed rabbits, aortas were examined morphologically and immunohistologically 16 weeks after cholesterol feeding. Areas with lesions in the surface of aortic intima covered 50% to 80% of intima in cholesterol-fed rabbits. Figure 2A and 2B depicts the histological appearance of vessel walls of rabbits that received chow and cholesterol diets, respectively. Intima of normal vessel walls constituted a monolayer endothelium and a little connective tissue, but aortic lesions of rabbits fed

![Figure 1. Serum cholesterol levels in rabbits. The values of serum cholesterol were measured every 2 or 4 weeks by an enzymatic procedure. Serum cholesterol levels in rabbits fed a 0.2% cholesterol diet (shaded bars) were significantly higher than in those (open bars) receiving the chow diet.](image)

![Figure 2. Cell compositions in atherosclerotic lesions. Rabbits receiving a chow or cholesterol-enriched diet for 16 weeks were killed, and aortic tissue fragments were frozen in liquid nitrogen or fixed in 4% buffered (pH 7.2) formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (A and B). Cryostat sections from aortic segments of rabbits fed a 0.2% cholesterol diet for 16 weeks (C through F) were labeled with normal mouse Ig as a negative control (C) or monoclonal antibodies against \(\alpha\)-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. Arrows indicate internal elastic lamina; arrowheads point to examples of positive-stained cells. Magnification ×250.](image)
a cholesterol-enriched diet were characterized by cell proliferation, foam cell accumulation, and lipid deposition in the intima (Figure 2B). To identify the main cellular components in atherosclerotic lesions, serial sections of the vessels were incubated with a battery of antibodies to specific cell markers. α-Actin–positive SMCs appeared in various stages of lesions, most frequently in advanced lesions (Figure 2D). Cells expressing the macrophage antigen identified by the RAM11 antibody were observed in all atherosclerotic lesions (Figure 2E), including early and advanced stages, most appearing as foam cells. Finally, T lymphocytes identified by monoclonal antibody L11/135 were frequently observed, especially in advanced lesions (Figure 2F).

ERK Hyperexpression and Activation in Atherosclerotic Lesions

From each group, 5 aortic specimens were immunohistologically stained with a monoclonal antibody against mammalian ERK1/2. Normal artery showed very weak staining, if any (Figure 3a). Nonspecific reactivity was minimal in the negative control labeled with normal mouse serum (Figure 3b), whereas the lesion-covered areas in intima from rabbits receiving a cholesterol-rich diet showed increased immunostaining intensity (Figure 3c). In the small lesions, some areas within the intima became positively stained, whereas fatty streaks displayed elevated ERK1/2 content in subendothelial regions. Heterogeneity of ERK1/2 staining became more evident in atherosclerotic plaques. Sites of increased ERK1/2 were mainly within the cap and base regions of the atherosclerotic plaque (Figure 3c).

Both ERK1 and ERK2 kinases are activated by dual phosphorylation of tyrosine and threonine residues in response to mitogenic or stress stimuli, In addition, tissues derived from atherosclerotic lesions are heterogeneous with respect to cell types (Figure 2). Therefore, we performed immunofluorescence double staining to identify the cells expressing activated ERK1/2 in lesions. Figure 4a through 4c shows data representing double staining with antibodies against phosphorylated ERK1/2 (a; green), α-actin (b; red), and counterstaining with Hoechst 33258 (c; blue). Typical double-positive cells are indicated by arrows, demonstrating a population of SMCs in lesions expressing activated ERK1/2. In addition, some macrophages were also positively stained with phosphorylated ERK1/2, indicating an activated or proliferating state.

There is evidence that ERK activation is required for passing through certain checkpoints in the cell cycle in proliferating cells in vitro. We performed experiments with immunofluorescence double staining with antibodies against phosphorylated ERK1/2 (a; green), PCNA (b; red), and counterstaining with Hoechst 33258 (c; blue). Figure 5 shows that the most PCNA-positive cells had higher levels of phosphorylated ERK1/2.

To further show that ERK1/2 proteins were increased in atherosclerotic lesions, protein extracts from tissues of normal intima/media and intima and media with lesions were analyzed by Western blot analysis. Abundant ERK1/2 pro-
Protein in atherosclerotic lesions were observed (Figure 6A). ERK proteins in intima with lesions were significantly higher than intima/media of control animals and media of cholesterol-fed rabbits (Figure 6A, bottom) when they were normalized with respect to actin levels of the same blots.

Western blot analysis using protein extracts from the arterial tissues and the antibody recognizing the phosphorylated ERK1/2 was also performed. The activated (phosphorylated) forms of p42 and p44 were identified, which showed marked increases in protein extracts of atherosclerotic lesion tissues (Figure 6B). These results demonstrated that ERK phosphorylation is present in lesions. Furthermore, ERK1/2 activity of protein extracts was also measured with MBP used as a substrate. Figure 6C shows the results of an experiment examining ERK1/2 activities in the vessel wall. Obviously, ERK1/2 activities were found at low levels in control vessels and media of cholesterol-fed rabbits but increased 3- to 5-fold in atherosclerotic lesions (Figure 6C).

Increased ERK Activities in Lesion-Derived SMCs

To compare cell proliferation and ERK activation between SMCs from atherosclerotic lesions and normal vessels, tissues were explanted onto gelatin-coated bottles, and SMC migration and/or proliferation from the tissues was evaluated microscopically. Data shown in Figure 7 are percentages of outgrowth SMCs around the tissue fragments. The results indicate that the migration/proliferation ability of SMCs from lesions was significantly higher than that of normal vessels.

To compare ERK activities in different types of SMCs, cellular protein extracts containing similar amounts of actin...
were used for immunoprecipitation with the specific antibody against ERK2, and kinase activities were measured on the basis of phosphorylation of basic myelin protein as a substrate. When lesion-derived SMCs were stimulated with LDL of normocholesterolemic or hypercholesterolemic rabbits, ERK2 activation was induced by both types of LDL at similar magnitudes (Figure 8A). However, ERK2 activities in lesion-derived SMCs stimulated with hypercholesterolemic LDL, PDGF, and serum were higher than those of SMCs derived from normocholesterolemic rabbits (Figure 8B). Taken together, these observations support the notion that alterations in ERK activation in the development of atherosclerosis of cholesterol-fed rabbits are due to changed sensitivity of SMCs to LDL and mitogen stimulation.

**Inhibition of ERK Activation and SMC Proliferation**

Because ERK-mediated signal pathways are crucial in mediating cell migration and proliferation, the effects of PD98059, a MAP kinase kinases 1/2 inhibitor, on LDL-stimulated ERK activation and SMC proliferation were investigated. A marked activation of ERK1/2 by LDL was found, which was inhibited by PD98059 in a concentration-dependent manner (Figure 9A); ERK2 kinase phosphorylation was completely abolished by 50 μmol/L PD98059. We had observed, by measuring [3H]thymidine incorporation, that LDL effectively induced SMC DNA synthesis. Figure 9B depicts PD98059 inhibition of SMC proliferation stimulated by LDL. A concentration of 50 μmol/L for treatment of SMCs completely abrogated SMC proliferation. Thus, blocking ERK-mediated signaling inhibits SMC proliferation induced by LDL.

**Discussion**

Activation of ERK kinase cascades is one of the major pathways for the regulation of proliferation and cell growth in various cultured cells.1–4,25,26 Reversible protein phosphorylation is the established mechanism of regulation of ERK kinases,27 an activity controlled by a family of dual-specificity protein kinases and a complex upstream cascade.4 A transient kinase activation or attenuation is seen in most in vitro cultured cells in response to chronic stimulation. In the present studies, we have demonstrated that in vivo, ERK activation is sharply elevated in lesions, which cannot be ascribed solely to phosphorylation of the proteins. Immunoblotting revealed a marked increase in the amount of ERK1/2 proteins from atherosclerotic lesions compared with normal vessel tissues or aortic media of cholesterol-fed rabbits.

Sustained expression and activation of ERK kinases in ERK-transfected fibroblasts resulted in oncogenicity associated with cell proliferation.28 Hyperexpression and activation...
of these kinases may play a main role in regulation of cell proliferation in the pathogenesis of atherosclerosis.

Chamley-Campbell et al.\(^9\) hypothesized that 2 distinct SMC phenotypes, contractile and synthetic, exist in the vessel wall and that SMCs in the atherosclerotic plaque differ from those in the normal tunica media.\(^30\) It has been established that SMCs in intimal lesions display increased levels of genes for growth factors,\(^14\) tumor necrosis factors,\(^31\) class II histocompatibility antigens,\(^32\) vascular cell adhesion molecule-1,\(^33\) and intercellular adhesion molecule-1.\(^34,35\) On the basis of these observations, Libby and Li\(^36\) called them "activated" SMCs. Our findings of selective or differential hyperexpression and activation of ERK protein kinases in atherosclerotic SMCs support the concept that the ERK level and activity in SMCs reflect a situation of gene expression, activation, and replication of this SMC population. These higher ERK activities from lesional SMCs can be maintained even in vitro culture for longer periods of time, further supporting the notion that SMCs from atherosclerotic lesions have been selected and differ from those from normal vessels.

Proliferation of vascular SMCs is a hallmark in the pathogenesis of atherosclerosis.\(^14\) LDL and oxidized LDL are mitogenic to cultured SMCs and have been demonstrated to activate ERK signal pathways in cultured cells in vitro.\(^37–40\) In the present study, we provide the first evidence that hypercholesterolemia can stimulate ERK expression and activation in the intima but not media or tissues (Figures 3 and 6) from other organs, including liver, kidney, brain, and heart (data not shown). Previously, we demonstrated that acute elevation in blood pressure induced by restraint or hypertensive agents resulted in MAP kinase activation in the media of the arterial wall.\(^41\) In the present experiments, we minimized the effect of animal handling on blood pressure fluctuation by daily conditioning of rabbits with intramuscular saline injection for 1 week before their death. This treatment was shown to be effective because kinase activities of the vessel wall from control rabbits and media from cholesterol-fed rabbits were of similar, and lower, levels. Such tissue-specific activation of ERK kinases induced by hypercholesterolemia may explain why the lesion is localized only in the arterial intima and may be due to different responses of various types of cells to lipids or LDL stimulation, ie, hypercholesterolemia induces atherosclerosis but not kidney or heart sclerosis. Thus, our findings could significantly enhance our understanding of the pathogenesis of atherosclerosis during hyperlipidemia.

Recent studies have focused on the signaling events in cultured cells from cardiovascular tissue, including myocytes and SMCs, which may provide a new strategy for therapeutic
intervention.\textsuperscript{3,25,42,43} Depletion of MAP kinases with an antisense oligodeoxyribonucleotide downregulates the phenylephrine-induced hypertrophic response in rat cardiac myocytes.\textsuperscript{44} Accumulating evidence indicates that MAP kinase phosphatase (MKP-1) specifically inhibits mitogen-activated induction of MAP kinases in cell lines.\textsuperscript{45–47} Lai et al.\textsuperscript{48} reported a reduction of MKP-1 expression in rat carotid arteries in response to balloon injury, which may be responsible for sustained activation of ERK2 during restenosis of the injured artery.\textsuperscript{49} In the present study, we demonstrate that inhibition of the ERK kinase activation by PD98059 abrogates SMC proliferation. The therapeutic effect of ERK antagonist or inhibitor on lesion formation should be addressed in future studies. Thus, understanding of the mechanisms serving to regulate MAP kinase activities could lead to new strategies for prevention or therapeutic intervention for atherosclerosis.

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