Role of p44/p42 MAP Kinase in the Age-Dependent Increase in Vascular Smooth Muscle Cell Proliferation and Neointimal Formation

Giuseppa Gennaro, Catherine Ménard, Edith Giasson, Sophie-Élise Michaud, Maria Palasis, Sylvain Meloche, Alain Rivard

Objective—Age-dependent increase in vascular smooth muscle cell (VSMC) proliferation is thought to contribute to the pathology of atherosclerotic diseases. In this study, we investigated the role of mitogen-activated protein kinases (MAPKs) on VSMC proliferation and neointimal formation in the context of aging.

Methods and Results—VSMCs were isolated from the aorta of young and old rabbits. The proliferative index after serum stimulation was significantly increased in old versus young VSMCs. This was associated with a significant and specific age-dependent increase in p44/p42 MAPK activation. Treatment with MEK inhibitor PD98059 successfully inhibited p44/p42 MAPK activities and VSMC proliferation. These results were confirmed in vivo using a model of balloon injury in rabbit iliac arteries. p44/p42 MAPK activities were rapidly induced by angioplasty in young and old animals. However, the levels of p44/p42 MAPK activities achieved in arteries of old rabbits were significantly higher than those of young rabbits. This was associated with a higher cellular proliferative index and a significant increase in neointimal formation in old animals. Local delivery of PD98059 in old rabbits successfully inhibited p44/p42 MAPK activities after angioplasty, which led to a significant reduction in cellular proliferation and neointimal formation in treated animals.

Conclusions—Our study suggests for the first time that increased p44/p42 MAPK activation contributes to augmented VSMC proliferation and neointimal formation with aging. p44/p42 MAPK inhibition could represent a novel therapeutic avenue against atherosclerotic diseases. (Arterioscler Thromb Vasc Biol. 2003;23:204-210.)

Key Words: atherosclerosis ■ aging ■ vascular smooth muscle cell proliferation ■ mitogen-activated protein kinase

Abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) play important roles in the pathophysics of atherosclerotic diseases. Previous studies have shown that, in marked contrast to most cell types, VSMCs isolated from old animals replicate more actively than corresponding cultures from young animals. Moreover, aging has been associated with an increased proliferative response of VSMCs after balloon angioplasty, and this response seems to be a function of the arterial segment rather than the host environment. Taken together, these findings suggest that age-dependent increase in VSMC proliferation may contribute to the increased prevalence and severity of atherosclerosis in the elderly.

The cellular and molecular mechanisms responsible for the enhanced proliferation of old VSMCs remain largely undefined. We have recently shown that augmented cyclin A expression via the action of the AP1 transcription factor c-fos contributes to the age-dependent increase in VSMC proliferation. However, the upstream elements involved in that signaling pathway are presently unknown. Mitogen-activated protein kinases (MAPKs) are a family of protein-serine/threonine kinases that include at least 4 distinctly regulated groups in mammals: extracellular signal–related kinases (ERK) 1/2 (or p44/p42 MAPK), Jun amino-terminal kinases (JNK1/2/3), p38 proteins, and ERK5. These enzymes phosphorylate different intracellular proteins and play important roles in regulating gene expression, cell proliferation, cell survival and death, cell motility, and cell differentiation. MAPKs are activated by a variety of stimuli, including growth factors, cytokines, antigens binding to T- and B-cell receptors, hormones that bind to G protein–coupled receptors, lipid compounds, and mechanical forces such as fluid shear stress and stretch. Moreover, MAPKs (p44/p42 MAPK and JNK) and MAP kinase phosphatase (MKP-1) have been reported to be activated in the arterial wall after balloon injury in different animal models. This suggests that MAPKs could represent a link between arterial injury and VSMC proliferation in atherosclerotic diseases.
In the present study, we investigated the effect of aging on MAPK activation after serum stimulation of VSMCs in vitro and in response to arterial injury in vivo. In both cases, we found that the induction of p44/p42 MAPK activities was significantly enhanced in old compared with young animals. In contrast, activities of MAPKs p38 and JNK were similar in young and old animals. We also demonstrate that the inhibition of p44/p42 MAPK activities can prevent the age-dependent increase in VSMC proliferation and neointimal formation. Thus, our study illustrates for the first time a specific signal transduction pathway by which increased p44/p42 MAPK activity contributes to greater VSMC proliferation and neointimal formation with aging. Our results suggest that p44/p42 MAPK could play a significant role in the physiopathology of atherosclerotic diseases, especially in the context of aging.

Methods
The Methods section can be found online at http://www.atvb.ahajournals.org.

Results
Effect of Aging on Rabbit VSMC Proliferation and Apoptosis
We compared the proliferative capacity of VSMCs isolated from the aorta of young or old rabbits using cellular counts and the MTS assay. The standard curves for the MTS assay were almost identical in young and old VSMCs (see Figure IA, available online at http://www.atvb.ahajournals.org). Using these curves to convert optical density measurements to cell number, we found a 80% increase in the proliferative activity of old versus young VSMCs after 24 hours of serum stimulation (Figure 1A, *P < 0.001). Importantly, the age-dependent increase in cellular proliferation was not found in other cell types, such as rabbit fibroblasts (Figure 1A) and rabbit myoblasts (data not shown), suggesting that this is a specific characteristic of VSMCs. We also investigated the effect of aging on the rate of apoptosis in VSMCs using FITC-Annexin V/PI dual color flow cytometry (see Figure IB, available online at http://atvb.ahajournals.org). Although a few cells (6% to 8%) were found to be apoptotic after 24 hours of serum stimulation, the level of apoptosis was similar in young and old VSMCs. This indicates that the age-dependent increase in cell number is not attributable to a decreased rate of apoptotic cell death in old VSMCs.

Comparison of MAPK Activation in Young and Old VSMCs
We used phospho-specific antibodies to compare the activation of MAPKs in serum-stimulated VSMCs harvested from young or old rabbits (Figure 1B). p44/p42 MAPK activities were very low or undetectable in quiescent VSMCs. After serum stimulation, maximal p44/p42 MAPK activities were seen at 30 minutes both in young and old VSMCs. However, p44/p42 MAPK activities were significantly higher in old compared with young VSMCs at 5 and 30 minutes after serum stimulation. In contrast, although both p38 and JNK activities were shown to be induced by serum, no significant difference in activities could be detected between young and old VSMCs. Moreover, the age-dependent increase in p44/p42 MAPK was specific for VSMCs, because a similar level of p44/p42 MAPK activation was found in young and old rabbit fibroblasts (Figure 1B) and myoblasts (data not shown). To confirm the effect of aging on p44/p42 MAPK activities in VSMCs, we performed specific kinase assays on cell lysates obtained at different time points after serum stimulation. As shown in Figure 1C, there was a significant increase in p44/p42 MAPK activities in old versus young VSMCs at 5 minutes (55% increase, *P = 0.02) and 30 minutes (47% increase, **P = 0.003) after serum stimulation.
Activities after treatment with PD98059 were still significantly higher in old versus young VSMCs at all the time points studied (100% increase at 5 minutes, 61% increase at 30 minutes, and 29% increase at 2 hours after serum stimulation, \( P<0.001 \)). We then evaluated the effect of p44/p42 MAPK inhibition on VSMC proliferation (Figure 2B). Treatment with PD98059 led to a 60% and 63% inhibition of the proliferative index of young and old VSMCs, respectively (\( P<0.001 \)). However, the residual proliferative activity after treatment with PD98059 was still significantly higher in old versus young VSMCs (\( P<0.001 \)). Treatment with PD98059 resulted in a 6% to 9% increase in the absolute rate of apoptosis in young and old VSMCs (see Figure IIB online at http://atvb.ahajournals.org). Our results suggest that the reduced cell number with PD98059 treatment is mostly attributable to decreased cellular proliferation and, to a lesser extent, increased rate of apoptosis.

**Effect of Aging on p44/p42 MAPK Activation After Angioplasty**

To evaluate the effect of aging on p44/p42 MAPK activation after arterial injury in vivo, we used a rabbit model of iliac artery angioplasty. Arteries harvested at different time points after angioplasty showed a rapid induction of p44/p42 MAPK activity, as revealed by immunoblotting with phospho-specific antibody and specific p44/p42 MAPK kinases assays (Figure 2A). p44/p42 MAPK activity was maximal at 30 minutes, decreased by 4 hours, and remained at low levels at days 3 and 7 after angioplasty (data not shown). However, p44/p42 MAPK activities induced by arterial injury were significantly higher in old versus young rabbits. At 30 minutes after angioplasty, old animals showed a 85% increase in p44/p42 MAPK activity compared with young animals (\( P<0.001 \)).

**Inhibition of p44/p42 MAPK Activity by Local Delivery of PD98059**

To confirm the role of p44/p42 MAPK on the age-dependent increase in VSMC proliferation and neointimal formation in vivo, we used a channel balloon to locally deliver 500 \( \mu \)g of PD98059 or diluent alone immediately after iliac artery angioplasty in old rabbits. In preliminary experiments, this dose was shown to maximally inhibit p44/p42 MAPK activity without being toxic to the animal. Local treatment with PD98059 successfully inhibited p44/p42 MAPK activation after arterial injury (Figure 3B). At 30 minutes after angioplasty (time of maximal p44/p42 MAPK activation), local treatment of old animals with PD98059 resulted in a significant 50% reduction in p44/p42 MAPK activity (\( P=0.004 \)) compared with injured arteries locally treated with the diluent alone.

**Effect of p44/p42 MAPK Inhibition on VSMC Proliferation and Neointimal Formation After Angioplasty**

VSMC proliferation was evaluated in young and old rabbits by BrdU incorporation at 7 days after angioplasty, a time point associated with high proliferative activity in that model. At that early time point, however, the neointimal layer was often very thin or even absent, especially
in animals locally treated with PD98059. Accordingly, the BrdU analysis was restricted to the media for adequate comparison between the different animal groups. As shown in Figure 4, aging was associated with a significant increase in VSMC proliferative index (25% vs 11.5% in old and young animals, respectively, P<0.01). Local treatment with PD98059 in old animals resulted in a 46% decrease in the VSMC proliferative index (P<0.05), with a residual level that was comparable to that of young rabbits (13.5% vs 11.5%, P=NS).

Neointimal formation was evaluated in young and old rabbits 14 days after angioplasty (Figure 5A). As shown in Figure 5B, the intima to media (I/M) ratio was significantly higher in old compared with young rabbits (0.55±0.06 versus 0.31±0.03, P=0.02). Local delivery of PD98059 in old animals led to a 40% decrease in the I/M ratio at 14 days after angioplasty (P=0.004). The amount of neointimal formation in old rabbits locally treated with PD98059 was similar to that of young rabbits (0.33±0.05 versus 0.31±0.03, P=0.87). Figure 5C demonstrates that the differences in I/M ratios observed were attributable to the specific effect of aging and PD98059 treatment on neointimal areas, because medial areas were found to be similar in the different animal groups.

Discussion

Although it is well established that aging is associated with an increased risk of atherosclerotic diseases,9-11 little is known about age-related mechanisms causing cardiovascular dysfunction and enhanced atherosclerosis. There is much evidence suggesting that abnormal VSMC proliferation could contribute to the phsiopathology of atherosclerosis in the elderly.19 However, the mechanisms involved in this age-dependent increase in VSMC proliferation are unknown. To our knowledge, our study is the first to identify a specific signal transduction pathway (p44/p42 MAPK), which enhanced activity is contributing to the age-dependent increase in VSMC proliferation and neointimal formation.

We used a rabbit model of arterial injury and evaluated vascular responses in young and old animals. For in vitro studies, VSMCs were isolated from the aorta of young or old rabbits and used at early passages. We found that aging was associated with a significant increase in VSMC proliferation, both in vitro (cell counts/MTS assay) and in vivo (BrdU incorporation, neointimal formation). We also demonstrated that the age-dependent increase in cell number was not attributable to a decreased rate of apoptosis in old VSMCs. These results extend those previously reported in the rat model of vascular injury.3-8 We then compared MAPK activation in young and old rabbits, because these enzymes are important mediators of cell proliferation13 and have been shown to be rapidly activated after arterial injury.14-18 Our results indicate that the activation of p44/p42 MAPK after serum stimulation and arterial injury is significantly enhanced in old compared with young animals. This age-dependent increase in MAPK activation seems to be specific for p44/p42 MAPK, because similar p38 and JNK activities were found in both groups of animals. Moreover, age-dependent increase in p44/p42 MAPK activation and cellular proliferation were not found in other cell types, such as rabbit fibroblasts and rabbit...
myoblasts, suggesting that these are specific characteristics of VSMCs.

To confirm the role of p44/p42 MAPK in the age-dependent increase in VSMC proliferation and neointimal formation, we used the MEK inhibitor PD98059 to suppress p44/p42 MAPK activation in vitro and in vivo. Interestingly, it was not possible to completely abolish p44/p42 MAPK activation in VSMCs isolated from old rabbits (Figure 2A), even when higher doses of PD98059 were used (data not shown). This suggests that aging is associated with a partial resistance to PD98059 in rabbit VSMCs. Nevertheless, p44/p42 MAPK activities in old VSMCs treated with PD98059 were reduced to levels well inferior to those of untreated young VSMCs. This resulted in a dramatic inhibition of cellular proliferation after serum stimulation in VSMCs isolated from old animals. We then evaluated the possibility of using this strategy to prevent the age-dependent increase in neointimal formation after arterial injury in vivo. Using a local delivery technique with the channel balloon, direct infusion of PD98059 in the arterial wall of old animals after angioplasty led to a 50% reduction of p44/p42 MAPK activities. This early inhibition of p44/p42 MAPK activities after angioplasty resulted in a significant decrease in VSMC proliferation and neointimal formation at days 7 and 14 after balloon injury, respectively. In fact, local arterial treatment with PD98059 in old animals reduced VSMC proliferation and neointimal formation to levels similar to those of young animals. To our knowledge, the present study is the first to demonstrate the efficacy of local MEK inhibitor (PD98059) administration to prevent neointimal formation after arterial injury. Because p44/p42 MAPK activity is rapidly induced after angioplasty, early inhibition with PD98059 could be necessary to influence cellular proliferation and neointimal formation. This would be consistent with the fact that in a previous study using the rat model of carotid injury, local administration of PD98059 at the time of arterial injury reduced medial cell replication at 2 days, whereas a similar treatment at day 6 after arterial injury did not block intimal cell replication.18 It is interesting to note that the inhibition of

Figure 4. Effect of p44/p42 MAPK inhibition on VSMC proliferation in vivo. Old rabbits were locally treated with either PD98059 (500 μg/artery) or diluent alone immediately after angioplasty. A, Photomicrographs of histological sections (H&E staining, magnification ×400) are shown. Arrows indicate BrdU-labeled nuclei. B, VSMC proliferation in the media was measured by calculating the BrdU index as follows: (BrdU-labeled nuclei)/(total nuclei)×100. Bars are BrdU index, mean±SEM, n=4 per group. *P<0.005 vs young; **P<0.05 vs old controls. LD indicates local delivery.

Figure 5. Effect of p44/p42 MAPK inhibition on neointimal formation. Young and old rabbits were locally treated with PD98059 (500 μg/artery) or diluent alone immediately after angioplasty and were killed 14 days later. A, Photomicrographs of representative histological sections (H&E staining, magnification ×400) are shown. Arrows indicate internal and external elastic membranes. B, Neointimal formation was evaluated by calculating the I/M ratio. Mean±SEM, n=8 to 11 per group. *P<0.001 vs young; **P<0.05 vs old controls. C, Intimal and medial areas by planimetric analyses. Mean±SEM, n=8 to 11 per group. *P<0.001 vs young; **P<0.01 vs old controls. LD indicates local delivery.
p44/p42 MAPK activities that we obtained using PD98059 and the channel balloon was not complete. This could be attributable to inhomogeneous distribution of the drug administered via the channel balloon or to a relative resistance of old VSMCs to PD98059, as described in vitro. Development of novel strategies to maximally inhibit p44/p42 MAPK activities after arterial injury could potentially lead to a more robust effect on VSMC proliferation and neointimal formation.

The age-dependent increase in p44/p42 MAPK activities that we describe in VSMCs is in marked contrast to what is found in other cell types. For instance, reductions in MAPK activities or defects in MAPK signaling with aging have been described in lymphocytes,21,22 enterocytes,23 neural cells,24 hepatocytes,25 melanocytes,26 and fibroblasts.27 The exact mechanisms by which VSMCs behave differently from other cell types remain to be determined. Heterogeneous populations of VSMCs have been described in the vessel wall and within the media.28–34 Because p44/p42 MAPK activation has been shown to protect against apoptosis in certain situations,35 it is possible that aging is associated with a natural selection of VSMC populations (or clones) that can express higher levels of p44/p42 MAPK activities. In these VSMC populations, higher levels of p44/p42 MAPK activities would not only promote cell survival during aging but would also be at least partly responsible for the enhanced cellular proliferation and neointimal formation in response to injury. Interestingly, p44/p42 MAPK activation has recently been shown to be involved in the determination of VSMC phenotype.36,37 Therefore, it is possible that the enhanced activation of p44/p42 MAPK in old VSMCs contributes to the phenotypic modulation from the differentiated to the dedifferentiated one, a phenotype that has been associated with cell proliferation and migration and the progression of atherosclerosis.1,2

In summary, our results suggest that augmented p44/p42 MAPK activation is at least in part responsible for the increase in VSMC proliferation and neointimal formation with advanced age. This signaling pathway could contribute to explain the increased prevalence and severity of atherosclerosis in the elderly. We propose that p44/p42 MAPK inhibition could represent a novel therapeutic target against atherosclerotic diseases, especially in the context of aging.

Acknowledgments

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References


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A

![Graph showing Optical Density (490nm) vs. Number of VSMCs for Young and Old groups.]

B

![Dot plots showing AnnexinV vs. PI for Young and Old groups in Control and Mitomycin C (10μg/ml) conditions.]

Gennaro et al., Data Supplement Figure I
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B

Control

- Young
  - PI
    - 7.5%
- Old
  - PI
    - 5.8%

PD98059 (50µM)

- Young
  - PI
    - 13.5%
- Old
  - PI
    - 15.0%

Gennaro et al., Data Supplement Figure II