Ras Induces Vascular Smooth Muscle Cell Senescence and Inflammation in Human Atherosclerosis

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Background—Vascular cells have a finite cell lifespan and eventually enter an irreversible growth arrest, cellular senescence. The functional changes associated with cellular senescence are thought to contribute to human aging and age-related vascular disorders. Ras, an important signaling molecule involved in atherogenic stimuli, is known to promote aging in yeast and cellular senescence in primary human fibroblasts. The aim of this study was to investigate the role of Ras-induced vascular smooth muscle cell (VSMC) senescence in atherogenesis.

Methods and Results—We introduced an activated ras allele (H-rasV12) into human VSMCs using retroviral infection. Introduction of H-rasV12 induced a growth arrest with phenotypic characteristics of cellular senescence, such as enlarged cell shapes and increases in expression of cyclin-dependent kinase inhibitors and senescence-associated β-galactosidase (SA-β-gal) activity. Activation of Ras drastically increased expression of proinflammatory cytokines, in part through extracellular signal-regulated kinase activation. To determine whether Ras activation induces cellular senescence in vivo, we transduced the adenoviral vector encoding H-rasV12 into rat carotid arteries injured by a balloon catheter. Introduction of Ras into the arteries enhanced vascular inflammation and senescence compared with mock-infected injured arteries. Moreover, SA-β-gal–positive VSMCs were detected in the intima of advanced human atherosclerotic lesions and exhibited increased levels of extracellular signal-regulated kinase activity and proinflammatory cytokine expression.

Conclusions—Our results suggest that atherogenic stimuli mediated by Ras induce VSMC senescence and vascular inflammation, thereby contributing to atherogenesis. This novel mechanism of atherogenesis may provide insights into a new antisenescence treatment for atherosclerosis. (Circulation. 2003;108:2264-2269.)

Key Words: aging ■ inflammation ■ atherosclerosis

Activation of Ras protein has been demonstrated to promote cell proliferation and transformation in immortal cell lines derived from various types of mammalian cells and thus has been thought to contribute to tumorigenesis in humans.1,2 In yeast, however, loss-of-function mutations in the Ras signaling pathway have been shown to extend longevity, which indicates that Ras activity negatively controls lifespan.3 Recently, in primary human fibroblasts, constitutive activation of the Ras signaling pathway has been reported to provoke cellular senescence, originally defined as a phenotype of arrested cells at the end of the replicative lifespan.4–6

Cellular senescence is accompanied by a specific set of changes in cell function, morphology, and gene expression. A number of studies have indicated that many of the changes in senescent vascular cell behavior are consistent with known changes seen in age-related vascular diseases including atherosclerosis, which suggests that these changes in cellular phenotype may contribute to atherogenesis.7 In addition, it has been reported that enlarged vascular cells that resemble senescent cells in vitro are frequently found in human atherosclerotic plaque and that in vitro growth properties of vascular cells isolated from atherosclerotic lesions are impaired compared with normal lesions, which implies that vascular cell senescence might occur in vivo.8,9

Consistent with this notion, we have demonstrated previously that senescent vascular endothelial cells are predominately localized in the plaque of human atherosclerosis but not in normal lesions and that vascular cell senescence results in endothelial dysfunction.10 Given that various atherogenic stimuli, including growth factors and oxidative stress, are mediated by Ras activity,11–12 it is assumed that activation of Ras promotes vascular cell senescence, thereby contributing to the pathogenesis of human atherosclerosis.

In the present study, we provide evidence that activation of Ras is involved in atherogenesis by inducing vascular smooth
muscle cell (VSMC) senescence. Constitutive activation of Ras promoted cellular senescence and drastically induced expression of proinflammatory cytokines in the primary cultures of human vascular cells, in part through extracellular signal-regulated kinase (ERK) activation. Introduction of Ras into the injured arteries enhanced vascular inflammation and senescence. Furthermore, activation of ERK and vascular inflammation were associated with VSMC senescence in human atherosclerosis, which suggests that Ras activity plays an important role in regulating VSMC lifespan and function in vivo.

Methods

Tissue Specimens
Advanced human atherosclerotic samples were obtained during surgery from 6 patients who had abdominal aortic aneurysm or peripheral arterial disease with their permission and subjected to senescence-associated β-galactosidase (SA-β-gal) staining within 3 hours after excision. Gastroepiploic arteries were obtained from 3 patients with an age range of 32 to 40 years who underwent gastric resection because of gastric cancer and used as normal control samples. The studies on human samples were approved by our institutional review board.

Histology
SA-β-gal activity was examined in tissue as described previously. Immunohistochemical analyses of the frozen sections (6 μm) were performed as described previously. Antibodies used were as follows: antibodies to phospho-ERK and interleukin (IL)-1β (Santa Cruz), anti-α-smooth muscle actin antibody for VSMCs (Pharmingen), and anti-CD68 antibody for macrophages (DAKO).

Retroviral Infection
Primary cultures of human aortic VSMCs were purchased from BioWhittaker and cultured according to the manufacturer’s instructions. Retroviral vector encoding H-rasV12 (pBabeh-rasV12) was the kind gift of Dr S.W. Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Retroviral stocks were generated by transient transfection of packaging cell line (PT67, Clontech) with the empty vector, pBabe (mock), or pBabeh-rasV12 as described previously.

In Vivo H-rasV12 Gene Transfer
A cDNA fragment encoding the H-rasV12 gene was cloned into a shuttle plasmid (pShuttle, Clontech), the excised fragment derived from the shuttle plasmid was then cloned into pAdeno-X (Clontech), and high-titer adenoviral stock was generated according to the user manual for Adeno-X Expression System. Rat carotid injury model from the shuttle plasmid was then cloned into pAdeno-X (Clontech), and high-titer adenoviral stock was generated according to the user manual for Adeno-X Expression System. Rat carotid injury model was prepared as described previously. After denudation, the H-rasV12 adenoviral vector or the empty adenoviral vector (5 × 10⁷ pfu/mL) was introduced into injured arteries. Two weeks after transduction, injured arteries were perfused with saline to remove blood cells from the lumen and analyzed for vascular inflammation. The experimental protocol for the present study was designed in accordance with the “Guide for Animal Experimentation,” Chiba University. Methods for Western blot and Northern blot are available in the Online Data Supplement.

Results

Activation of Ras Promotes VSMC Senescence
We first determined whether activation of Ras induces cellular senescence in VSMCs. We introduced an activated ras allele (H-rasV12) into human VSMCs using retroviral infection and compared these with VSMCs infected with the empty vector, pBabe (mock). Transduced cells were then purified by use of puromycin for 4 days. After selection, 3 × 10⁶ cells were seeded onto a 100-mm-diameter dish on the fifth day (designated day 0) and subjected to analyses for cell morphology, cell growth, and gene expression. H-rasV12-transduced VSMCs became flat and enlarged by day 3 (top, Ras) compared with VSMCs infected with empty vector, pBabe (top, Mock), which resulted in increased activity of SA-β-gal (bottom, β-gal). B, Expression of cell-cycle regulatory proteins. Whole-cell lysates were extracted from H-rasV12–transduced (Ras) or mock-transduced (Mock) VSMCs on day 0 and examined for cell-cycle regulatory proteins by Western blot analysis. C and D, Expression of proinflammatory molecules. Total RNA samples or whole-cell lysates were extracted from H-rasV12–transduced (Ras) or mock-transduced (Mock) VSMCs on day 3 and analyzed for proinflammatory cytokines and chemokines by ribonuclease protection assay (C) and Western blotting (D). Expression levels of GAPDH or actin served as internal controls. Similar results were obtained from 3 independent experiments.
by H-rasV12, we examined expression of cell-cycle regulatory proteins. We found that Ras activation resulted in elevated expression of p53 (2-fold, \( P < 0.05 \)) and p21 (17-fold, \( P < 0.01 \)) as well as p16 (5-fold, \( P < 0.01 \); Figure 1B and Data Supplement Figure II), which suggests that Ras-induced growth arrest is different from quiescence, because neither p53 nor p16 accumulates during quiescence.

**Ras Activation Induces Proinflammatory Cytokines**

Atherosclerosis is characterized by the recruitment of monocytes into arterial walls, and this process involves various proinflammatory molecules such as cytokines and chemokines.\(^{16} \) To investigate the role of Ras-induced cellular senescence in atherogenesis, we examined expression of proinflammatory cytokines and chemokines in human VSMCs. Introduction of H-rasV12 drastically induced expression of proinflammatory cytokines and chemokines such as IL-1\( \alpha \) (11-fold, \( P < 0.05 \)), IL-1\( \beta \) (50-fold, \( P < 0.01 \)), IL-6 (12-fold, \( P < 0.05 \)), IL-8 (77-fold, \( P < 0.01 \)), and monocyte chemoattractant protein (MCP)-1 (6-fold, \( P < 0.05 \)) as demonstrated by ribonuclease protection assay and Western blot analysis compared with mock-infected VSMCs (Figures 1C and 1D and Data Supplement Figure III). These results suggest that activation of Ras may induce vascular inflammation in human atherosclerosis. Expression of proinflammatory cytokines was also elevated in VSMCs undergoing replicative senescence compared with young cell populations, although the increased levels were <10-fold (Data Supplement Figure IV).

**Role of ERK Activation in Ras-Induced VSMC Senescence and Inflammation**

We next investigated the potential downstream signaling pathways that are activated by H-rasV12 to promote VSMC senescence. A number of signaling cascades have been implicated in cell survival and cell growth induced by Ras.
activation, including mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt, and p38. H-rasV12-transduced VSMCs were analyzed for cell growth in the presence of specific kinase inhibitors: PD98059 for MEK/ERK, LY294002 for PI3K/Akt, or SB203580 for p38. Inhibition of each signaling cascade by the kinase inhibitor was verified by Western blot analyses for phospho-ERK (PD98059), phospho-Akt (LY294002), and phospho-ATF-2, which is known to be phosphorylated by p38 (SB203580; Figure 2A). Introduction of H-rasV12 resulted in growth arrest compared with mock-infected cells (P<0.001), and treatment with PD98059 but neither LY294002 or SB203580 significantly ameliorated the growth-inhibitory effects of Ras activation in VSMCs (P<0.001 versus Ras; Figure 2A). In addition, inhibition of the MEK/ERK signaling pathway prevented morphological changes induced by Ras activation, whereas inhibition of either the PI3K/Akt or p38 pathway had no effect (Figure 2B), which suggests that the MEK/ERK signaling pathway is involved in Ras-induced VSMC senescence. This idea is further supported by Western blot analyses for cell-cycle regulatory proteins. Increased expression of p53, p21, and p16 by H-rasV12 transduction was effectively inhibited by PD98059 (P<0.05 for p53 and p21, P<0.01 for p16 versus Ras) but not by either LY294002 or SB203580 (Figure 2C and Data Supplement Figure V). To elucidate the signaling pathways that contribute to Ras-induced cytokine expression, we performed ribonuclease protection assay in H-rasV12–infected VSMCs treated with the kinase inhibitors. Treatment with either PD98059 or SB203580 but not LY294002 significantly reduced expression levels of IL-1β (P<0.01 versus Ras) in Ras-infected VSMCs (Figure 2D, upper panel, and Data Supplement Figure VI). In contrast, all kinase inhibitors examined had no effect on expression of MCP-1 (Figure 2D, lower panel, and Data Supplement Figure VI). Thus, these data suggest that the MEK/ERK signaling pathway is important for Ras-induced cell growth arrest, but there are other pathways for vascular inflammatory response elicited by Ras activation.

Activation of Ras Induces VSMC Senescence and Inflammation In Vivo
To determine whether Ras activation induces cellular senescence and inflammation in vivo, we transduced the adenoviral vector encoding H-rasV12 (AdenoRas) or the empty vector (mock) into rat carotid arteries injured by a balloon catheter. We chose a rat carotid injury model for analysis of vascular inflammation because it is known that accumulation of macrophages is minimally involved in lesion formation in this model. Two weeks after transduction, injured arteries were analyzed for SA-β-gal activity. Whereas only a little SA-β-gal activity was found in mock-infected injured arteries infected with empty vector (Mock), transduction of AdenoRas into injured arteries increased SA-β-gal activity (Ras). Double staining for α-smooth muscle actin (brown) and SA-β-gal activity (blue) of AdenoRas-infected injured arteries indicates that these β-gal-positive cells were VSMCs in outer layer of intima (I) and media (M; right). Original magnification was ×1000. Scale bar=10 μm. B, Immunohistochemistry for macrophages in injured arteries. Two weeks after vascular injury and infection with empty vector or AdenoRas, arteries were perfused with saline to remove blood cells from lumen and analyzed for vascular inflammation. Accumulation of macrophages (brown) was markedly enhanced in AdenoRas–infected injured arteries (Ras) compared with injured arteries infected with empty vector (Mock). Arrowhead indicates disruption of media in AdenoRas–infected arteries. Original magnification was ×100. Scale bar=100 μm.

(data not shown). Furthermore, the area of accumulated macrophages in the intima was increased markedly (×8-fold, P<0.001) in AdenoRas–infected injured arteries compared with mock-infected injured arteries (Figure 3B and Data Supplement Figure VII), which indicates a causal relationship between Ras activation and vascular inflammation. Disruption of medial layers, one of the pathological features of human atheroma, was observed frequently in AdenoRas–infected injured arteries, whereas it was never detected in mock-infected injured arteries, further implicating a critical role of Ras activation in atherogenesis.

Senescent VSMCs in Advanced Human Atherosclerotic Plaque
To investigate the possible role of Ras-induced VSMC senescence in human atherosclerosis, we first examined SA-β-gal activity to locate senescent VSMCs in advanced human atherosclerotic plaque. Many of the intimal cells exhibited granular blue stainings in the cytoplasm that were quite similar to those of senescent cells in culture reported previously (Figure 4A).13 Double staining for α-smooth muscle actin and SA-β-gal activity identified SA-β-gal–positive cells as VSMCs in the intima (Figure 4A). No signal

A

Mock

Ras

α-SMA / β-gal

B

Mock

Ras

Figure 3. Activation of Ras induces VSMC senescence and vascular inflammation in vivo. A, SA-β-gal activity in injured arteries. Left panel shows photograph of injured arteries stained with β-gal staining. Whereas only a little SA-β-gal activity was found in injured arteries infected with empty vector (Mock), transduction of AdenoRas into injured arteries increased SA-β-gal activity (Ras). Double staining for α-smooth muscle actin (brown) and SA-β-gal activity (blue) of AdenoRas–infected injured arteries indicates that these β-gal-positive cells were VSMCs in outer layer of intima (I) and media (M; right). Original magnification was ×1000. Scale bar=10 μm. B, Immunohistochemistry for macrophages in injured arteries. Two weeks after vascular injury and infection with empty vector or AdenoRas, arteries were perfused with saline to remove blood cells from lumen and analyzed for vascular inflammation. Accumulation of macrophages (brown) was markedly enhanced in AdenoRas–infected injured arteries (Ras) compared with injured arteries infected with empty vector (Mock). Arrowhead indicates disruption of media in AdenoRas–infected arteries. Original magnification was ×100. Scale bar=100 μm.
was detected in a control staining with nonimmune IgG (data not shown). To ascertain whether SA-β-gal–positive cells in the intima were senescent VSMCs, we performed a double staining for p53 and SA-β-gal activity (blue) in intima and media. Many intimal cells exhibited granular blue stainings in cytoplasm that are colocalized with immunoreactivity of α-smooth muscle actin (Intima). In contrast, few SA-β-gal–positive VSMCs were observed in media (Media). Nuclei were counterstained with hematoxylin and are shown in purple. Original magnification was ×1000. Scale bars=10 μm. All samples examined provided similar results. B, Activation of ERK in senescent VSMCs in human atherosclerosis. Cross sections of atherosclerotic samples were double-stained with β-gal activity and anti-phospho-ERK. Activated ERK (brown) was detected in nucleus of β-gal–positive VSMCs in intima (Intima), whereas few medial VSMCs exhibited ERK activation (Media). Original magnification was ×1000. Scale bars=10 μm. Insets show immunostaining for α-smooth muscle actin of serial sections.

Figure 4. Senescent VSMCs in advanced human atherosclerosis. A, Double staining for α-smooth muscle actin (brown) and SA-β-gal activity (blue) in intima and media. Many intimal cells exhibited granular blue staining in cytoplasm that are colocalized with immunoreactivity of α-smooth muscle actin (Intima). In contrast, few SA-β-gal–positive VSMCs were observed in media (Media). Nuclei were counterstained with hematoxylin and are shown in purple. Original magnification was ×1000. Scale bars=10 μm. All samples examined provided similar results. B, Activation of ERK in senescent VSMCs in human atherosclerosis. Cross sections of atherosclerotic samples were double-stained with β-gal activity and anti-phospho-ERK. Activated ERK (brown) was detected in nucleus of β-gal–positive VSMCs in intima (Intima), whereas few medial VSMCs exhibited ERK activation (Media). Original magnification was ×1000. Scale bars=10 μm. Insets show immunostaining for α-smooth muscle actin of serial sections.

Discussion

It has been demonstrated that various molecules including growth factors, vasoactive peptides, and oxidative stress are induced during lesion formation and regulate numerous critical cell functions, thereby contributing to atherogenesis. These stimuli function as mitogens for VSMCs through the signaling cascades that activate Ras. Inhibition of Ras activity has been reported to prevent intimal formation after vascular injury, which suggests a critical role of Ras activation in VSMC proliferation. In the present study, the cell number of Ras-infected VSMCs was 1.5-fold more than that of mock-infected VSMCs on the third day after infection (T. Minamino, unpublished observation, 2002). However, Ras-infected cells were virtually growth arrested by day 3 (the eighth day after infection), whereas mock-infected cells were being proliferated (Figure 2A). Thus, it is likely that introduction of Ras initially promotes VSMC proliferation, and when constitutively activated, Ras induces VSMC senescence. This notion was further supported by the fact that senescent VSMCs were detected in advanced human atheroma but not in early lesions of atherosclerosis.

The present data provide evidence that constitutive activation of Ras induces vascular inflammation and senescence in vitro and in vivo. Consistent with our findings, functional inhibition of Ras has been demonstrated to suppress expression of proinflammatory molecules, thereby reducing lesion formation in apolipoprotein E-deficient mice. Moreover, angiotensin II, an important atherogenic molecule that activates the Ras signaling pathway, has been demonstrated to promote VSMC senescence and vascular inflammation. We found that treatment of human VSMCs with proinflammatory cytokines did not induce premature senescence (T. Minamino, unpublished observation). Thus, it is likely that increased...
expression of proinflammatory molecules is a result of cellular senescence rather than a cause. Vascular inflammation is known to promote degradation of extracellular matrix by various proteinases such as collagenases and gelatinases and by inhibition of matrix production.\(^{21}\) Therefore, it is assumed that decreased cellularity and enhanced inflammation associated with VSMC senescence may contribute to plaque vulnerability.

The growth signals mediated by Ras are known to promote aging in yeast.\(^{3}\) The present results suggest that the signaling pathway of cellular aging is conserved in human VSMCs and that constitutive mitogenic stimuli in the lesions induce a senescent phenotype in VSMCs, thereby contributing to atherogenesis. Gain-of-function mutation of Ras has been found frequently in human malignancy, whereas introduction of oncogenic Ras into human primary cultures results in cellular senescence. Thus, Ras-induced senescence is thought to be an antitumorigenic mechanism in normal somatic cells.\(^{22}\) Ras-induced growth arrest may have beneficial effects early in life to decrease the incidence of cancer, but late in life, this mechanism may promote the accumulation of senescent cells in a body, which results in age-associated diseases such as atherosclerosis.

In summary, we demonstrated the role of Ras-induced VSMC senescence in vitro and in vivo. Introduction of senescence by activation of Ras resulted in vascular inflammation, whereas inhibition of senescence reduced the inflammatory response. Activation of ERK and increased expression of proinflammatory cytokines were detected in senescent VSMCs in human atherosclerosis. We propose a novel mechanism of atherogenesis whereby senescence of VSMCs is induced by Ras, which in turn results in the induction of proinflammatory cytokines and chemokines, leading to further vascular inflammation. Thus, our results will provide insights into a novel antisenescence treatment for atherosclerosis.

Acknowledgments

This work was supported by grants from Takeda Medical Research Foundation, Takeda Science Foundation, Japan Heart Foundation, Mochida Memorial Foundation, Uehara Memorial Foundation, Mitsubishi Pharma Research Foundation, and the Ministry of Education, Science, Sports, and Culture of Japan (to Drs Minamino and Komuro). We thank Dr Lowe for pBabeHrasV12.

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_Circulation_. 2003;108:2264-2269; originally published online October 13, 2003; doi: 10.1161/01.CIR.0000093274.82929.22

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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