Abstract—Using an in vitro model of a conditionally immortalized cell line, we investigated how human vascular smooth muscle cells (VSMCs) are affected by the expression of simian virus 40 (SV40) large T antigen (LT antigen), which binds to cell cycle regulators, such as the tumor suppressor protein p53. Cells were obtained after infection of saphenous vein–derived VSMCs with a nonreplicative retroviral vector containing a temperature-sensitive (ts) mutant of SV40 LT antigen and were shown to have maintained some characteristics and responses of VSMCs. Under permissive temperature conditions (36°C), the increased rate of cell proliferation was shown to be associated with expression of LT antigen and with LT-antigen binding to and inactivation of p53. p53 inactivation failed to block apoptosis induced by serum withdrawal or by UV irradiation. Downregulation of LT-antigen expression at the nonpermissive temperature (39°C) was shown to be associated with growth arrest, increased expression of the cell cycle inhibitor p21WAF1/CIP1, increased MDM2-promoter activity, and differential expression of MDM2 gene products, suggesting that p53-induced transcription/transactivation may be involved in VSMC cell cycle control but not necessarily apoptosis. The established SMC line HVTs-SM1 may be a useful model for the study of processes involved in myointimal hyperplasia and cellular aging, as well as for the study of cell cycle control in general. (Arterioscler Thromb Vasc Biol. 2000;20:636-644.)

Key Words: vascular smooth muscle ■ SV-40 ■ cell line ■ p53 ■ MDM2 ■ p21WAF1/CIP1

V ascular smooth muscle cell (VSMC) proliferation has long been considered to be a key event in the remodeling of the vascular wall during development and following vascular injury in such diseases as atherosclerosis and vascular restenosis after invasive intervention.1,2 More recently, the precise role of proliferation in atherogenesis has received more critical attention,2 and other processes, such as contraction, cell migration, and apoptosis, have also been proposed to be involved. The mechanisms regulating cell cycle control in VSMCs remain largely unknown.2 Primary VSMCs, especially of human origin, are difficult to study in vitro: their availability is limited, they grow slowly, and they have a limited life span. Furthermore, cell responses may vary between cell lines depending on the donor, disease, or tissue.2,3 Recently, there has been interest in in vitro models of established VSMC lines obtained from animal or human primary cells by transfection with SV40 LT antigen or other viral oncogenes.4–7 However, although there are some reports of immortalized cell lines derived from animal cells, human cells in general have been more difficult to immortalize.

We describe here some of the cellular characteristics of a SMC line derived from human saphenous vein that was established by infection with a temperature-sensitive (ts) SV40 LT antigen. Rapid proliferation of cells was associated with LT antigen expression and LT antigen–dependent inactivation of G1/S checkpoint control via inactivation of such cell cycle regulators as p53.8 Downregulation of LT antigen expression under nonpermissive temperature conditions was shown to be associated with growth arrest, the appearance of morphological characteristics typical of senescent cells, and upregulation of the expression of growth regulator(s) downstream of p53, such as p21WAF1/CIP1.9 Transformed cells showed high levels of apoptosis that increased after serum withdrawal or DNA damage by UV irradiation. Because this cell line maintains some characteristics of human VSMCs, it may provide a useful in vitro model to study the regulation of phenotype and to investigate changes in cell cycle control involved in cellular aging, cell death, and other processes contributing to vascular disease.

Methods

Reagents, Antibodies, and Plasmids
All culture media were from Gibco Life Technologies/BRL. [Methyl-3H]thymidine (740 GBq/mmol) was from ICN Flow. Un-
fractionated porcine heparin (Paynes & Byrne) was a gift from Dr B. Mullan, National Institute of Biological Standards and Control, South Mimms, UK. Platelet-derived growth factor (PDGF)-BB, epidermal growth factor (EGF), and transforming growth factor (TGF)-β were from Life Technologies. All other reagents used, unless otherwise specified, were from Sigma. Primary and secondary antibodies were from DAKO A/S. The p53 (non–cross-reactive) human specific monoclonal antibody (mAb) DO-1, the p21 WAF1/CIP1 antibodies were from DAKO A/S. The p53 (non–cross-reactive) antibodies were from Sigma. Primary and secondary antibodies were from DAKO A/S.

Cell Culture Conditions: Transfection and Isolation of Conditionally Immortalized Human VSMCs

Human SMCs were isolated from explants derived from normal saphenous vein from patients undergoing coronary artery bypass as previously described. Cells that migrated from these explants were subcultured at a split ratio of 1:2 in DMEM containing 15% FCS. Equal numbers of cells from 12 separate lines derived from human saphenous vein–derived SMCs with similar proliferative responses were pooled at passage 2, subcultured, and used at passage 3 to 4 and are referred to in this study as normal or untransformed VSMCs. Actively growing primary VSMCs obtained from a single middle-aged patient were infected at passage 4 with conditioned medium previously described. Cells that migrated from these explants were irradiated with UV-C (36 W), 200 J/cm², and the independent reporter plasmid CMB–luc reporter plasmid by the calcium phosphate precipitation method. An equivalent amount of CMV–MDM2-luc reporter plasmid by the calcium phosphate precipitation method. An equivalent amount of CMV–MDM2-luc reporter plasmid by the calcium phosphate precipitation method. An equivalent amount of CMV–MDM2-luc reporter plasmid by the calcium phosphate precipitation method. An equivalent amount of CMV–MDM2-luc reporter plasmid by the calcium phosphate precipitation method. An equivalent amount of CMV–MDM2-luc reporter plasmid by the calcium phosphate precipitation method. An equivalent amount of CMV–MDM2-luc reporter plasmid by the calcium phosphate precipitation method.

Plasmid Transfection and Luciferase Assay

HTV-SM1 cells plated in dishes 3 cm in diameter (10/5 dish) in 10% FCS/DMEM were transfected 24 hours later with 1 to 2 µg of MDM2-luc reporter plasmid by the calcium phosphate precipitation method. An equivalent amount of CMV–MDM2-luc plasmid was cotransfected for normalization of transfection efficiency. The transfected cells were incubated at 36°C or 39°C, and at the indicated time points, they were washed 3 times with PBS, lysed in 100 µL of lysis buffer (Promega UK Ltd), and assayed for reporter activity of luciferase and β-galactosidase.

DNA Synthesis and Cell Proliferation

Normal and HTV-SM1 cells were plated at 36°C in 96-well plates at 10⁴ cells/well in 10% FCS/National Cancer Tissue Culture (NCTC)-109 medium. Twenty-four hours later, the cells were washed 3 times with PBS and growth-arrested (72 hours) in serum-free (SF) medium. DNA synthesis induced by the various stimuli was measured 30 hours after stimulation with 5 µCi [methyl-³H]thymidine that was added 6 hours before the DNA was harvested, as previously described.

UV Irradiation and Cell Apoptosis

Normal and HTV-SM1 cells were irradiated at 50 mJ/cm² in an XL-1000 UV crosslinker (Spectronics Corp) and then incubated at 36°C in 10% FCS/DMEM. At the indicated time points, they were trypsinized and counted. In parallel, the detached and adherent cells from each of 2 additional wells were collected for flow cytometry, washed with PBS, fixed in 70% ice-cold ethanol, and centrifuged at 2000 rpm (10 minutes, 4°C). Cells and apoptotic bodies in each pellet were resuspended in PBS containing 50 µg/mL propidium iodide and 12 µg/mL RNase A and incubated in the dark (30 minutes, room temperature). Flow cytometry was performed in a
We investigated whether the transformed HVTs-SM1 cells retained cytoskeletal proteins typical of SMCs. The majority of transformed cells stained positive for smooth muscle \( \alpha \)-actin. The decorated filaments appeared to be more cytoplasmic and irregular in the transformed cells, whereas in normal cells they traversed the cell along its long axis (Figure 2, A1 and A2). There were no observable differences in the strength of smooth muscle \( \alpha \)-actin staining and filament distribution when either normal or transformed VSMCs were incubated at 39°C for 24 to 48 hours. Staining for vimentin was positive in both cell types, but compared with normal cells, the filaments of the transformed cells were more sparse, less organized, and spread from perinuclear bundles to the periphery of the cell (Figure 2, B1 and B2). Antibody staining for von Willebrand factor and desmin was negative for either normal or transformed cells. The transformed cells showed weak and mainly perinuclear rod-like staining for smooth muscle myosin heavy chain (Figure 2, C2), whereas in normal VSMCs, the staining was observed in organized filaments along the long axis of the cell (Figure 2, C1).

LT-Antigen Expression and Expression of Cell Cycle Regulatory Molecules in HVTs-SM1 Cells

Previous reports on cells immortalized with SV40 have identified that the expression of a functional LT antigen is required for the maintenance of the immortalized phenotype.\(^{15}\) Those studies have also established that SV40 LT antigen binds to wild-type p53 and extends its half-life.\(^{8,20}\)

We therefore investigated whether HVTs-SM1 cells expressed LT antigen conditionally and whether LT-antigen expression had an effect on p53 protein level. Indirect immunofluorescence with anti–LT-antigen or with anti-human specific wild-type p53 antibodies in cells growing at 36°C showed positive nuclear staining for both antigens in all cells (Figure 3). LT-antigen and p53 nuclear immunofluorescence staining were reduced to almost background levels when cells were transferred to 39°C (Figure 3, A2 and B2), and normal cells grown under similar conditions showed no nuclear staining with either antibody. Similarly, immunoblotting studies showed elevated levels of LT antigen and p53 in cells growing at 36°C but not in cells cultured at 39°C (Figure 4). Levels of LT antigen and p53 were unaffected by the presence or absence of 10% FCS, and p53 was found to coimmunoprecipitate with LT antigen (Figure 4). It should be noted that the HVTs-SM1 cells had to be cultured for 5 to 7 days at 39°C before the protein levels of LT antigen and p53 were reduced below detection limits of the antibodies used.

We also examined the transcription of molecules that are known to be regulated by p53, such as p21 and MDM2, because it has been documented that LT-antigen binding to p53 may adversely affect its functions.\(^{20}\) The specific mAb to the cell cycle inhibitor p21 detected a 21-kDa protein band only when the transformed cells were cultured at 39°C (Figure 4). Taking into account the observed temperature-dependent LT-antigen and p53 protein expression profiles and the coimmunoprecipitation of LT antigen with p53, these results are in keeping with the proposal that p53-induced p21 expression may be inhibited by LT-antigen binding to p53.\(^{9}\)

To investigate the effect of LT-antigen expression on the transcriptional regulation of MDM2 by p53, HVTs-SM1 cells were transfected with an MDM2-luciferase reporter plasmid.
The luciferase activity profile obtained showed a 4-fold increase in MDM2-promoter activity when the cells were cultured at 39°C rather than at 36°C (Figure 5). Furthermore, Western blot analysis of HVTs-SM1 lysates from cells cultured at the 2 temperatures indicated that MDM2-protein levels were also reduced in HVTs-SM1 cells cultured at permissive temperature (Figure 5, inset).

**Growth and Survival of HVTs-SM1 Cells**

We then investigated the growth rate and survival of HVTs-SM1 cells. At 36°C, the serum growth-response curves of transformed (Figure 6A) and normal (Figure 6D) cells plated at 5×10^4 cells/cm^2 appeared sigmoidal. The transformed cells, however, grew more rapidly (doubling time, 49±9 hours; n=4; *P*<0.001) and reached a density ∼6-fold higher than that of normal cells (doubling time, 132±36 hours [n=12]; passage 3). Under permissive temperature conditions, the serum-dependent proliferation of HVTs-SM1 cells, like that of normal cells, appeared to slow down through contact inhibition (Figure 6A versus 6D). Anti-BrdU antibody labeling and acridine orange staining showed that cell division as well as cell death by apoptosis was taking place in HVTs-SM1 cells concurrently during this period (data not shown). After a long incubation period (3 to 4 months), normal VSMCs continued to proliferate very slowly and form a densely packed multilayer, whereas the transformed cells did not form such a multilayer.

Unlike nontransformed cells, serum withdrawal under permissive conditions did not cause the HVTs-SM1 cells to growth-arrest (>90% of the cells were BrdU-positive 24 hours later). However, the cells were not able to sustain continuous proliferation; their number declined (Figure 6C), and acridine orange staining demonstrated many apoptotic cells in this period (Figure 6, E and F). In contrast, serum...
withdrawal from normal SMCs induced only a small loss of cells within the first 24 to 48 hours. The remaining cells appeared to survive in a growth-arrested state for at least a week (Figure 6D).

The temperature change to 39°C appeared to have no effect on the serum-dependent proliferation of HVTs-SM1 cells in the first few days. However, cell number declined 5 to 7 days later (Figure 6B), and acridine orange staining indicated increased apoptosis. The fate of surviving cells depended on their density. If the cells did not become very sparse after the period of rapid cell death, they continued to proliferate very slowly and remained viable for at least 3 to 4 months. Flow cytometry indicated that the majority of cells accumulated at G1 as well as G2 phases of the cell cycle (Table), in agreement with previous reports.15 With time, there was a significant increase of cell size and appearance of perinuclear vacuoles, and these cells resembled in vitro senescent or atherosclerotic cells.18,19 Provided that the temperature was shifted back to 36°C before the cells became very sparse, they were able to quickly resume as vigorous a growth in response to serum as that of cells that had not been exposed to the nonpermissive temperature.

We also investigated whether HVTs-SM1 cells retained responses to smooth muscle growth factors and heparin. As shown in Figure 7A, under permissive temperature conditions, induction of HVTs-SM1 cells by 10% FCS had a large, positive effect on thymidine incorporation, whereas induction with PDGF and EGF had only a small positive effect; TGF-β had no apparent effect. Heparin (150 μg/mL) inhibited thymidine incorporation induced by serum by ≈50% and that induced by PDGF and EGF to a lesser extent (Figure 7A). Heparin inhibition of serum-induced DNA synthesis was broadly comparable to that we have previously observed in normal VSMCs,3 and no further effect was seen after an increase in the heparin concentration up to 5-fold. Serum-induced cell proliferation was inhibited by 25% to 30% when cells were cultured in the presence of 150 μg/mL heparin for 7 days (Figure 7B). When cells were cultured in the presence of 0.5% serum, addition of heparin (150 μg/mL) resulted in a small net reduction in cell number (Figure 7B versus Figure 6C).

**LT-Antigen Transformation Increases SMC Sensitivity to UV Irradiation**

Flow cytometry of UV-irradiated (50 mJ/cm²), normal SMCs cultured at 36°C showed no significant time-dependent increase in the percentage of apoptotic cell bodies (control, 0 time: 10%; 24 hours after irradiation: 7%), suggesting that the cells are resistant to UV irradiation. The majority of cells remained in G1 phase of the cell cycle, with only a small proportion of cells traversing S phase (Table), in keeping with the slow proliferation rate of normal SMCs. In contrast, the transformed cells showed a 6-fold increase in the percentage of apoptotic bodies as early as 6 hours after irradiation (control, 0 time: 6%; 6 hours after irradiation: 34%). This was accompanied by a time-dependent increase in degraded DNA.
and a decrease in the percentage of cells in G1 phase. UV irradiation had no significant effect on the proportion of cells traversing S phase. However, 24 hours after irradiation, there was an increase in the proportion of cells in G2 phase (Table). Compared with nonirradiated HVTs-SM1 cells, there was a 40% to 50% reduction in cell numbers 48 to 50 hours after irradiation; this was accompanied by a period of growth arrest and then recovery (Figure 8). Early apoptosis of UV-irradiated HVTs-SM1 cells was also confirmed by detection of DNA fragmentation 4 hours after irradiation (data not shown).

Figure 4. Immunoblot analyses of LT-antigen, p53, and p21 protein expression in HVTs-SM1 cells. LT antigen: Western blots of lysates from cells grown for 9 days in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 10% FCS at 36°C (lanes 1 and 3) or at 39°C (lanes 2 and 4). p53: Western blots of lysates from cells grown for 9 days in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 10% FCS at 36°C (lanes 1 and 3) or at 39°C (lanes 2 and 4). p53/LT antigen: For detection of LT antigen–p53 complex, lysates from cells cultured for 5 days at 36°C (lanes 1 and 3) or at 39°C (lanes 2 and 4) were first treated with anti-p21 mAb (lanes 1 and 2; control) or anti–LT-antigen mAb (lanes 3 and 4), and the immunoprecipitates were then subject to Western blotting with anti-p53 mAb, p21: Western blots of HVTs-SM1 cells grown for 7 days at 36°C (lane 1) or at 39°C (lane 2), transformed human aortic SMCs that do not express p21 (negative control) cultured at 36°C and 39°C (lanes 3 and 4, respectively), positive controls, human lymphoma cells (lane 5), and mouse fibroblast cells (lane 6).

Discussion
In vitro aging and senescence limits the life span of primary human VSMCs. This and the interindividual and intrindividual variability of cellular responses to growth factors/inhibitors emphasizes the need for established cell lines. However, in contrast to rodent cells, only a few such human cell lines have been established by viral infection. In a previous study that used SV40 infection of human VSMCs, the maximum life span reported was ∼60 PDs. Using a ts replication-defective SV40 vector, we have been able to establish an immortal (life span >200 PDs) human VSMC line that we have named HVTs-SM1. These cells are conditionally immortalized, because their proliferative phenotype is expressed only under the permissive temperature. Under nonpermissive temperature conditions, the cells growth-arrest and eventually lose viability in a density-dependent manner, implying the presence of an autoregulatory mechanism for
survival. The transformed cells are smaller and have lost the typical spindle morphology that characterizes normal VSMCs. Nevertheless, the cells continue to express smooth muscle–specific cytoskeletal proteins, including myosin heavy chain and α-actin. We have also shown that at permissive temperature, HVTs-SM1 cells retain, to some extent, their response to such SMC growth factors as PDGF-BB and to such growth inhibitors as heparin. However, DNA synthesis in response to PDGF-BB was 2- to 3-fold lower than in normal cells, possibly as the result of LT-antigen–induced downregulation of PDGF receptor expression.

SMCs play an important role in the pathogenesis of atherosclerosis, vascular graft occlusion, and restenosis after angioplasty. Although the precise role of smooth muscle proliferation and apoptosis in the development of these diseases, particularly atherosclerosis, is debated, it is likely that these processes contribute, at least at some stage, to their pathogenesis. HVTs-SM1 cells may be a useful model to study the role of growth regulators in normal and pathological VSMC proliferation, cell death, and other aspects of cell cycle control.

SV40 LT antigen is known to have transforming and tumorigenic potential, because it is able to target and inactivate several molecules involved in the control of cell cycle progression, including the tumor suppressor molecules Rb and p53. p53 mediates the transcription of the cyclin-dependent kinase inhibitor p21WAF1, and this is a key event in cell growth arrest and in cell apoptosis due to DNA damage. p21 regulates the activity of Rb by inhibiting its phosphorylation, the subsequent release of the E2F family members, and finally the cell’s entry to S phase. p53 is also known to interact with the tumor progression factor MDM2, which promotes p53 degradation and can inhibit p53 transactivation and transrepression. Interestingly, both p53 and MDM2 have been reported to be expressed in SMCs and macrophages in human atherosclerotic tissue, and high levels of p53 in association with cytomegalovirus infection were seen in a study of coronary restenosis. Our results indicate that the rapid and continuous proliferation of HVTs-SM1 cells under permissive temperature conditions is associated with the expression of SV40 LT antigen and with LT-antigen–induced downregulation of PDGF receptor expression.

Flow cytometric analyses of UV-irradiated normal VSMCs and HVTs-SM1 cells

<table>
<thead>
<tr>
<th>Time After UV Irradiation</th>
<th>Normal Vein SMCs, %</th>
<th>HVTs-SM1 Cells, %</th>
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<tbody>
<tr>
<td>Zero time (control)</td>
<td>G1      70 1 18 65 11 17</td>
<td></td>
</tr>
<tr>
<td>6 hours</td>
<td>G1      71 3 18 47 9 10</td>
<td></td>
</tr>
<tr>
<td>12 hours</td>
<td>G1      68 4 20 37 12 14</td>
<td></td>
</tr>
<tr>
<td>24 hours</td>
<td>G1      67 6 19 29 8 29</td>
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</tbody>
</table>

Flow cytometric determinations of the time-dependent frequency distribution of cell phases (G1, S, G2) of UV-irradiated normal VSMCs and HVTs-SM1 cells. Subconfluent cells plated overnight in 6-well plates in 10% FCS-NCTC-109 at 36°C were UV-irradiated with 50 mJ/cm² and either harvested immediately or after incubation in fresh 10% FCS-NCTC-109 medium for the indicated time periods. Harvested cells were fixed in 70% ethanol and stained with propidium iodide before analysis. Results are from 1 of 2 representative determinations.

Figure 7. DNA synthesis and proliferation of HVTs-SM1 cells induced by serum and growth factors in the presence and absence of heparin. A, Cells plated at 5×10⁴ in 96-well plates at 36°C were growth-arrested for 48 to 72 hours in SF medium and then induced with either 10% FCS or 5 ng/mL growth factor in medium only, in the absence (open bars) or presence (solid bars) of 150 μg/mL heparin. B, Time-dependent proliferation of cells plated at 5×10⁴ in 35-mm-diameter plates. Cells were incubated with 10% FCS (circles) or 0.5% FCS (squares) in the absence (open symbols) or presence (solid symbols) of heparin (150 μg/mL). Results represent the mean±SD of quadruplicate determinations of 2 experiments.

Figure 8. Cell proliferation of UV-irradiated HVTs-SM1 cells. UV-irradiated (open symbols) and nonirradiated (solid symbols) cells cultured in 10% FCS-NCTG at 36°C for the indicated time points were harvested and counted. Results are the mean±SD of triplicate determinations of 2 experiments.
reported, direct binding of LT antigen to Rb may release E2F transcription factor(s) and thus contribute further to the deregulation of cell growth arrest and to apoptosis. Deregulation of cell cycle control at the level of p53 and at the level of growth regulators that depend on p53 activity in HVTs-SM1 cells is further suggested by the fact that when LT antigen is no longer functional because of a temperature shift to 39°C, the cells were able to growth-arrest. Under these conditions, the cells express high levels of p21, as is observed in senescent cells in vitro. Furthermore, cells remained viable and could be rescued and resume proliferation even several weeks later after return to the permissive temperature.

Apoptosis of VSMCs has been observed in vascular remodeling during development and may be another important mechanism in the modulation of cellularity in vascular disease. We observed that proliferation of HVTs-SM1 cells at the permissive temperature was accompanied by apoptosis. Compared with normal cells, apoptosis of transformed cells was increased by serum withdrawal, suggesting that the cells are more dependent on serum factors for survival than for proliferation. It was recently proposed that p53 is a potent inducer of apoptosis when Rb is inhibited in smooth muscle. However, the data from our system, in which both Rb and p53 are inactivated by SV40 LT antigen, imply the presence of a p53-independent pathway for apoptosis. This inference is supported by the conclusions of a recent study that examined the effect of p53 inactivation on atherogenesis in apolipoprotein E–knockout mice, an animal model for atherosclerosis. However, the cell death observed on transfer of cells to the nonpermissive temperature may be attributable to the slow loss of SV40 LT-antigen expression. UV irradiation induced rapid apoptosis in HVTs-SM1 cells grown at the permissive temperature, whereas normal cells did not undergo detectable apoptosis after UV irradiation. The reason for this is unclear, but it may be a result of their slow proliferation rate, which allows DNA repair to take place. This question requires further study, and examination of the effects of more pathophysiologically relevant mediators, such as oxidized lipoproteins, oxysterols, and nitric oxide, may be of particular interest. Apoptosis after DNA damage by UV irradiation has been also associated with increased p53 activity, but from the results of the present study, it appears that p53-induced transcriptional activity is not required for UV-induced apoptosis. Overall, a possible hypothesis is that in HVTs-SM1 cells, E2F1 overexpression (due to inactivation of Rb by LT antigen) can induce apoptosis independently of p53, as previously shown in Saos-2 cells, which are null for p53 and lack a functional Rb. Further studies will be necessary to confirm this hypothesis.

In conclusion, we have shown that the established HVTs-SM1 cell line can be used as an in vitro model to study specific perturbations of cell proliferation. This model of human VSMCs offers opportunities to establish a better understanding of the processes and molecules involved in the aging and senescence of the vasculature and in the control of the cell cycle in human VSMCs.

Acknowledgments

This work was supported by the Thrombosis Research Trust (UK), the British Heart Foundation (UK), the British Council, the UK Home Office, and the Greek-Britain Scientific and Technological Collaboration. D. Kletsas was supported by a FEBS fellowship. We are grateful to M.J. O’Hare for the transfection of cells and K. Gallagher for assistance with tissue culture. We also thank H. Pratsinis and members of Clinical Pharmacology, Imperial College, for their helpful comments and assistance.

References


p53, p21WAF1/CIP1, and MDM2 Involvement in Proliferation and Apoptosis in an In Vitro Model of Conditionally Immortalized Human Vascular Smooth Muscle Cells
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doi: 10.1161/01.ATV.20.3.636
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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