Mechanical stress-induced DNA damage and rac-p38MAPK signal pathways mediate p53-dependent apoptosis in vascular smooth muscle cells¹

MANUEL MAYR, YANHUA HU,* PIERRE HAINAUT, † AND QINGBO XU§,2
Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, Austria; *Institute for Pathophysiology, University of Innsbruck, Innsbruck, Austria; †International Agency for Research on Cancer, Lyon, France; and §Department of Cardiological Sciences, St. George’s Hospital Medical School, London, UK

SPECIFIC AIMS
We recently provided evidence that mechanical stress induces smooth muscle cell (SMC) apoptosis, an early event in the development of arteriosclerosis. To further scrutinize the molecular mechanisms of mechanical stress-induced SMC apoptosis, the present study was designed to investigate signal pathways that regulate gene expression leading to SMC death.

PRINCIPAL FINDINGS

1. Mechanical stress induces p53 activation
To elucidate whether mechanical stress induces p53 activation in SMCs, we analyzed DNA binding activity of p53 by electrophoretic gel mobility shift assay (EMSA). Aortic SMCs were subjected to cyclic strain as described previously. Tensile stimuli with 15% elongation of original size (1 Hz, 6 h) resulted in p53 activation (Fig. 1C). Similar results were obtained for human, mouse, and rat SMCs. The specificity of the binding was controlled by competition using cold and mutant oligonucleotides as well as p53/H11002/SMCs. In accordance with the EMSA experiments, Western blot analyses revealed that levels of p53 increased in the cytoplasm and cell nuclei in response to mechanical stress.

2. p38 mitogen-activated protein kinase (MAPK) contributes to mechanical stress-induced p53 activation
Our previous findings indicated that p38 MAPK may be involved in transducing signals leading to cell death. Pretreatment with SB202190, a specific inhibitor of p38 MAPKs, significantly reduced mechanical stress-induced p53 activation. Stable transfection of SMCs with a dominant negative rac 1 (rac1 N17), a member of the ras superfamily of small GTP binding proteins and a key upstream signal transducer in p38 MAPK signaling, inhibited p53 activation after mechanical stress vs. vector transfected cells. To obtain direct evidence of p53 phosphorylation by p38 MAPK, p38 MAPK from mechanical stressed SMCs was isolated and a kinase assay was performed using GST-p53 as a substrate. Phosphorylation of GST-p53 paralleled p38 MAPK activation and peaked after 10–30 min of mechanical stress. Pretreatment with the p38 MAPK inhibitor SB202190 markedly inhibited GST-p53 phosphorylation.

3. p53 activation by oxidative DNA damage
It has been demonstrated that grafting veins to arteries or exposing SMCs to pulsatile stretch increases oxidative stress. Therefore, we used a FITC-labeled fluorescent probe to detect 8-oxoguanine, a well-established surrogate for oxidative DNA damage. No staining was observed in unstressed SMCs, but oxidative nucleotide modifications were present 3–6 h after mechanical stress (Fig. 1A). The appearance of 8-oxoguanine paralleled the production of reactive oxygen species in stressed SMCs as demonstrated by dihydrorhodamine 123 staining (Fig. 1B). If SMCs were preincubated with the amino steroid U-74389G, an antioxidant inhibiting lipid peroxidation, mechanical stress-induced p53 activation was abrogated in EMSA experiments (Fig. 1C). However, U-74389G did not influence p38 MAPK activation (Fig. 1D).

4. Increased expression of Bcl-2 proteins in response to mechanical stress
The expression of Bcl-2 proteins was assessed in subcellular extracts of stressed and unstressed SMCs. Increased protein levels of Bax were detected in the cytosolic and mitochondrial fraction after mechani-
that p53−/− SMCs lost their ability to express Bax in response to mechanical stress (Fig. 2D).

5. SMC apoptosis is p53 dependent and associated with mitochondrial dysfunction

Since Bcl-2 family members are known to control the opening of mitochondrial transition pores, we measured the mitochondrial potential (Δψ) after mechanical stress by use of JC-1, a potential-sensitive fluorescent dye that concentrates in mitochondria. Mitochondrial depolarization was observed in p53+/+, but not in p53−/− SMCs. In wild-type cells, mitochondrial depolarization coincided with a leakage of cytochrome c into the cytosol and a significant increase in apoptosis as identified by

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**Figure 1.** Oxidative DNA damage and p53 activation. *A*) Rat SMCs were subjected to cyclic mechanical strain for 0.5 and 6 h (15% elongation, 1 Hz), fixed with paraformaldehyde, and incubated with an FITC-labeled probe for 8-oxoguanine (Biotrin) at 4°C overnight. Oxidative DNA damage was visualized by confocal microscopy in stressed SMCs. *B*) Oxidative stress was measured simultaneously by the presence of the oxidant-sensing fluorescent probe dihydrodihydromine 123. *C*) SMCs were preincubated with 100 μM of the antioxidants U-74389G (UG) for 1 h, stressed, and nuclear extracts were harvested for EMSA experiments. Filled arrows indicate specific binding, *Nonspecific binding; open arrows indicate free probe. *D*) SMCs were serum-starved for 3 days and stressed for 10 min at 15% elongation in the presence or absence of U-74389G. Protein extracts were prepared and analyzed for p38 MAPK phosphorylation by Western blot.

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**Figure 2.** Bcl-2 family protein expression after mechanical stress. Subcellular protein extracts were prepared by differential centrifugation 24 h after the onset of mechanical stress and separated on 12% SDS-polyacrylamide gel, transferred to membranes, and probed using antibodies to Bax, Bcl-2, and Bcl-xL. *A*) Bcl-2 family protein expression in cytosol, mitochondria and nucleus of stressed SMCs (Fig. 2C). Western blot analysis of total protein extracts revealed
annexin V labeling. Consistent with our previous results, SMCs of p53−/− mice were resistant to mechanical stress-induced apoptosis.

CONCLUSION

A common feature of vascular diseases is altered or elevated biomechanical stress. For instance, spontaneous atherosclerotic lesions are prone to locate in the branch where cyclic strain is elevated. Vein vessels do not develop atherosclerosis in their normal low-pressure environment, but accelerated atherosclerosis is observed when veins are grafted to arteries, where the vessels bear increased biomechanical forces. Obviously, biomechanical stress is a crucial factor in atherogenesis.

In vein graft arteriosclerosis, we demonstrated earlier that biomechanical stress is primarily responsible for SMC apoptosis and that MAPKs partially mediate the process of mechanical stress-induced signaling. The present study provides the first evidence that mechanical stress is a novel factor for p53 activation, which is initiated by oxidative DNA damage and rac-p38MAPK activation in SMCs (Fig. 3). We demonstrated that apoptosis occurs as a result of mitochondrial dysfunction due to changes in the ratio of pro- and antiapoptotic Bcl-2 family members. Because of the importance of p53-dependent apoptosis and mechanical stress in the pathogenesis of vascular diseases, our data provide a link between mechanical force applied to vascular SMCs and cell death (Fig. 3).

Based on recent observations in our mouse model of vein graft arteriosclerosis, loss of p53 accelerated neointima formation, which was primarily attributed to SMC accumulation. Fewer apoptotic cells were detected in p53-deficient vein grafts, suggesting that p53 is required for SMC apoptosis in vivo. Therefore, our results suggest that mechanical stress exerts its role in cell apoptosis via p53 activation in the development of vascular diseases.