Transfer of Wild-Type p53 Gene Effectively Inhibits Vascular Smooth Muscle Cell Proliferation In Vitro and In Vivo

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Abstract—Wild-type p53 (wt-p53), a key protein in cell cycle regulation, inactivates the G1 cyclins through direct activation of p21Waf-1/Cip-1/Sdi-1. Persistent vascular smooth muscle cell (VSMC) proliferation following vascular interventions hinders the benefits of these therapeutics. Using the hemagglutinating virus of Japan/liposome–mediated gene transfer method, we examined the inhibitory effect of overexpression of exogenous wt-p53 on VSMC proliferation in vitro and in vivo. We assessed the proliferative activity of human p53 cDNA–transduced bovine VSMCs by DNA synthesis assay, flow cytometry, and cell proliferation assay. p53 gene transfer reduced thymidine incorporation of VSMCs stimulated by platelet-derived growth factor-BB (P<.001). The p53-transduced VSMCs underwent synthetic phase depletion (mean, 8.02% versus 33.7% of control; P<.001) and transient G2/M accumulation 2 days after gene transfection, and in almost all cells, G1 arrest occurred (mean, 92.6% versus 79.3% of control; P<.001) 5 days later. The wt-p53 gene transfection also inhibited the VSMC proliferation (P<.001) with no detectable induction of apoptosis. Cell death of p53-transduced VSMCs was induced only by additional treatment with an apoptosis-stimulating reagent, doxorubicin. The verification of apoptosis was made by DNA ladder, flow cytometry, and electron microscopy. In vivo transfection of p53 cDNA inhibited neointimal formation after balloon injury in rabbit carotid arteries, without apoptotic stimuli (P=.01). Thus, overexpression of the p53 gene in the injured arterial wall inhibits the proliferation of VSMCs in vitro and in vivo. This novel concept, including not only exogenous but also endogenous p53 overexpression in the vessel wall, may be one approach worth exploring in the treatment of patients with restenosis occurring after vascular interventions. (Circ Res. 1998;82:147-156.)

Key Words: wild-type p53 • cell cycle arrest • hemagglutinating virus of Japan/liposome • smooth muscle • apoptosis

Wild-type p53 tumor suppressor protein (wt-p53), a nuclear molecule, regulates the cell cycle of neoplastic or nonneoplastic cells by activating the G1 cyclin–suppressive proteins, such as p21Waf-1/Cip-1/Sdi-1. Overexpression of this nuclear protein is also attributed to cell cycle arrest or programmed cell death (apoptosis), and the latter process markedly enhances the cytotoxicity of anticancer agents in different cell lines. On the other hand, persistent VSMC migration and proliferation and the deposition of extracellular matrix, either after PTCA or at sites of anastomosis after peripheral vascular surgery, remain critical problems to be overcome. Speir et al reported that smooth muscle cell proliferation in restenotic human coronary arteries after PTCA might be induced by inhibition of p53 function by interacting with immediate-early 84-kD protein (IE-2) of human CMV. This suggests that p53 may possibly play a critical role in VSMC proliferation after vascular injury. Although the role of CMV-IE in coronary stenotic lesions is controversial, the concept of intentionally inducing supportive wt-p53 expression in most VSMCs in vivo seems reasonable. Several studies indicated that low-dose irradiation reduced neointimal formation after balloon injury or stenting; however, the molecular mechanism involved in reduction of intimal thickening in vivo remained to be determined. Since low-dose irradiation is an inducer of endogenous p53 gene expression, the inhibitory effect of irradiation on VSMC proliferation would depend, to some extent, on p53 expression.

To evaluate the therapeutic benefit of induction of wt-p53 on growth control of VSMCs, we transduced human wt-p53 cDNA into cultured bovine VSMCs in vitro and in balloon-injured rabbit carotid arteries in vivo using the HVJ/liposome-mediated gene transfer method. We also examined the efficiency of growth inhibition related to p53-dependent cell

Received February 14, 1997; accepted October 29, 1997.
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Selected Abbreviations and Acronyms

BSS = balanced salt solution
CMV = cytomegalovirus
DOX = doxorubicin
HMG = high-mobility group
HVJ = hemagglutinating virus of Japan
NR = neutral red dye
PAI-1 = plasminogen activator inhibitor-1
PDGF-BB = platelet-derived growth factor-BB homodimer
PTCA = percutaneous transluminal coronary angioplasty
t-PA = tissue plasminogen activator
u-PA = urokinase plasminogen activator
VSM = vascular smooth muscle
VSMC = VSM cell
wt-p53 = wild-type p53 tumor suppressor protein

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cycle arrest and the effect of an anticancer reagent, DOX, on the p53-overexpressing VSMCs. Apoptotic stimuli were also included as a positive control study for the induction of apoptosis. We then evaluated the possible application of this novel strategy for treatment of vascular disorders related to restenosis.

Materials and Methods

Cell Culture

Bovine VSMCs were isolated from aortic media by the explanting of the bovine aortic wall after the endothelium and adventitia had been carefully removed. These cells were grown on plastic culture dishes (Falcon Labware) in DMEM supplemented with 10% FCS (Flow Laboratories). The VSMCs were confirmed by morphological features and by immunohistochemistry, using anti–smooth muscle cell α-actin monoclonal IgG (Sigma Chemical Co) (data not shown). VSMCs from the sixth to eighth passages were used for the following in vitro experiments.

Construction of Human wt-p53 cDNA Expression Vector

Human wt-p53 cDNA (php53cl) was kindly provided by Dr T. Takahashi, Aichi Cancer Center Research Institute, Japan. The Xho I–Xho I fragment of p53 cDNA, which contains the entire p53 coding region, was inserted downstream from the promoter/enhancer of human CMV immediate-early gene of pRc/CMV (Invitrogen), known as pRc/CMV-wt-p53. pRc/CMV without cDNA and pSV–B galactosidase (Promega Corp) were used as negative controls for in vitro or in vivo studies.

Preparation of HVJ/Liposomes

Procedures used for the preparation of HVJ/liposomes were as documented. Briefly, 64 μg of HMG nuclear protein extracted from calf thymus (HMG-1 and -2 mixture, Wako Pure Chemicals) and 200 μg of plasmid DNA were mixed and incubated at 20°C for 1 hour in 200 μL of BSS (140 mmol/L NaCl, 5.4 mmol/L KCl, and 10 mmol/L Tris-HCl, pH 7.6). Phosphatidylserine (sodium salt), from calf thymus (HMG-1 and -2 mixture, Wako Pure Chemicals) and 200 mg of proteinase K (Boehringer-Mannheim) were added to the mixture. The mixture was incubated at 37°C for 30 minutes and then incubated with 1 μg/mL of proteinase K (Boehringer-Mannheim) for 1 hour at 4°C and treated with 15% for 1 hour at 4°C and treated with 15% formamide, 10% FCS (Flow Laboratories). The VSMCs were confirmed by morphological features and by immunohistochemistry, using anti–smooth muscle cell α-actin monoclonal IgG (Sigma Chemical Co) (data not shown). VSMCs from the sixth to eighth passages were used for the following in vitro experiments.

In Vitro Gene Transfer

The VSMCs seeded on dishes were rinsed twice with BSS, after which HVJ/liposome solution was added. The dishes were kept at 4°C for 5 minutes and 37°C for 30 minutes and were then washed with fresh medium and incubated in a CO2 incubator at 37°C with 10% FCS/DMEM. Two days after transfection, the cells were treated for 3 hours in the presence or absence of DOX (Kyowa Hakko Co Ltd). These cells were then used for the experiments described below.

RNA Extraction and Northern Blotting for Human p53 Transcript in VSMCs

Total cellular RNA from the pRc/CMV-wt-p53–transferred VSMCs was isolated using the acid guanidinium thiocyanate–phenol–chloroform extraction method and was then electrophoresed on a 1.0% agarose-formaldehyde gel and transferred overnight to a nylon membrane (GeneScreen) by the capillary blotting in 20× SSC. After prehybridization, the filters were hybridized overnight with random (α-32P) dCTP–labeled probes at 42°C. The bands were then visualized by autoradiography.

In Situ Hybridization

The digoxigenin-labeled antisense and sense ribonucleotide probes were synthesized in vitro by SP6 or T7 RNA polymerase (GIBCO-BRL) from digested pRc/CMV-wt-p53 with HindIII or ApaI. The VSMCs seeded on slide chamber plates were fixed with 4% paraformaldehyde for 1 hour at 4°C and treated with 1 μg/mL of proteinase K (Boehringer-Mannheim) for 15 minutes at 37°C. One hundred microliters of hybridization solution (50% formamide, 10 mmol/L Tris-HCl, pH 7.6, 200 μg/mL tRNA, 1× Denhardt’s solution, 600 mmol/L NaCl, 0.25% SDS, and 1 μL/mL EDTA) containing digoxigenin-labeled ribonucleotide probes was then applied on each slide; thereafter, hybridization was performed for 16 hours at 50°C in a wet chamber. After washing and blocking treatment, 500X diluted anti-digoxigenin Fab (Boehhringer-Mannheim) was applied for 30 minutes at room temperature. The reaction products were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride, according to the standard procedures given in the nonradioactive RNA Labeling and Detection Kit (Boehringer-Mannheim).

Immunocytochemical and Immunohistochemical Detection of wt-p53 Protein

The p53 protein synthesis of the gene–transferred VSMCs in vitro and in vivo was examined, using immunocytochemical and immunohistochemical techniques. Mouse anti-human p53 monoclonal IgG (PAb 1801, Oncogene Science Inc) was the primary antibody used. Nonimmunized mouse IgG1 (MOPC21, Cappel Products) instead of PAb 1801 was used for the negative control study. The cultured VSMCs were fixed with ice-cold acetone for 10 minutes. The gene–transferred rabbit arteries, which were fixed in 4% paraformaldehyde and embedded in paraffin, were sectioned for 5 μm and autoclaved for 1 minute at 120°C and 2 atm after deparaffinization. After blocking with 3% skim milk for 30 minutes and incubation with primary antibody for 1 hour, the cells were washed with 0.01 mol/L phosphate buffer and then reacted with biotinylated rabbit anti-mouse IgG+IgA+IgM (Histofine kit, Nichirei Co). Thirty minutes later, the cells were treated with the avidin–biotin–peroxidase complex (Histofine kit) for 15 minutes at room temperature. Reaction products were developed in 3,3′-diaminobenzidine tetrahydrochloride containing 0.1% H2O2. As negative controls, nonimmunized mouse serum was used instead of primary antibody and pRc/CMV vector plasmid–transferred VSMCs. The arterial segments for in vivo gene transfer efficiency were sectioned into five serial ones at 5-mm intervals per animal (total, 15 sections of three animals in each group, respectively). These sections were embedded in paraffin, and 5-μm-thick sections were used for studies on the immunohistochemistry of p53.
DNA Synthesis Assay
The cells in growth phase were seeded at 30% confluence per well and were incubated with the 0.5% FCS/DMEM for 30 hours. VSMCs treated with HVJ/liposomes with pRc/CMV or pRc/CMV-wt-p53 were then incubated with 0.5% FCS/DMEM, supplemented with or without 10 ng/mL of recombinant human PDGF-BB (Biomedical Technologies Inc) at 37°C for 24 hours. The cells were pulsed for 8 hours with 0.5 μg of [3H]thymidine (Amer sham Co) per milliliter of medium. After washing in PBS, the cells were extracted with cold 5% trichloroacetic acid. The radioactivity of resulting precipitates was determined in a liquid scintillation counter (LSC-3500, Aloka Co).
This experiment was repeated three times.

Flow Cytometry for Cell Cycle Analysis
Cell cycle analysis was assayed by flow cytometry. The pRc/CVM-wt-p53 vector was transferred into VSMCs at 30% confluence in 75-mm2 flasks by HVJ/liposomes, using the same procedure described above. Two or 5 days later, the cells were collected after trypsin treatment. The cells were treated with 0.1% Triton X and 0.5% RNase A, and then propidium iodide was added. All samples were passed through 70-μm mesh before the flow cytometry analysis, which was performed on a FACStar Plus (Becton Dickinson) flow cytometer; 30,000 nuclei were noted in each analysis. The percentage of cells in each phase of the cell cycle was estimated using the program of CellFIT cell-cycle analysis, version 2.0 (Becton Dickinson). Three separate experiments were performed with three different populations of cells.

Estimation of Cell Viability by NR Assay
All procedures were performed in almost the same manner as reported by Borenfreund et al. Briefly, the VSMCs were seeded into 96-well microtiter plates at 30% confluence. Two or 5 days after the transfection procedures, we added to each well 0.2 mL of fresh medium containing 40 μg/mL NR (Nakarai Tesque), which was passed through a 0.22-μm filter just before use to remove the possible aggregates. Incubation of cultures with NR solution was then continued for 3 hours at 37°C. The cells were then rapidly washed with PBS and fixed with 0.5% formalin/1% CaCl2 (vol/vol), and the NR incorporated into the viable cells was released into the supernatant with 0.2 mL of 1% acetic acid/50% ethanol. Absorbance was photometrically recorded at 540 nm, and the data, expressed as percentage of the untreated controls (mean±SD), were used for an index of the ratio of viable cells.

Analysis of DNA Fragmentation
The collected cells were treated with 240 μL of lysis solution (400 μg/mL proteinase K, 400 μg/mL RNase, and 1% SDS in 10 mmol/L Tris-HCl with 1 mmol/L EDTA, pH 8.0) for 30 minutes at 37°C. Then, 300 μL of NaI solution (6 mol/L NaI, 13 mmol/L EDTA, 0.5% sodium-N-laurylsarcosinate, 10 mg/mL glycogen, and 26 mmol/L Tris-HCl, pH 8.0) was added, and the preparation was incubated at 60°C for 15 minutes. Thirty micrograms of DNA was applied in each lane and electrophoresed in a 1% agarose gel.

Transmission Electron Microscopy
Four days after gene transfer, the VSMCs were trypsinized, collected, and fixed with 3% glutaraldehyde. After postfixation with 2% osmium tetroxide, the cytosin cells were dehydrated in a graded series of ethanol and embedded in Epon 812 (TAAB Co Ltd). The ultrathin sections were cut, stained with uranyl acetate, and examined under a JEOL 1200 EX transmission electron microscope (Nippon Denshi Ltd) at 80 kV.

Histological Examination
Immediately after the rabbits were killed, the common carotid artery, which had been dissected gently and carefully, was cannulated with an 18-gauge catheter. The arterial segments were rinsed with normal saline for 10 minutes and perfused with 4% formaldehyde for 10 minutes at a sustained positive pressure of 150 mm Hg in vivo, and then additional fixation was applied for 6 hours. The arterial segments were sectioned into five or six serial sections at 5-mm intervals. These sections (5 μm thick) were embedded in paraffin and stained with hematoxylin–eosin and elastica van Gieson for light-microscopic examination. The intimal and medial area of five sections, at 5-mm intervals per one artery, were quantified using a Nikon COSMOS-ZONE image analyzer (Nikon Co Ltd). The average value of each intima-to-media ratio derived from the same segment was used.

Statistical Analysis
All values are expressed as mean±SD. The data were analyzed by one-way ANOVA, and where appropriate, Student’s t test with Scheffe’s adjustment for multiple comparisons was used.

Results
Human p53 Gene Expression and Protein Synthesis in Gene-Transferred VSMCs
In the Northern blot analysis, the gene expression of p53 was not detected in the control pRc/CVM-transduced VSMCs but was evident in the pRc/CVM-wt-p53 gene-transferred VSMCs, using a human p53 cDNA probe (Fig 1A). In addition, the entire cell population of VSMCs transferred with pRc/CVM-wt-p53 gave positive cytoplasmic signals for p53 mRNA, as assessed by in situ hybridization, using a digoxigenin-labeled cRNA antisense probe (Fig 1B, a), whereas the sense probe for the p53-transfected cells (Fig 1B, b) and the antisense probe for the control vector-transferred cells (Fig 1B, c) were both negative.
By use of PAB 1801 and immunoperoxidase methods, the human p53 protein was detected in the nuclei of VSMCs as a light brown stain (Fig 2a, arrows). In contrast, there was a lack of stain in cells that were transfected with pRc/CVM-wt-p53 and treated with nonimmunized mouse IgG1 (Fig 2b) or
transfected with the vector without cDNA and reacted with PAb 1801 (Fig 2c).

Inhibition of DNA Synthesis due to wt-p53 Gene Transfer
As shown in Fig 3, under a low serum concentration (0.5%) without PDGF stimulation, the baseline value of the thymidine uptake by untreated VSMCs was 12568.1 ± 1696.2 cpm/well. High-dose PDGF-BB stimulation (10 ng/mL) significantly increased the DNA synthesis of untreated or HVJ/liposome vehicle–treated VSMCs 3-fold (43518.3 ± 1593.9 and 42776.1 ± 2089.9 cpm/well, respectively), whereas the p53 gene transfer inhibited thymidine uptake (21915.5 ± 2886.2 cpm/well, P < .001).

Figure 1. A, Northern blot analysis for gene expression of human wt-p53 gene-transferred VSMCs. Total RNA was extracted 2 days after gene transfer, and 10 μg of total RNA was electrophoresed in each lane. [α-32P]dCTP-labeled probes synthesized from the Xba I–Xba I fragment of pHSV3cl were hybridized to the transferred membrane. Intense p53 gene expression was evident in the right lane (pRc/CMV-wt-p53 gene-transferred VSMCs), whereas no apparent band was detected in the left lane (pRc/CMV control vector–transferred VSMCs). B, In situ detection of gene expression of human wt-p53 on VSMCs by a nonradioactive in situ hybridization technique, using a digoxigenin-labeled probe. a, In situ hybridization with an antisense cRNA probe for the human wt-p53–transferred VSMCs. The entire cell population of VSMCs showed a strong positive signal (dark purple) in the cytoplasm. b, In situ hybridization using a sense cRNA probe for the wt-p53–transferred VSMCs. c, In situ hybridization using a digoxigenin-labeled antisense cRNA probe for the control vector (pRc/CMV)–transferred VSMCs. No positive signal can be seen in either b or c of panel B. These experiments were repeated three times with similar results.

Figure 2. Immunocytochemical detection of human wt-p53 protein on pRc/CMV-wt-p53–transferred bovine VSMCs. Two sections (a and c) were reacted with anti-human p53 monoclonal antibody (PAb 1801), and the remaining one (b) was treated with nonimmunized mouse IgG1 (isotype-matched). All sections were counterstained with hematoxylin. a, wt-p53–expressing VSMCs gene-transferred by HVJ/liposomes. Light brown staining is recognized in the nuclei (arrows). b, wt-p53–transduced VSMCs treated with HVJ/liposomes that reacted with nonimmunized mouse IgG1. No positive reaction was observed in this specimen. c, Control vector–transduced VSMCs treated with HVJ/liposomes. No positive signal was detected. These experiments were repeated three times with similar results.

Figure 3. Inhibitory effect of transfected p53 cDNA on incorporation of [3H]thymidine in cultured VSMCs. Each group contained four samples. Untreat indicates untreated cells; vehicle, cells treated with HVJ/liposome vehicle without plasmid DNA; and p53, pRc/CMV-wt-p53–transferred cells treated with HVJ/liposomes. This experiment was repeated three times with similar results.

Effects of wt-p53 Gene Expression and/or DOX Treatment on VSMC Proliferation
Because wt-p53 overexpression is well known to enhance chemosensitivity, we examined the effects of wt-p53 gene transfer and/or the anticancer reagent DOX on cell proliferation, using a rapid and highly sensitive NR chemosensitivity assay (Fig 4).

First, we determined the dose-dependent effect of DOX on wt-p53–transferred or –untransferred VSMCs. The cells used in this study were resistant to DOX, and 20% of growth inhibition (versus untreated control) was observed 3 days after treatment even with a high concentration of DOX (2 μg/mL, Fig 4A). On the other hand, wt-p53 gene transfer itself decreased the cell number (63.8 ± 8.62% versus untreated control), and high-dose DOX (2 μg/mL) led to the markedly decreased viable cell number of the p53 gene-transferred VSMCs 5 days after transfer (12.1 ± 3.42% versus untreated control, Fig 4A). On the basis of these findings, we used this dose of DOX in the following experiments.

Figure 4. Effects of DOX treatment on VSMC proliferation.
Flow Cytometric Analyses

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Results are shown after cell cycle analysis for the effects of wt-p53 gene transfer. Cells were collected at 2 or 5 days after gene transfer, and 30,000 propidium iodide–labeled nuclei were encountered by FACStar Plus (Becton Dickinson). Three separate experiments were performed. Values are mean±SD.

*P<.001 vs no-treatment group; †P<.001 vs no-treatment group or vehicle-treated group.

Next, we examined the time course of the coupled effects of wt-p53 gene transfer and DOX treatment on VSMC proliferation, and the results are given in Fig 4B. The untreated VSMCs proliferated gradually (218.85±19.43% at 2 days and 364.49±50.00% at 5 days after gene transfer, Fig 4B). The HVJ/liposome vehicle–treated VSMCs also showed a gradual proliferation (178.34±29.60% at 2 days and 310.87±21.03% at 5 days after treatment), which was the same level as the untreated control. On the other hand, growth of wt-p53–transduced cells was almost arrested during the experiment (116.04±27.90% at 2 days and 121.83±12.81% at 5 days after transfection). Moreover, DOX treatment (2 μg/mL) 2 days after wt-p53 gene transfer significantly reduced the cell number 3 days after drug treatment (45.17±16.39%).

Cell Cycle Analysis of VSMCs by Flow Cytometry

In the case of the 10% serum–stimulated proliferation of VSMCs, a FACScan (Becton Dickinson) demonstrated that 33.7±1.25% of the cells were in S phase and 9.8±0.95% of the cells were in G2/M phase (Table). The VSMCs treated with HVJ/liposome vehicles showed a mild decrease in number in the S phase (19.2±0.78%). Three days later (a total of 5 days after transfection), cell population of the untreated or vehicle-treated VSMCs in S or G2/M phase reached similar levels. On the other hand, wt-p53–transduced cells was almost arrested during the experiment (116.04±27.90% at 2 days and 121.83±12.81% at 5 days after transfection). Moreover, DOX treatment (2 μg/mL) 2 days after wt-p53 gene transfer significantly reduced the cell number 3 days after drug treatment (45.17±16.39%).

VSMCs was due to a direct effect of the HVJ/liposome particles. DOX treatment demonstrated S-phase depletion (2.0±0.30%) and G2/M accumulation (20.1±3.09%) 3 days after treatment.

Figure 4. A, Dose-dependent effects of DOX on either wt-p53 gene-transferred or nontransferred VSMCs. The number of cells was calculated 5 days after gene transfer, and the data were expressed as relative cell number per untreated control. Cell growth of control vector plasmid (pRc/CMV)–transferred VSMCs (□) was slightly inhibited, in a dose-dependent manner. On the other hand, cell number of the wt-p53 gene–transferred VSMCs (■) was remarkably and dose-dependently reduced by DOX treatment. B, NR assay for cell proliferation. All cell numbers were expressed in relative percentages compared with those of VSMCs at day 0. Untreated or HVJ/liposome–treated VSMCs proliferated and reached a subconfluent state 5 days after gene transfer. The proliferation of wt-p53 gene–transferred VSMCs was arrested at almost the same level on days 0 or 2. ■ indicates untreated; ●, HVJ/vehicle–treated; ○, wt-p53 gene–transferred; and ▪, wt-p53 gene–transferred with DOX treatment.

Figure 5. DNA histograms of VSMCs treated with DOX and wt-p53 gene transfer. Small fragments under the 20 counts of propidium iodide fluorescence intensity for FACSscan (Becton Dickinson) parameter 2, containing small DNA fragments and some cell debris, were omitted from the histograms. Both adherent cells and nonadherent cells show peaks of fragmented DNA, suggesting apoptosis (thin arrows, A). Both S-phase depletion (open arrow) and G2/M accumulation (thick solid arrow) are noted.
On the other hand, the combined effect of p53 gene transfer and DOX treatment was also assessed by FACScan. DNA histograms of VSMCs treated with DOX 5 days after wt-p53 gene transfer is demonstrated in Fig 5. Fragments at 20 counts of propidium iodide fluorescence intensity, containing small DNA fragments and cell debris, were omitted from the histograms. Both adherent and nonadherent cells showed peaks of fragmented DNA, thus suggesting apoptosis (thin arrows, A). In addition, both S-phase depletion (open arrow) and G2/M accumulation (thick solid arrow) were also associated in these adherent cells.

Evidence of Apoptosis due to wt-p53 Gene Transfer and/or DOX Treatment

We assumed that a markedly decreased cell number induced by treatment of wt-p53 gene transfer combined with DOX was due to apoptosis, and we searched for convincing evidence of apoptosis on VSMCs.

Four days after gene transfer, the cells were collected, cellular DNA was extracted, and agarose gel electrophoresis was performed to analyze DNA fragmentation. The results are demonstrated in Fig 6. In the untreated, HVJ/liposome vehicle–treated, wt-p53 gene-transferred, and DOX-treated groups, there was no evidence of DNA fragmentation. On the other hand, the combined effect of wt-p53 gene transfer and DOX treatment on apoptosis was confirmed by DNA ladder formation.

We also examined ultrastructure of these cells by transmission electron microscopy (Fig 7). Control cells treated with HVJ/liposome vehicles had an almost normal appearance with dense lysosomes (Fig 7a, thin arrow), thereby indicating entrapped lipids of vector components. On the other hand, the coupled treatment of p53 gene transfer and DOX resulted in various ultrastructural features in the VSMCs. Some VSMCs treated with DOX after p53 gene transfer showed a peripheral condensation of chromatin (Fig 7b, thick arrows), a blebbing of the cellular membrane (Fig 7c, open arrow), and the disappearance of villi. These early cellular changes indicated apoptosis. On the other hand, a large number of VSMCs had a fragmented chromatin, so-called “apoptotic bodies” (Fig 7d). These findings are considered to represent definitive evidence of apoptosis in VSMCs.

Effect of wt-p53 Gene Transfer on Neointimal Formation In Vivo

The inhibitory effect of p53 gene transfer on balloon-injured rabbit carotid arteries was examined. Four days after gene transfer, arterial segments (n=3, each groups) were studied immunohistochemically to detect transgene expression (Fig 8). In the wt-p53 gene–transduced arterial segments, brown signals were frequently evident in the nuclei of the medial VSMCs using PAb 1801, an anti-human p53-specific monoclonal antibody (Fig 8a), whereas no apparent signal was observed using nonimmunized mouse IgG1 for primary antibody as a negative control on serial sections (Fig 8b). In addition, no nuclear signal was detected with PAb 1801 in the medial VSMCs of mock-transfected injured arteries (Fig 8c). The p53–positive cell ratio in the medial cells was estimated to be \( \approx 32.3 \pm 11.5\% \).
Histological examination was made on arterial sections harvested 4 weeks after injury. Severely thickened neointima was noted in balloon-injured rabbit carotid arteries treated with HVJ/liposomes containing pRc/CMV control vector (Fig 9a and 9b), with containing LacZ expression vector (pSV-β-galactosidase), or with BSS only (data not shown). On the other hand, pRc/CMV-wt p53–transferred arteries showed a reduced neointimal area (Fig 9c and 9d).

These arterial sections were evaluated quantitatively using an image analyzer (Fig 10). Five sections spaced 5 mm per artery (each group contains total 25 sections) were examined. The medial areas of both groups did not differ (BSS, 3.24 ± 0.36 mm²; pSV-β-galactosidase, 3.25 ± 0.27 mm²; pRc/CMV, 3.22 ± 0.59 mm²; and pRc/CMV-wt p53, 3.29 ± 0.50 mm²; n=5 for each group), whereas the intimal area of the p53 gene-transferred arteries was remarkably reduced (BSS, 3.45 ± 1.01 mm²; pSV-β-galactosidase, 3.56 ± 0.70 mm²; pRc/CMV, 3.62 ± 0.89 mm²; and pRc/CMV-wt p53, 0.56 ± 0.11 mm²; n=5 for each group; P<.01).

Carotid arterial sections from the mock-transfected control arteries had an intima-to-media ratio of 0.96 ± 0.13 (BSS, n=5), 1.05 ± 0.15 (pSV-β-galactosidase, n=5), and 1.006 ± 0.36 (pRc/CMV, n=5). On the other hand, wt-p53 transfection resulted in an intima-to-media ratio of 0.176 ± 0.05 (n=5), a >80% reduction compared with the control vessels treated with vector only (P<.01).

**Discussion**

In the present study, we confirmed wt-p53–dependent growth inhibition on VSMCs via cell cycle arrest at G1 or G2/M, without massive cell death, and we demonstrated that the combined treatment of p53 overexpression and apoptotic stimuli induces apoptosis in vitro. We also demonstrated that p53 gene transfer itself effectively inhibits neointimal formation after balloon-induced injury in vivo without any apoptotic stimulus. These findings suggest a role for the p53 molecule.
in the regulation of VSMC proliferation and apoptosis and possible implications for restenosis therapy. However, several important issues, especially differences among the studies of other investigators and our present study, should be addressed.

We detected no endogenous expression of the p53 gene by Northern and in situ hybridization or of p53 protein by immunocytochemistry in the control bovine VSMCs, as shown in Figs. 1, 2, and 8. On the other hand, Bennett et al. noted in Northern blots endogenous p53 mRNA in control rat VSMCs. The nucleotide sequence homology between human p53 and bovine p53 (83%), H. Komori, N. Ishiguro, M. Horiuchi, M. Shinagawa, Y. Aida, unpublished data, 1995; GenBank accession No. D49825) is higher than that between human and rat (78%)10; thus, the human p53 probe, including the entire coding region used in the present study, could detect endogenous p53, and this expression was too low in control cells to detect by Northern analysis. In addition, as shown by Bennett et al, the sensitivity of immunohistochemistry is too weak to detect unstable wt-p53 protein, whereas p53 gene and protein expression could be confirmed by Northern or Western analyses. Regarding these findings, the effect of endogenous p53 gene expression does not seem to be significant in our present study.

There was a discrepancy between gene transfer efficiencies in the p53 protein detection rate in the present study and LacZ gene used in our previous study.13 It is suggested that the instability of protein of wild-type, not mutant, p53 also simian virus 40 large-T antigen, adenoviral E1A, or human prolong and easily detected immunocytochemically when p53 protein than for LacZ. The half-life of p53 protein is is that the half-life of wt-p53 is much shorter than that of the gene used in our previous study.13 It is suggested that the instability of cell proliferation controlled by p53 may be a paradox. The immunohistochemically detected human wt-p53 protein was estimated to be ≈32% for the nuclei, as described in “Results,” whereas with LacZ gene transfer, it was >80% of the transduction rate in our previous study.11 One explanation is that the half-life of wt-p53 is much shorter than that of the mutant p53, which may explain the lower detection rate for p53 protein than for LacZ. The half-life of p53 protein is prolonged and easily detected immunocytochemically when the cells coexpress other p53-binding viral protein, such as simian virus 40 large-T antigen, adenoviral E1A, or human CMV IE-2,1 or cells including a germ-line mutation of p53. Bennett et al.12 reported that the p53 gene and protein expression were detected by Northern and Western analyses, respectively, in normal VSMCs or in the VSMCs transduced by retroviral vector alone, whereas p53 staining was seen in normal VSMCs or VSM-vector cells. However, they further reported that staining in VSMC-myc or VSM-E1A cells determined by immunocytochemistry was obvious and that increased amounts of p53 protein in E1A-coexpressing cells without an increased expression of mRNA is evidence of stabilized p53 protein. In the present study, high-level gene expression of p53 driven by the CMV enhancer/promoter was achieved by exogenous p53 gene transfer and would result in an ≈2.5-fold lower detection rate of wt-p53 protein in the nuclei of rabbit VSMCs, which do not express such viral protein. Although we were unable to assess the role of the undetectable endogenous p53, the observations of a strongly expressed exogenous wt-p53 gene do provide significant and important implications regarding VSMC growth control.

Flow cytometry analysis in the present study demonstrated transient G2/M accumulation of wt-p53 gene-transferred VSMCs. Wt-p53 is considered to be a potential inhibitor of cyclin-dependent kinase–cyclin A, D, and E complexes and results in cell growth arrest in the G1 phase (G1 arrest), and it has yet to be elucidated whether this nuclear protein can affect other phases of the cell cycle. Stewart et al12 reported that the antiproliferative activity of p53 is involved in regulation of the cell cycle at the G1/M restriction point as well as transit through G1/S and the initiation of DNA synthesis. They suggested that p53 may control the expression of either p21Cip1/Waf1 or another related cdc2/cyclin B kinase–interacting protein at the G1/M part of the cell cycle, and their data strongly support our results. We also demonstrated by flow cytometry that a mild S-phase decrease was induced by the HVJ/liposome vehicle compared with no treatment. This was also observed in the VSMCs transferred with pRc/CMV control plasmid by HVJ/liposomes, and it may be that the slight and transient inhibition of cell cycle progression induced by vehicles affects G1/S transition.

The wt-p53–mediated apoptosis may contribute to reduction of intimal thickening in p53-overexpressing vessels. However, there are conflicting differences regarding the role of p53 in VSMC apoptosis among the results of recent studies, including our present study. In the present study and that of Katayose et al.22 G1 and G2/M accumulation was found to be induced in human or bovine VSMCs by p53 gene transfer, whereas Bennett et al17 found that in normal rat VSMCs transduced with p53 there were no alterations in mitosis, no change in the number of cells in S phase, no accumulation in G1, and no cell cycle arrest. However, our present study has shown that another apoptotic stimulus was necessary to induce massive cell death in bovine VSMCs, whereas p53 gene transfer itself resulted in apoptosis in human VSMCs.22 More recently, Johnson et al.26 indicated that exogenous p53 induced massive apoptosis of human VSMCs, whereas rat VSMCs were resistant to p53 transfection, despite the induction of endogenous p21 after p53 gene transfer in both cell species. Although it can be hazardous to compare results obtained under different experimental conditions, some discussions can be made. First, it is possible that the expression level of exogenous p53 may affect its biological properties, such as cell cycle arrest or apoptosis, in VSMCs. Katayose et al.22 Johnson et al.23 and our team used a stronger CMV promoter than that of the retroviral long-terminal repeat used by Bennett et al. This was evident by the detection of wt-p53 protein driven under the CMV promoter in the present study; Bennett et al detected wt-p53 protein driven by long-terminal repeat. A high level of gene expression may be necessary for p53 to act as a transcription factor, because p53 requires oligomerization (tetramer) to bind its consensus sequences.24 In addition, the observations of Johnson et al.23 also suggest that VSMC responses to wt-p53 expression may be varied and species dependent. All these findings suggest that the biological actions of p53 molecule may be more complex and different; hence, further examination is required to clarify its role in VSMC proliferation.

How does the wt-p53 molecule prevent VSMC proliferation in vivo? Cell cycle arrest, apoptosis, other biological effects, or effects induced by p53 overexpression may suppress VSMC proliferation after vascular injury in animal models or in the human artery in vivo. The overexpression
of p21/Waf-1/Cip-1/Sdi-1 inhibited neointimal formation, an ≈50% reduction rate after balloon angioplasty in the rat carotid artery.25 We noted a reduction of intimal thickening of ≈80% by p53 gene transfection, without apoptotic stimuli. The wt-p53 molecule upstream from p21 is expected to have beneficial biological actions, including not only cell cycle control via p21 but also other effects via related genes. There are reports suggesting the potential superior usefulness of wt-p53 to p21 in VSMC growth control in vivo. Plasmin generation catalyzed by u-PA and t-PA proved necessary for migration of VSMCs26 in vivo, and PAI-1 strongly interferes with this process. Another study showed that wt-p53 represses the transcription of u-PA and t-PA and activates the PAI-1 gene expression, and it was suggested that cellular expression of wt-p53 may contribute not only to the growth of neoplastic cells but also to their migration.27 Moreover, p53 suppresses the activity of various promoters of proto-oncogenes, such as c-fos28,29 and c-jun,28 and overexpression of these genes is frequently observed in the early phase of vascular injury.30,31 These both cell-static and paracrine mechanisms induced by p53 may be more pertinent to address the problem of VSMC proliferation than considering only cell-static strategy using p21 as a cyclin inhibitor. In the present study, we used DOX for apoptotic stimulus as a positive control for the induction of apoptosis on VSMCs. Can a greater reduction in neointima be expected by the additional administration of DOX in wt-p53–expressing vessels? It may be possible, but such is not practical for clinical use. Our present study showed that a high concentration of DOX (2 μg/mL) was required to induce apoptosis of VSMCs; however, >0.5 μg/mL of systemic DOX concentration might result in unexpected side effects in mice (Kyowa Hakko, Co Ltd, unpublished data, 1971). Thus, our results suggest that overexpression of wt-p53 itself is sufficient and that additional DOX administration is not required to suppress the neointimal formation in vivo. One limitation of the present study is that we were unable to address the contribution of apoptosis in inhibiting VSMC proliferation via wt-p53 overexpression during the repair process after injury.

During the intimal repair process after vascular injury, the gene expression of c-fos and the ornithine decarboxylase gene are upregulated promptly,30 which means that activation of the signal transduction pathway in VSMCs, as induced by various growth factors that bind to tyrosine kinase–type receptors, is completed in 1 day. Bromodeoxyuridine– or bromodeoxyuridine–incorporated cells were usually observed for 1 or 2 weeks in the balloon-injured arteries.31 These findings suggest that prominent stimulation of VSMC proliferation subsides within ≈14 days after vascular injury. Hence, the early end and short-term inhibition of VSMC proliferation can be effective in minimizing chronic neointimal formation. Recombinant gene expression in the vessel wall transduced by HVJ/liposomes continued for at least 14 days,13 which explains the successful delivery of wt-p53 cDNA and the suppression of intimal thickening following injury. However, the efficacy of gene delivery of HVJ/liposomes to human vessels is not clearly defined, and it seems difficult for vector particles to penetrate all medial layers, especially those covered with an extensive atheromatous lesion. Feldman et al32 have reported the low efficiency of catheter-based and adenovirus-mediated atherosclerotic arterial gene transfer. Their study also suggests that atherosclerotic changes in target vessels markedly reduce the efficiency of penetration of vector particles. Thus, we are also dubious about gene therapy strategy for restenosis, using the present gene delivery system. An advanced device that could effectively deliver the vector particle into all the layers of the atherosclerotic vessel wall is absolutely needed to prevent restenosis by gene therapy.

Nevertheless, our data also suggest the usefulness of endogenous induction of wt-p53 on VSMC growth inhibition in the vessel wall with a pathological condition. Several studies revealed that low-dose irradiation could sufficiently inhibit neointimal formation after balloon injury33 or stenting.34 Waksman et al34 used a short duration of irradiation (28 to 38 minutes before stenting; total, 14 Gy) in their porcine experiments. Since irradiation promptly induces p53 gene expression after DNA damage,19,20 it is strongly suggested that the transient expression of p53 may be one mechanism involved in inhibition. From this point of view, our present data are consistent with the suggestion that irradiation may possibly control VSMC growth via a p53–dependent mechanism.

In summary, since wt-p53 gene transfer inhibited VSMC proliferation in vitro and in vivo, p53 overexpression may have beneficial effects toward the control of restenosis after vascular injury. Our results serve as part of a theoretical basis of irradiation therapy after vascular interventions. In vivo overexpression of exogenous or endogenous wt-p53 in the targeted vessel wall, by gene transfer or by irradiation, shows some promise for clinical application.

Acknowledgments

This study was supported in part by a grant for Intractable Vasculitis from the Ministry of Health and Welfare and a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (Nos. 04454180 and 06454186). Dr Yonemitsu is the recipient of a Research Fellowship from Uehara Memorial Bio-Medical Research Foundation. We thank Y. Baba for excellent technical assistance with flow cytometry analyses and Drs Y. Maehara and M. Yamamoto, Cancer Center, Kyushu University Hospital, for critical comments and fruitful discussions. We are also grateful to R. Fukuda for excellent technical support in performing cell cultures and in vitro gene transfer and S. Yugawa for technical assistance with preparation of this manuscript.

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p53 Inhibits VSMC Growth In Vitro and In Vivo

Transfer of Wild-Type p53 Gene Effectively Inhibits Vascular Smooth Muscle Cell Proliferation In Vitro and In Vivo
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Circ Res. 1998;82:147-156
doi: 10.1161/01.RES.82.2.147

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