Overexpression of p21Waf1 Induces Apoptosis in Immortalized Human Vascular Smooth Muscle Cells

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To understand the role of the cell cycle regulatory protein in the control of smooth muscle cell (SMC) proliferation, we tested the overexpression of p21Waf1, a cyclin-dependent kinase inhibitor, in human normal (MS9) and immortalized SMCs (ISS10) transfected with ori-minus simian virus 40 DNA, using an adenovirus-mediated system. In MS9, overexpression of p21Waf1 resulted in the inhibition of cell cycle progression at the G1/S boundary without apoptosis. On the other hand, in ISS10, overexpression of p21Waf1 induced marked apoptosis. In these cells, immunohistochemistry revealed that overexpressed p21Waf1 was localized in the nucleus. No differential expression pattern of either p53 or SV40T was observed in p21Waf1- and control gene (β-galactosidase)-infected cells. Old-passaged ISS10 cells eventually showed growth arrest and a senescent-like phenotype. Immunohistochemistry revealed that p21Waf1 was localized in the cytoplasm of the early-passaged cells, but was found in the nucleus of the old-passaged cells. Our data suggested that nuclear accumulation of p21Waf1 plays a role in the cell death of immortalized SMC, which carries dysfunction of the cell cycle regulatory proteins such as p53. This culture model may be useful for studying the process of SMC proliferation, cell death, senescence, and cell cycle regulation. J Atheroscler Thromb, 2003; 10: 239–245.

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Introduction

The proliferative activity of smooth muscle cells (SMCs) changes in the process of vascular diseases such as atherosclerosis (1). The total amount of SMC mass in such injured vessels is also negatively regulated by cell death, including that by apoptosis (2, 3). It is known that cell cycle regulatory proteins play an important role in the determination of SMC proliferation and cell death (4, 5); however, the details of the mechanism regulating the cell cycle in SMCs remain unclear (6).

In the progression of the SMC cell cycle, activation of cyclin-dependent kinases (CDKs), followed by the phosphorylation of the retinoblastoma gene product (Rb) has been demonstrated (7). The kinase activity of CDKs is negatively regulated by CDK inhibitors such as p16Ink4 (8), p21Waf1 (9–11), and p27Kip1 (12). Initially, p21Waf1 has been reported as a downstream target of the p53, a tumor suppressor gene (9–11). It has been recognized in both p53-dependent and p53-independent processes as being crucial for the induction of p21Waf1 (13). p21Waf1 has been shown to directly bind with cyclin and CDK complexes, and it is known to prevent cell-cycle progression at the late G1 phase. p21Waf1 also binds with...
DNA polymerase δ co-factor, a proliferating cell nuclear antigen (PCNA) (14). Previous studies by our group and others have demonstrated that p21Waf1 can inhibit the cell cycle progression of SMCs in vitro and in vivo (15–18). Induction of p21Waf1 also inhibits SMC migration (19). These lines of evidence suggest that p21Waf1 expression renders SMCs cytostatic; however, it has also been reported that p21Waf1 acts as an assembly factor for growth factor-induced SMC proliferation (20). In addition, p21Waf1 expression has been demonstrated in neointimal proliferating SMCs in cases of human atherosclerosis (21, 22). The roles played by p21Waf1 in SMC apoptosis remain controversial (18, 23). Moreover, p21Waf1 potentially regulates differentiation (24) and the process of senescence (25). Thus, p21Waf1 may play a role in cell cycle control of SMCs as well as in certain specific cellular functions; the details of such mechanism remain to be more fully explored.

We have established an immortalized SMC cell line by transfection with ori-minus simian virus 40 (SV40) DNA (26). Induction of SV40 large T antigen in SMCs inactivates p53, a major inducer of p21Waf1, and increases susceptibility to apoptosis (27). In this study, we performed an adenovirus-mediated overexpression of p21Waf1 in both normal and immortalized SMCs in order to better understand the role of cell cycle regulatory proteins in SMC proliferation, apoptosis, and senescence.

**Materials and methods**

**Recombinant adenoviruses**

The construction and preparation of an adenovirus vector expressing human p21Waf1 (Adp21) has been previously described (18). For the control, AdLZ encoding bacterial β-galactosidase was used in each experiment. Working stocks of viruses were prepared by large-scale infection of 293 cells (American Type Culture Collection), and stored in aliquots at −80°C (18). For the infection with adenovirus vectors, the cells were incubated for 24 hours in DMEM containing 2% fetal bovine serum (FBS) (Filtron Pty Ltd., Brooklyn, Australia) with the vectors at the indicated multiplicity of infection (MOI), which was determined by a conventional plaque assay. Transfection efficiencies were tested by *in situ* X-Gal staining and at least 80% of the cells tested positive for β-galactosidase 24 h after infection with 100–200 MOI of AdLZ. There were no significant differences between AdLZ-infected cells and mock-infected cells as regards cell morphology or concentration of lactate dehydrogenase in the conditioned media, which suggested a low level of cellular toxicity at the maximal MOI applied in this series of experiments (data not shown).

**Cell culture**

Human aortic SMCs (MS9) and immortalized SMCs with transfected with ori-minus SV40 DNA (ISS10) were isolated as previously reported (22, 26). The cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% FBS together with antibiotics (100 U/ml penicillin and 100 µg/ml of streptomycin) (Life Technologies, Grand Island, NY) at 37°C in a humidified 5% CO2–95% air atmosphere. Normal SMCs from 5 to 10 passages, and approximately 30 passages (early) to 60 passages (old) of ISS10 cells were subjected to each of the following experiments.

**Assessment of proliferation, cell cycle distribution, and apoptosis**

The cell number was estimated by hand counting with a hemocytometer after trypan blue exclusion. For the flow-cytometric analysis of cell-cycle distribution (18), cells were harvested by trypsin-EDTA (Life Technologies) and fixed with 70% ethanol. Cells were then stained with 10 µg/ml of propidium iodide (Sigma, St Louis, MO) and incubated with 0.1 µg/ml of RNase/DNase free (Roche, Mannheim, Germany), in phosphate buffered saline (PBS) (pH7.4). The whole-cell extract was analyzed using a FACScan analyzer (Becton-Dickenson, San Jose, CA) and ModFitLT curve fitting software (Verity Software, Topsham, ME) on a Macintosh computer (Apple, Cupertino, CA). The probability of apoptosis was tested by means of terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL), as previously described (28). The multitude of newly generated 3′-OH ends by DNA fragmentation was identified visually using an *in situ* cell death detection kit (Roche). A positive control was established by incubating the specimens in DNase I/RNase-free (Roche) for 10 minutes before the reaction with TdT enzyme. For the assay, the cells inoculated on a cover slip were washed with PBS, fixed in 4% paraformaldehyde in PBS, and treated with 0.2% Triton X-100 in citrate buffer. Samples were incubated with TdT and fluorescein-labeled dUTP, counterstained with propidium iodide, and then observed with a fluorescence microscope (Leica, Wetzlar, Germany). Percentages of apoptotic cells were estimated by counting a total of 300 cells from random fields.

**Western blot analysis**

Western immuno-blotting was performed as previously described (29). The whole-cell lysate in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was sheared three times through a 23-gauge needle. Equal amounts of the samples normalized by cell number were subsequently resolved by SDS-PAGE, and transferred to PVDF membranes (Immobilon™, Millipore, MA). For the detection of p21Waf1, p53, and SV40 T antigen, the electronically
transferred membranes were incubated with a 1:1000 dilution of a rabbit polyclonal anti-p21Sdi1 (a synonym of p21Waf1, as well of p21Cip1) (Pharmingen, San Diego, CA), a mouse monoclonal anti-p53 antibody (clone DO7, Dako Japan, Tokyo Japan), or a mouse monoclonal anti-SV40 T (Oncogene Research Products, Cambridge, MA), respectively. The blots were then incubated with a peroxidase-conjugated goat anti-rabbit or mouse IgG (Organon Teknika Corp., Westchester, PA) and were visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). To confirm that equal amounts of the proteins were subjected to Western blotting analysis, the membranes were re-probed with an antibody against β-actin (Sigma, St Louis, MO). Densitometric analysis was performed with N.I.H. image software, and the relative ratio to the β-actin expression was calculated in each sample.

Immunohistochemical examination
The cells were grown on a glass cover slip and cultured as described above. To examine the expression of p21Waf1, p53, and SV40 T antigen, the cells were fixed with 4% paraformaldehyde in PBS (pH7.4) for 10 minutes and were then incubated with a 1:100 dilution of mouse monoclonal anti-p21Waf1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53 antibody (clone DO7, Dako Japan), or a mouse monoclonal anti-SV40 T (Oncogene Research Products), respectively, followed by incubation with a 1:1,000 dilution of FITC-conjugated rabbit anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA). After counterstaining with 0.1 µg/ml of propidium iodide (Sigma), the specimens were observed with an Olympus fluorescence microscope.

Statistical analysis
Experimental groups were compared by analysis of variance (ANOVA) and, when appropriate, by Student’s t-test. All data are expressed as the mean ±SD. A level of P<0.05 was accepted as statistically significant.

Results

Overexpression of p21Waf1 inhibited proliferation of normal and immortalized human SMCs
The Western blot analysis revealed that infection with Adp21, but not with AdLZ, dose-dependently induced the expression of p21Waf1 protein in normal and immortalized human SMCs (Fig. 1). In both SMC cell lines, the overexpression of p21Waf1 inhibited proliferation, and cell number accumulation was reduced with 200 MOI of Adp21 by 45% in MS9 and by 75% in ISS10, respectively (Fig. 2A). Flow cytometric analysis showed that the overexpression of p21Waf1 reduced S-phasic entry in both cells. In the MS9 cells, Adp21 infection did not alter the percentages of sub-G1 populations indicating the

Fig. 1. Overexpression of p21Waf1 in normal (MS9) and immortalized (ISS10) human SMCs. The cells were infected with 50-200 MOI of AdLZ or Adp21; otherwise, they were sham-infected (NV). The cell lysates, derived from an equal number of cells (1 × 10⁶ cells), were analyzed by Western blotting. The blots were probed with an antibody against human p21Waf1, and then were reprobed with an antibody against β-actin. The results are also expressed in relative densitometric units of p21Waf1/β-actin, with the value of sham-infected cells being 1.0.

Fig. 2. Inhibition of SMC proliferation by exogenous p21Waf1. (A) Cell counting assay. MS9 and ISS10 cells were infected with 50-200 MOI of AdLZ or Adp21; otherwise, they were sham-infected (NV). The cell number was hand-counted 2 days after infection. Results are expressed as the percentages of infected cells in relation to the control-uninfected cells. The values shown are the mean ±SD (n=3). NS: not significant. (B) Flow cytometric analysis. MS9 and ISS10 cells were infected with 200 MOI of AdLZ or Adp21, and flow cytometric analysis was performed 1 day after infection. The cell cycle phase distributions were estimated using curve-fitting software, and the estimated populations of cells in the S phase and sub-G1 populations are shown.
ratio of apoptotic cells (30). A small percentage (2–3%) of the sub-G1 population was spontaneously appeared in ISS10, and this percentage markedly increased following the overexpression of p21Waf1 (Fig. 2B).

**Overexpression of p21Waf1 induced apoptosis in immortalized human SMCs without leading to changes in the nuclear expression of either p53 or SV40 T antigen**

ISS10 cells overexpressing p21Waf1 revealed bizarre morphologies such as nuclear swelling, multinucleation, and elongation of the cytoplasm, in association with low proliferative activity. Overexpression of p21Waf1 dose-dependently increased the percentages of apoptotic cells in ISS10, but not in MS9 (Fig. 3). Upon immunohistochemical analysis, ISS10 revealed strong expression of p53 and SV40 T antigen in the nuclei. Adenovirus-mediated induction of exogenous p21Waf1 resulted in the nuclear accumulation of p21Waf1 protein. Overexpression of p21Waf1 induced a high frequency of apoptosis and cells with bizarre morphology; these cells nonetheless maintained strong expression of p53 and SV40 T antigen in the nuclei. Western blotting revealed that neither p53 expression nor SV40 T antigen expression changed remarkably with or without overexpression of p21Waf1 (Fig. 4).

**Old-passaged ISS10 cells showed a senescent-like phenotype with the nuclear accumulation of p21Waf1**

SV40 T-antigen-immortalized ISS10 cells have been shown to extend their life span and are capable of maintaining a high rate of proliferation by about 50 passages (26). At more than 50 passages, the cells gradually re-

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**Fig. 3.** Apoptosis of ISS10 cells induced by p21Waf1. (A) ISS10 cells were infected with 200 MOI of AdLZ or Adp21, and were then further cultured for 2 days. Morphology was observed by phase-contrast microscopy (upper panel), and representative TUNEL-positive cells (lower panel) are shown. (B) Semi-quantitative analysis of apoptosis. MS9 and ISS10 cells were infected with 50-200 MOI of AdLZ or Adp21; otherwise, they were sham-infected (NV). The percentages of TUNEL-positive cells were estimated by counting a total of 300 cells from random fields. Data are shown as the mean ±SD (n=3). NS: not significant.

**Fig. 4.** Expression of p53 and SV40 T antigen in p21Waf1-overexpressing ISS10 cells. (A) Immunohistochemistry. ISS10 cells were infected with 200 MOI of AdLZ or Adp21, and were then cultured for 2 additional days. Immunohistochemical analysis for p21Waf1, p53, and SV40 T antigen (SV40T) was performed. Each slide was incubated with FITC-conjugated secondary antibody, followed by counterstaining with propidium iodide. (B) Western blot analysis. ISS10 cells were infected with 200 MOI of AdLZ or Adp21; otherwise, they were sham-infected (NV). The cell lysates, derived from an equal number of cells (1X10⁵ cells), were analyzed by Western blotting. The blots were probed with primary antibodies against p53 or SV40T, and then were reprobed with an antibody against β-actin. The results are expressed in relative densitometric units of p53 or SV40T/β-actin, with the value of sham-infected cells being 1.0.
revealed a senescent-like phenotype and failed to divide. Although a small number of apoptotic cells were observed in early-passaged ISS10 (ca. 30 passages), numerous apoptotic cells were observed in old-passaged cells (ca. 60 passages or more). Immunohistochemically, a weak signal for p21Waf1 was seen in the peri-nuclear cytoplasm in early-passaged cells. On the other hand, strong p21Waf1 expression was detected in the nucleus of old-passaged cells (Fig. 5).

Discussion

Apoptosis of ISS10, immortalized SMCs with SV40 T antigen, by exogenous p21Waf1 and its implication in human atherogenesis

Consistent with previous reports (15–18), adenovirus-mediated overexpression of p21Waf1 inhibited SMC proliferation by the suppression of cell cycle progression at the G1/S boundary (Fig. 2). Induction of apoptosis by exogenous p21Waf1 was observed in ISS10 cells (a SMC cell line immortalized with SV40 T antigen), but such apoptosis was not observed in normal SMCs (Fig. 3). Human diploid cells derived from normal tissues undergo senescence and ultimately cell death (31). Transformation by SV40 increases the growth potential and extends the life span of these cells (32). SV40 large T antigen binds with and inactivates cell cycle inhibitory genes such as p53 and RB (33-35). Alternatively, high levels of p53 unbounded with T Antigen, the presence of T antigen/p53 complexes, and/or other events may account for the process of immortalization (36). ISS10 cells revealed an abundant expression of p53 in the nucleus, in association with SV40 T antigen (Fig. 4). This finding may suggest that p53 is continuously inactivated in these cells, irrespective of the presence of exogenous p21Waf1; otherwise, p21Waf1 possibly induces apoptosis in the cells carrying the dysfunction of p53.

The inflammatory response plays a crucial role in human vascular diseases (37, 38). Infection by any of several pathogens (eg. herpes simplex virus, cytomegalovirus (CMV), Epstein-Barr virus, Hemophilus influenzae, and Chlamydia pneumoniae) have been suggested to play a role in atherogenesis (39). It has previously been demonstrated that one of the immediate-early gene products of human CMV, IE2-84, binds to and inhibits p53 transcriptional activity, suggesting that the dysfunction of p53-mediated pathways is caused by CMV infection in human arteries (40, 41). Cytostatic gene therapy has been proposed as a possible treatment in cases of accelerated atherosclerosis and restenosis; such strategy would involve the modification of cell cycle regulatory proteins, including p21Waf1 (15–17). In the present study, overexpression of p21Waf1 induced apoptosis in immortalized SMCs with inactivated p53, but not in normal SMCs. Thus, it is possible that that exogenous p21Waf1 not only inhibits proliferation, but also induces apoptosis in human arteries infected with specific pathogens such as CMV that potentially inactivate p53. Although the infectious mechanisms contributing to atherogenesis remain unclear, it is possible that therapeutic interventions involving cell cycle regulatory proteins could be useful in the future. Further study will be needed for understanding the roles of endogenous and exogenous p21Waf1 in the regulation of SMC mass in diseased arteries.

Senescence of immortalized SMCs and p21Waf1

Immortalized human diploid cells containing SV40 extend the life span until the cells ended in the stage of crisis, which is characterized by massive degeneration of the cultural viability (32). Crisis is considered as a period in which the cell number remains constant or declines because successful cell division is balanced by cell death, which has proposed to be a senescence in SV-40 infected cells (42). Mechanisms on crisis have not been clearly determined; however, the upregulated expression of p21Waf1 has been observed in the cells in crisis (43). Consistently, we observed massive nuclear accumulation of p21Waf1 in the old-passaged ISS10 cells showing a senescent-like phenotype (Fig. 5). Interestingly, early-passaged cells showed weak immunoreactivity for p21Waf1 in the cytoplasm. Recently, it has been demonstrated that cytoplasmic p21Waf1 acts as an inhibitor of apoptosis in human monocytes (44). Moreover, p21Waf1 is potentially phosphorylated by Akt, a serine/threonine kinase that mediates cell-survival signaling in various types of cells, resulting in the enhanced stability of p21Waf1 protein and cell survival activity in human glioma cells (45). Thus, topographic organization of
p21Waf1 may regulate proliferation, senescence, and cell death in ISS10 cells.

In the present study, we performed the overexpression of p21Waf1, a cell cycle inhibitor, in normal and immortalized SMCs in vitro. p21Waf-1 effectively inhibited SMC proliferation; however, apoptosis was only observed in the immortalized SMCs. Our data suggested that the status of cell cycle regulatory proteins such as p53 is an important factor in determining the effect of exogenous p21Waf1. The nuclear accumulation of p21Waf1 may play a role in the cell death of immortalized SMCs bearing a dysfunction along the p53 pathway as induced by SV40 T antigen. This culture model is potentially useful for gaining a better understanding of the processes of SMC proliferation, cell death, and senescence. In addition, the present culture model may help clarify the mechanism of crisis of immortalized cell lines.

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References


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