Mechanical Stretch Induces Phosphorylation of p38-MAPK and Apoptosis in Human Saphenous Vein

Jacqueline Cornelissen, Johanna Armstrong, Cathy M. Holt

**Objective**—Failure of saphenous vein grafts remains a major limitation of coronary bypass surgery. The aims of the present study were to determine whether pressure distension of human saphenous vein induces the activation of p38-MAPK and to determine its role in apoptosis.

**Methods and Results**—Phosphorylated p38 was detected at basal levels in human saphenous vein obtained immediately after harvesting. Distended saphenous vein showed significantly higher levels of phosphorylated p38 compared with control vein ($P<0.01$) and nondistended saphenous vein maintained for 3 and 6 hours after harvesting (both $P<0.01$). Apoptosis in distended and nondistended vein was significantly higher at 24 hours compared with control vein, with distended vein showing increased apoptosis compared with nondistended saphenous vein at all time points investigated ($P<0.001$). Immunolocalization showed co-localization of phosphorylated p38 and apoptosis. Inhibition of p38 activity reduced the apoptotic index of cultured vascular smooth muscle cells by 72.1%±1.2% and cultured distended saphenous vein segments by 72.7%±0.9%.

**Conclusions**—Pressure distension of intact human saphenous vein induces activation of p38, and this is associated with apoptosis. Inhibition of p38 kinase activity in saphenous vein smooth muscle cells and intact vein reduces apoptosis. These findings contribute to our understanding of the mechanisms of saphenous vein graft failure. (Arterioscler Thromb Vasc Biol. 2004;24:451-456.)

**Key Words:** apoptosis ■ p38-MAPK ■ smooth muscle cells ■ human saphenous vein

A utologous saphenous vein (SV) is the commonest conduit used for peripheral and coronary artery bypass grafting. However, 30% to 50% of vein grafts become occluded by 10 years.$^1$ The causes of vein graft occlusion are thrombosis in the early stages, and later the proliferation and migration of intimal smooth muscle cells along with the deposition of extracellular matrix leading to myointimal hyperplasia.$^1$ The precise cellular mechanisms involved in these processes remain unclear; however, surgical trauma to the vessel, including the stretch that occurs during manual distension before implantation, as well as implantation of the vein into the arterial circulation may be important.

Apoptosis is reported to occur in pathological SV grafts, where it is thought to be involved in the transformation of a smooth muscle cell-rich myointimal thickening toward a fibrous cell-poor intimal thickening.$^{2,3}$ Apoptosis has also been shown to occur in tissue remodeling, associated with longitudinal stretch (axial strain) in rabbit carotid arteries.$^4$ We have previously shown that apoptosis is induced in pressure-distended human SV and is associated with increased vessel wall expression of the transcription factor c-fos, a downstream component of various signaling cascades.$^5$ In cultured porcine vascular smooth muscle cells (VSMCs), cyclic stretch has been shown to cause an increase in apoptosis, accompanied by a sustained activation of the mitogen-activated protein kinases (MAPK) JNK and p38.$^6$ In vitro studies using cultured smooth muscle cells have shown that mechanical stretch can activate ras/rac/p38 signal pathways$^7$ and p38 phosphorylates p53, which is responsible for mechanical stress-induced apoptosis.$^8$ In addition, the application of biomechanical stress has been shown to increase apoptosis in mouse vein grafts, and this occurs via p38 activation.$^9$

MAPKs are proline-directed serine/threonine kinases activated by dual phosphorylation on threonine and tyrosine residues in response to a wide variety of extracellular stimuli. The JNK and p38 MAPK signaling cascades are activated in response to cellular stress and certain cytokines via the activation of $G$ protein coupled receptors$^{10}$ and are known to be involved in apoptosis.$^{11}$ Overexpression of MAPKs upstream of p38, such as MKK-3, leads to sustained activation of p38 and apoptosis.$^{12,13}$ However, ERK-MAPK is downregulated during apoptosis and upregulated during cellular proliferation.$^{14,15}$ It has been suggested that the dynamic balance between growth-factor–activated ERK and stress-activated JNK/p38 pathways may be important in determining cell survival.$^{14}$
Recent evidence suggests that MAPKs may be activated during stretch-induced injury to the vessel wall in vivo and contribute to the development of neointimal hyperplasia. For example, ERK1 has been shown to be activated after mechanical stretch of porcine carotid arteries. Sustained activation of p38 has also been identified after balloon vascular injury in rabbits, and inhibition of p38 activity using SB239063 reduced the vascular response to injury. Other p38 inhibitors have also been investigated and have been shown to reduce neointimal hyperplasia after endothelial denudation in the rat carotid artery.

The activation of p38 and its association with apoptosis have not previously been demonstrated in intact segments of human vascular tissue. The aims of the present study were to investigate whether p38 is activated after pressure distension of intact segments of human SV and to establish whether this was linked to the induction of apoptosis.

**Methods**

**Isolation, Preparation, and Maintenance of Human SV**

Freshly isolated segments of the long SV were obtained, after informed consent, from patients undergoing coronary artery bypass grafting and were immediately snap-frozen or fixed for control, (C); nondistended (ND); or distended (D) for 2 minutes at 200 to 250 mm Hg as previously described. ND and D segments were then maintained in serum-free culture medium for 1, 3, 6, and 24 hours and then treated similar to controls.

**Western Analysis and Immunohistochemistry for Phosphorylated p38 and p53**

Please see online Methods section at http://atvb.ahajournals.org for further details.

**Detection of Apoptosis Using TUNEL and Double Staining for Apoptosis With SMC Phenotype or Phosphorylated p38**

Detection of DNA fragments in sections of SV was performed using the ApopTag apoptosis detection kit (Intergen Company). Double labeling for apoptosis and SMC phenotype or phosphorylated p38 were performed on SV sections after performing the TUNEL procedure.

**Electrophoretic Detection of Internucleosomal DNA Fragmentation**

Fragmented DNA extracted from snap-frozen segments of SV or VSMCs was analyzed using Suicide Track DNA Ladder Isolation Kit (CN Biosciences).

**Detection of Reactive Oxygen Species**

The oxidative fluorescent dye, dihydroethidium (DHE), was used to determine production of superoxide.

**Inhibition of VSMC and Intact SV Apoptosis With p38 Kinase Inhibitor SB203580**

SV VSMCs were preincubated in the absence or presence of SB203580, exposed to hydrogen peroxide, then washed and incubated at 37°C for 24 hours. TUNEL analysis was then performed on VSMC cytospins. For experiments on intact human SV, segments were either snap-frozen after excision; nondistended and incubated for 24 hours; distended and incubated for 24 hours; or preincubated with, and then distended with, SB203580, followed by incubation with SB203580 for 24 hours.

**Results**

**Detection of Phosphorylated p38 and p53 in Distended Human SV**

All control segments of SV showed minimal immunohistochemical staining for phosphorylated p38 (Figure 1B). In distended and nondistended segments, phosphorylated p38 staining levels increased in the endothelial and medial layers of the vessel wall over 24 hours, whereas staining within the intimal layer remained low (Figure 1C through 1F). At all time points investigated phosphorylated p38 staining in the adventitia and microvasculature endothelium remained elevated (Table, available online at http://atvb.ahajournals.org). There was no significant difference between any of the time points or between the nondistended and distended groups within these areas of the vessel wall. Cellular phenotyping
revealed that activation of p38 was localized mainly to the medial VSMCs (Figure 1G), the monocytes/macrophages (Figure 1H), and the endothelial cells of the luminal layer and microvasculature (data not shown). The medial layer was further subdivided into inner longitudinal and central circular medial layers, and these were quantified separately (Table, available online at http://atvb.ahajournals.org). Overall, distended segments showed significantly higher levels of phosphorylated p38 compared with control segments (P < 0.01), with 3- and 6-hour time points significantly different from the equivalent nondistended time points (P < 0.01). The differences in levels of phosphorylated p38 between distended and nondistended SVs were significantly different in the central circular layer (P < 0.05) and the inner longitudinal layers (P < 0.01). Maximal phosphorylated p38 levels were observed at 6 hours after distension with a ranked score median (interquartile range) of 2.5 (2.34, 2.64), compared with 1.5 (1.33, 1.6) for nondistended 6-hour segments. Phosphorylated p38 levels in both treatment groups decreased at 24 hours to similar levels (P = NS), with a ranked median of 2.0 (1.59, 2.25) for distended and 1.67 (1.45, 1.67) for nondistended segments. (For definition of grading system, see http://www.ahajournals.org.)

Western analysis of all SV segments showed the presence of a single band specific for total p38, and a further single band representing phosphorylated p38 was also detected in all specimens (Figure I, available online at http://atvb.ahajournals.org). After densitometry and correction for protein loading, the intensity of the band for phosphorylated p38 was consistently lower in control segments of SV when compared with distended and nondistended specimens. The phosphorylated p38-to-total p38 ratio was greater for the distended compared with nondistended group at 6 hours, but this difference was nonsignificant (data not shown).

Immunostaining for p53 showed very low levels of basal staining in control segments of human SV, and also in segments preincubated with, and then distended with, 1.0 μmol/L SB203580, a specific inhibitor of p38 kinase activity. Nondistended SV showed slightly elevated levels, and in distended vein clear nuclear staining in a proportion of the medial and adventitial cells was identified (Figure II, available online at http://atvb.ahajournals.org).

Apoptosis After Distension of Human SV
TUNEL staining revealed a small proportion of apoptotic cells within control segments of SV, compared with a greater degree of apoptosis present in later time points (Figure 2A through 2C). Apoptotic labeling indices (LI) (mean ± SEM) in nondistended (ND) and distended (D) SV segments were significantly higher at 24 hours compared with control SV [C] (2.97 ± 1.42 ND versus 0.96 ± 0.26 C; P < 0.05) and (11.67 ± 2.85 D versus 1.24 ± 0.51 C; P < 0.001) (Figure III, available online at http://atvb.ahajournals.org). In addition, distended SV showed significantly higher apoptotic LI compared with nondistended SV at all time points investigated (P = 0.001). The presence of apoptosis in distended and nondistended SV at 24 hours was further confirmed by electrophoretic detection of DNA fragments, with the absence of detectable apoptosis in control SV (data not shown).

Double staining for apoptosis and SMCs (Figure 2D) localized the presence of apoptosis predominantly to the medial SMCs. Furthermore, phosphorylated p38 double staining with SMC (Figure 1G) or TUNEL (Figure 2E) revealed that phosphorylated p38 co-localized to apoptotic VSMCs, although not all cells expressing phosphorylated p38 were TUNEL-positive.

Production of Reactive Oxygen Species in Distended Human SV
Dihydroethidium enters cells and is oxidized to ethidium bromide in the presence of superoxide (O$_2^-$). Control sections of human SV showed little evidence of reactive oxygen species (ROS) (Figure 3A). Nondistended sections of SV showed low levels of ROS in the medial layers and the endothelial cells of the luminal layer and microvasculature (Figure 3B). Segments of SV that were preincubated, distended, and then incubated with 1.0 μmol/L SB203580 showed greatly reduced levels of ROS (Figure 3C) compared with distended sections of SV, which showed elevated levels of ROS in the media and adventitia (Figure 3D).

Inhibition of Phosphorylated p38 Kinase Activity and Apoptosis in VSMC and Human SV
The activation of p38 and its involvement in the induction of VSMC apoptosis was assessed in SV VSMCs preincubated with p38 kinase inhibitor SB203580. VSMC apoptosis was significantly elevated 24 hours after treatment with hydrogen peroxide (H$_2$O$_2$) compared with untreated control VSMCs.
Apoptotic LI (mean ± SEM) were 4.61% ± 0.71% (0.1 mmol/L H₂O₂) or 7.85% ± 1.05% (0.5 mmol/L H₂O₂), compared with 0.45% ± 0.11% in control VSMCs (P < 0.05) (Figure 4B). VSMCs preincubated with 1.0 μmol/L or 10.0 μmol/L SB203580 and then treated with 0.1 mmol/L H₂O₂ showed a 57.9% ± 3.1% and 72.1% ± 1.2% reduction in apoptosis, respectively. VSMCs preincubated with 1.0 μmol/L or 10.0 μmol/L SB203580 and then treated with 0.5 mmol/L H₂O₂ showed 72.1% ± 1.2% and 77.7% ± 1.2% reduction in apoptosis, respectively. Preincubation with SB203580 significantly reduced levels of apoptosis in both H₂O₂ treatment groups (P < 0.05).

For additional confirmation that H₂O₂ induces SV VSMC apoptosis, cells were exposed to H₂O₂ at 0.1 or 0.5 mmol/L for 30 minutes, then cultured for a further 24 hours. The presence of apoptosis was confirmed by electrophoretic detection of DNA fragments in the supernatant of 0.5 mmol/L H₂O₂-treated VSMCs, with the absence of apoptosis in the untreated control VSMCs (data not shown).

Intact segments of human SV were either nondistended and maintained in culture for 24 hours, or preincubated, distended, and then incubated with 1.0 μmol/L SB203580 for 24 hours. Labeling indices for apoptosis were 2.31% ± 0.45% for nondistended and 3.20% ± 0.15% for SB203580-treated distended SV (P = NS). Phosphorylated p38 staining levels were low (mean staining: < 25%) in segments of vein after incubation with SB203580. Therefore, inhibition of phosphorylated p38 activity after incubation and subsequent distension with SB203580 resulted in a 72.7% ± 0.9% reduction in apoptosis, compared with untreated distended segments of SV with an average apoptotic LI of 11.7% ± 2.9% at 24 hours.

Discussion

The main findings of this study were that p38 is activated in human SV after mechanical stretch and that this is associated with apoptosis. This is the first time that such an observation has been noted in intact segments of human vessel. More than 30% of patients undergoing coronary bypass surgery require reoperative surgery or percutaneous intervention 12 years from the original procedure.²¹ The precise cellular mechanisms involved in this process are not entirely clear, although surgical trauma to the SV and its implantation into the arterial circulation may be important. Surgical distension of SV with heparinized blood or saline is performed during surgery to check for leakage from side branches, and distension pressures of >450 mm Hg have been observed.²² This type of

Figure 3. Reactive oxygen species (ROS) production in human SV. Control sections of human SV show very little evidence of ROS (A). Nondistended sections of SV at 24 hours after explant show low levels of ROS (red florescence) in the medial layers and the endothelial cells of the luminal layer and microvasculature (B). Segments of SV preincubated, distended, and then incubated with 1.0 μmol/L SB203580 show low levels of ROS at 24 hours (C). Distended sections of SV, at 24 hours after explant, show elevated levels of ROS in the media and adventitia (D). Scale bars represent 40 μM.

Figure 4. Inhibition of apoptosis in VSMCs after preincubation with SB203580. Human SV VSMCs were preincubated with 1.0 or 10.0 μmol/L SB203580, then exposed to 0.1 or 0.5 mmol/L H₂O₂, followed by incubation for 24 hours before cytospin preparation. Controls of VSMCs in 2% culture medium, 10.0 μmol/L SB203580, and 0.0003% DMSO (vehicle control) were also performed. A, TUNEL detection of apoptosis in cytospin preparations of adherent and nonadherent cells; (i) apoptosis-positive control cells treated with 2 mmol/L staurosporine (arrow); (ii) DNAse I generated positive control for TUNEL staining; (iii) negative control TUNEL staining; (iv) positive apoptotic staining of H₂O₂-treated VSMCs (arrow). B, TUNEL-positive cells were counted and represented as a percentage of the total cell count (Apoptotic LI) (mean ± SEM) (see http://www.ahajournals.org for further details). The data show that both concentrations of H₂O₂ significantly increase VSMC apoptosis (P < 0.05), and this is significantly reduced after incubation with the p38 kinase inhibitor SB203580 (P < 0.05).
distension has been shown to cause morphological and biochemical alterations in the vessel wall. In addition, several hemodynamic changes occur in the SV after implantation into the coronary circulation, including alterations in blood flow, transmural pressure, shear stress, and radial stress. In addition, interruption of the vasa vasorum during explantation of vein is likely to cause ischemic necrosis. However, Ma et al showed that ischemia alone caused a moderate increase in p38 activity, with reperfusion causing maximal activation, and our own studies have demonstrated viability of human SV up to 24 hours ex vivo. In this study, we identified the occurrence of apoptosis in human SV after mechanical stretch performed to simulate the surgical distension of SV. We have also identified a similar incidence of apoptotic cells in segments of SV that were continually distended for 3 hours (data not shown). Apoptosis has previously been identified in pathological specimens of human SV grafts and in a mouse model of venous bypass grafting. It is thought that the occurrence of apoptosis may be caused by mechanical forces on the vessel wall, and apoptosis has been demonstrated in various animal models of balloon injury.

The role of apoptosis in human SV grafts remains unclear. SV grafts exhibit enhanced deposition of extracellular matrix and a progressive increase in intimal fibrosis and a reduction in cellularity. This pathology is in agreement with the incidence of apoptosis as an early event in SV grafts. Proliferation is also known to occur in SV graft failure. It is possible that signaling molecules, for instance growth factors such as basic fibroblast growth factor, are released from apoptotic cells and these may subsequently cause the proliferative response characteristic of SV graft failure. In a previous study, apoptosis was seen to precede proliferation after balloon injury in pig coronary arteries, and the current study suggests a similar sequence of events may occur in vein grafts. In two recent studies, vein grafts were performed in protein kinase C and p53 null mice, with increased intimal lesions containing enhanced numbers of VSMCs and decreased apoptosis observed, suggesting that lesion size correlates with this event.

The pathways involved in the induction of apoptosis in human SV graft are unclear. We hypothesized that p38 activation may be responsible, because this has previously been shown to be involved in the apoptosis pathway. Support for this hypothesis has also been provided in a study by Mayr et al, who demonstrated that p38 is activated in mouse SV grafts and that activation occurs concurrently with apoptosis. In the current study, we have shown an increase in activated p38 in intact segments of human SV after mechanical stretch. Phosphorylated p38 was localized predominantly to the VSMCs and endothelial cells of the luminal layer and microvasculature, and specifically in close association with cells undergoing apoptosis.

Enhanced superoxide production has been demonstrated in a vascular injury model of rabbit vein grafts and recently linked to mechanical stress-induced oxidative DNA damage and p53 activation in murine SMCs. In this study, we identified enhanced production of ROS after pressure distension, which was abrogated after the treatment of SV segments with the specific p38 kinase inhibitor, SB203580. Furthermore, when cultured human SV VSMCs were preincubated with SB203580, a decreased apoptotic response to hydrogen peroxide stimulated p38 phosphorylation was observed, further implicating the involvement of p38 in human SV VSMC apoptosis.

The downstream mechanisms involved in p38 induced apoptosis in our model are likely to involve p53. Immunohistochemical analysis of distended and nondistended vein showed upregulation of p53, consistent with the findings of Mayr et al who demonstrated that pretreatment of VSMCs with SB202190 (a p38 inhibitor) caused the inhibition of mechanical stress-induced p53 activation and apoptosis. The same study also showed that the apoptotic response to cyclic strain was absent in murine p53null VSMCs.

Our studies revealed that although apoptosis was reduced after inhibition of p38 kinase activity with SB203580, apoptosis was still evident, suggesting that pathways other than p38 are also involved. A recent study showed that cyclic stretch of porcine VSMCs caused clustering of TNFR-1 and a sustained activation of JNK and p38, resulting in apoptosis. These investigators also showed that dominant-negative forms of p38 and c-jun, when tested individually, did not significantly attenuate apoptosis; however, when used together apoptosis decreased, suggesting that multiple signaling pathways are involved.

The findings of this study suggest that the early stages of SV bypass grafting may have important long-term consequences. Indeed, intervention at the time of grafting in the PREVENT trial showed that intraoperative transfection of human bypass vein grafts with E2F decoy, inhibited cell cycle gene expression, and improved patency rates at 6 months. Thus, it is likely that modulation of p38 activity and therefore apoptosis may also confer beneficial effects to patients undergoing coronary bypass surgery.

In summary, this study has demonstrated that mechanical stretch caused by pressure distension of intact human SV induces the activation of p38, and this is associated with apoptosis. Furthermore, inhibition of p38 kinase activity in human SV VSMCs and distended segments reduced apoptosis. These findings provide a likely mechanism for the induction of apoptosis as detected in human SV grafts and are important in providing a better understanding of the mechanisms of human vein graft failure.

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