Role of the p21 cyclin-dependent kinase inhibitor in limiting intimal cell proliferation in response to arterial injury

cell cycle/cyclin/vascular smooth muscle cells/gene therapy

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ABSTRACT Arterial injury induces a series of proliferative, vasoactive, and inflammatory responses that lead to vascular proliferative diseases, including atherosclerosis and restenosis. Although several factors have been defined which stimulate this process in vivo, the role of specific cellular gene products in limiting this response is not well understood. The p21 cyclin-dependent kinase inhibitor affects cell cycle progression, senescence, and differentiation in transformed cells, but its expression in injured blood vessels has not been investigated. In this study, we report that p21 protein is induced in porcine arteries following balloon catheter injury and suggest that p21 is likely to play a role in limiting arterial cell proliferation in vivo. Vascular endothelial and smooth muscle cell growth was arrested through the ability of p21 to inhibit progression through the G1 phase of the cell cycle. Following injury to porcine arteries, p21 gene product was detected in the neointima and correlated inversely with the location and kinetics of intimal cell proliferation. Direct gene transfer of p21 using an adenoviral vector into balloon injured porcine arteries inhibited the development of intimal hyperplasia. Taken together, these findings suggest that p21, and possibly related cyclin-dependent kinase inhibitors, may normally regulate cellular proliferation following arterial injury, and strategies to increase its expression may prove therapeutically beneficial in vascular diseases.

The definition of cell cycle regulatory proteins has greatly facilitated the molecular analysis of pathologic conditions related to cell proliferation. A variety of gene products originally defined in yeast have been shown to have mammalian homologues that alter the activity of cyclin-dependent kinases (CDK) and regulate cell cycle progression and senescence (reviewed in refs. 1–3). p21, also known as WAF1, CIP1, or SD11 (4–6), is a downstream target of the p53 tumor suppressor gene and has been implicated in abnormalities of cell proliferation. Expression of p21 directly inhibits the kinase activities of cyclin/CDK complexes in vitro (4–6). Induction of p53 in response to DNA damage results in G1 checkpoint arrest (7, 8), at which point DNA repair is accomplished prior to DNA replication in S phase.

p21 also inhibits proliferating cell nuclear antigen (PCNA) dependent DNA replication but not DNA repair in vivo (9) and is likely to affect proliferation of a variety of cell types. The expression of p21 in arteries and its regulation of cell growth during vascular remodeling have not been well characterized. Previous studies have suggested that overexpression of this gene product inhibits restenosis in injured rat carotid arteries (10), but the expression of endogenous cellular p21 relative to the proliferative response to arterial injury in vivo has not been defined. In this study, we have analyzed the effect of p21 on these cells in vitro and expression of recombinant and endogenous p21 in a porcine model of arterial balloon injury in vivo.

MATERIALS AND METHODS

Adenoviral Vector Construction and Purification. The recombinant adenoviral vector, ADV-p21, was constructed by homologous recombination between sub360 genomic DNA, an adenovirus type 5 (Ad5) derivative with a deletion in the E3 region, and a p21 expression plasmid, pAd-p21, which had the left-hand sequence of Ad5 genome but not E1A and E1B (11). An adenoviral vector lacking a p21 expression cassette, ADVΔE1, but containing the same viral elements, was used for control experiments. The structure of these replication-defective viruses was confirmed by Southern blot analysis. All recombinant viruses were propagated in 293 cells, cesium chloride purified, sterilized with a 0.45-μm filter, and diluted for storage in 13% glycerol-PBS solution to yield a final concentration of 1–3 × 10^{-12} viral particles/ml [0.8–5 × 10^{-10} plaque-forming units (pfu)/ml]. All stocks were evaluated for the presence of replication competent adenovirus by infection at a multiplicity of infection of 10 onto 3T3 cells, and none yielded replication-competent virus. A eukaryotic expression plasmid, pRc/RSVp21, was prepared by introduction of p21 cDNA from pRc/CMV-p21, kindly provided by D. Beach and G. Hanna (4, 12), into pRc/RSV (Invitrogen).

Cell Culture, Infection, and Cell Cycle Analysis. Porcine endothelial cells (EC) and vascular smooth muscle cells (VSMC) were infected with ADV-p21 or ADVΔE1 (multiplicity of infection-300/cell) for 1 h in Dulbecco's modified Eagle's medium (DMEM) and 2% fetal calf serum, and normal media was added after 1 h. Control cells were uninfected and carried in M199 with 10% fetal bovine serum. Twenty-four hours later, the cells were split into 6-well dishes at 6 × 10^4 cells per well. Cells were harvested at 0, 2, 5, 7, and 10 days, and cell numbers were measured by a hemocytometer. Cell viability was assessed by trypan blue exclusion.

For analysis of cell cycle, porcine EC and VSMC were infected at a multiplicity of infection of 300/cell with the ADVΔE1 or ADV-p21 vectors, harvested 2 days after infection, washed with phosphate-buffered saline (PBS) twice, and fixed in 70% ethanol for 30 min at 4°C. Control cells were uninfected and carried in M199 with 10% fetal bovine serum prior to fixation. The cells were treated with 1 unit of DNase-free RNase in 1 ml of PBS for 30 min at 37°C, resuspended in 0.05 mg/ml propidium iodide, and analyzed by flow cytometer using a FACScan model (Becton Dickinson) (11).

Abbreviations: BrdC, 5-bromo-2'-deoxyuracil; CDK, cyclin-dependent kinase; EC, endothelial cells; I/M, intima to media ratio; RT, reverse transcriptase; VSMC vascular smooth muscle cells; pfu, plaque-forming unit.

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Western Blot Detection of p21 Protein. Western blot analysis was performed to detect p21 expression in infected EC and VSMC and in injured and uninjured porcine arteries. To determine the cellular localization of p21, 293 or VSMC were transfected with CaPO4 with 5 μg of a control or pRC/RSVp21 plasmid, and nuclear and cytoplasmic extracts were prepared by standard methods. Samples were resolved on a 15% SDS/polyacrylamide gel and blotted onto nitrocellulose. p21 protein was visualized using an anti-peptide rabbit polyclonal antibody (no. sc-397; Santa Cruz Biotechnology) together with an anti-rabbit horseradish peroxidase conjugate secondary antibody and subsequent chemiluminescent detection (Amer sham).

Porcine Arterial Injury. Iliofemoral arteries from Yorkshire pigs were injured with a double-balloon catheter (C. R. Bard, Billerica, MA) (13), and animals were sacrificed 1 (n = 4 arteries), 7 (n = 4 arteries), 21 (n = 8 arteries), and 60 days (n = 4 arteries) after injury. Sham operated, uninjured iliofemoral arteries (n = 4 arteries) were studied as controls.

In Vivo Gene Transfer in Injured Porcine Arteries. Direct gene transfer was performed in injured iliofemoral arteries using a double balloon catheter (13). In each animal, both right and left iliofemoral arteries were infected with the same vector at a titer of 1 × 10^10 pfu/ml (dose of 7 × 10^6 pfu). In nontransfected control animals, the iliofemoral arteries were injured and saline was infused into the artery segment.

The artery segments infected with ADV-p21 (n = 28 arteries), ADV-ΔE1 (n = 28 arteries), or nontransfected controls (n = 8 arteries) were excised 7 or 21 days later. Each artery was processed in an identical manner (13). To evaluate intimal cell proliferation, animals sacrificed at 7 days received a 25 mg/kg intravenous infusion of 5-bromo-2'-deoxyuridine (BrdC) (Sigma), and immunohistochemistry was performed with an anti-BrdC antibody. A proliferation index was calculated as the ratio of labeled cells to total number of cells, using a microscope-based video image analysis system (Image One Systems; Universal Imaging, West Chester, PA). To assess the development of intimal thickening, intima and media cross-sectional areas were measured 21 days after gene transfer in four sections from each artery which spanned the 1.5-cm arterial region (Image One Systems). The four intima to media (I/M) area ratios were averaged to determine the I/M area ratio of each artery. All animal experiments were performed in accordance with National Institutes of Health guidelines and with approval of the University of Michigan Committee in the Use and Care of Animals.

Reverse Transcriptase (RT)–PCR Analysis. Total RNA was prepared using Trizol reagents (GIBCO/BRL) according to the manufacturer's protocol. PCR for the p21 gene was performed (14) in the presence or absence of RT with the primers: 5'-GAG ACA CCA CTC GAG GGT GAC TTC G-3' (sense); and 5'-GGG CAA ACA GAT GGC TGG CAA C-3' (antisense). The antisense primer was located in the 3' untranslated region of bovine growth hormone proximal to the polyadenylation site and was specific for recombinant p21 RNA and not endogenous porcine p21 RNA.

Immunohistochemistry. Immunohistochemical studies were performed with two polyclonal rabbit anti-human p21 antibodies, 1:500 dilution (no. sc-756 and no. sc-397; Santa Cruz Biotechnology); a monoclonal mouse anti-smooth muscle α-actin antibody, 1:500 dilution (Boehringer Mannheim); a monoclonal mouse anti-BrdC antibody, 1:1000 dilution (Amersham); a monoclonal SP11.8 amino peptide-specific type I procollagen antibody, 1:500 dilution (Hybrido ma Bank, University of Iowa); and a polyclonal goat anti-human von Willebrand factor antibody, 1:10,000 dilution (Atlantic Antibodies, Scarborough, ME). Control experiments were performed using purified rabbit serum, goat serum, a mouse IgG2a antibody, 1:100 dilution (Promega), or a secondary antibody alone which did not stain the arterial specimens. Specificity of

the rabbit polyclonal antibodies for porcine p21 was analyzed by immunostaining of injured porcine iliofemoral arteries with the anti-p21 rabbit polyclonal antibodies preabsorbed with 293 cells transfected with pRC/RSV-p21 plasmid or a pRSVhpAP plasmid encoding a reporter gene, human placental alkaline phosphatase.

Statistical Analysis. All values are expressed as mean ± SEM. Comparisons of intimal BrdC labeling index between ADV-p21 and ADV-ΔE1 arteries were made by two-tailed, unpaired t test. Comparisons of I/M area ratio between ADV-p21, ADV-ΔE1, and nontransfected arteries were made by ANOVA with Dunnett t test. Statistical significance was detected at the 0.05 level.

RESULTS

Expression of p21 Inhibits Vascular Cell Proliferation and Induces Cell Cycle Arrest in Vitro. To study the effects of p21 on vascular cell growth and cell cycle distribution, quiescent porcine EC and VSMC were infected in vitro with an adenoviral vector, ADV-p21, or a control vector containing an E1 deletion, ADV-ΔE1, and then stimulated to proliferate by incubation in 10% fetal bovine serum. p21 expression in these cells was confirmed by Western blot analysis (data not shown). Exposure of uninfected or ADV-ΔE1-infected cells to serum resulted in rapid proliferation of EC and VSMC. In contrast, expression of p21 in EC and VSMC resulted in inhibition of cell proliferation by >90% (Fig. 1A); these cells were still viable (>95%) as assessed by trypan blue exclusion. Expression of p21 in these cells also resulted in accumulation of cells in G0/G1 (Fig. 1 B and C), as assessed by propidium iodide staining. These data suggest that cells were arrested in cell cycle by p21 expression rather than p21 causing cell death.

p21 Is Induced in Balloon Injured Arteries in Vivo. To investigate the potential of p21 to regulate vascular cell growth in vivo, we first determined whether p21 expression is induced in injured arteries. Porcine iliofemoral arteries were injured by balloon angioplasty, and injured segments were analyzed 1, 7, 21, and 60 days later for p21 expression. These arteries were compared with normal, uninjured porcine iliofemoral arteries. Following balloon injury, smooth muscle cells proliferate within the intima, starting at 1 day after injury. Intimal smooth muscle cell proliferation peaks at 7 days, with 15–18% of cells dividing (13). Smooth muscle cell proliferation declines to <2% by 14 days. Intimal area continues to expand due to the synthesis of extracellular matrix from 7 to 21 days. After 21 days, the intima is fully formed.

In normal, uninjured porcine arteries, p21 was not detected by tissue immunostaining (Fig. 24) or by Western blot (Fig. 3, lane 5). One day after balloon injury, p21 protein was observed in <5% of intimal cells (Fig. 2B). However, at 7 days, p21 protein was detected in a significant percentage of intimal smooth muscle cells (Fig. 2C) and by Western analysis (Fig. 3, lanes 1 and 2). At 21 days, p21 expression was present in a majority of smooth muscle cells, particularly in lower regions of the intima, next to the internal elastic lamina (Fig. 2 D and E), in regions where cell proliferation was not present (Fig. 2F). p21 protein expression was also detected 21 days after injury by Western analysis (Fig. 3, lanes 3 and 4). Sixty days after injury, p21 expression was not present in the intima (Fig. 2G). To confirm that the rabbit polyclonal anti-human p21 antibodies cross-reacted with porcine p21, and that the p21 tissue staining was specific, the polyclonal antibodies to human p21 were preabsorbed with p21 or a negative control gene, human placental alkaline phosphatase. Preabsorption with extracts from p21 expressing cells eliminated immunostaining, in contrast to the control (Fig. 4 A versus B), confirming that the p21 reactivity was specific. Quantitative Western blot analysis revealed an estimated difference in expression of ≥16-fold in injured arteries (data not shown). Thus, p21
expression was specific and inversely correlated with smooth muscle cell proliferation. These findings suggest that p21 is expressed at increased levels at times when intimal cell proliferation is declining after arterial injury and that p21 may help to limit vascular cell proliferation in injured arteries.

From the immunohistochemical studies, it appeared that p21 protein was present in both the nucleus and cytoplasm. To confirm that p21 could be found in these fractions in intact cells, Western blot analyses were performed using nuclear and cytosolic extracts of porcine smooth muscle cells transfected

Fig. 2. In vivo expression of p21 in balloon injured porcine arteries. p21 is not detected in normal, quiescent porcine arteries (A). Following arterial injury, p21 protein is not observed in intimal cells at 1 day (B); p21 is present at 7 days (C), and strongly expressed at 21 days (D) and no. sc-756 (E). Cell proliferation was detected in <1% of intimal cells at 21 days (F). Sixty days after injury, p21 expression was not detectable. Control studies were performed on p21 transfected 293 cells with rabbit serum control antibody (H) and a p21 antibody [no. sc-756 (I)]. The black arrow denotes the internal elastic lamina.
with pRC/RSV-p21 plasmid (Fig. 5). The Western blot analysis revealed the presence of a considerable portion of p21 protein in cytosolic extracts, in addition to the nucleus (Fig. 5). While the functional significance of cytoplasmic p21 is not yet known, such a subcellular distribution is not surprising, given the cytoplasmic localization of some cyclin/CDK complexes at particular stages of the cell cycle (15, 16).

**Overexpression of p21 in Injured Arteries Limits the Development of Intimal Hyperplasia.** p21 was thus found to be expressed in injured arteries at times following balloon injury when intimal cell proliferation is declining, suggesting that it may limit vascular cell proliferation in injured arteries. We hypothesized that early overexpression of p21 after balloon injury might reduce intimal formation. To assess the direct effect of p21 on vascular cell growth in vivo, p21 vectors were introduced into porcine arteries immediately following injury. Porcine iliofemoral arteries were balloon injured and infected with ADV-p21 or ADV-ΔE1 (0.7 × 10^10 pfu). Previous studies have shown that adenoviral infection of porcine iliofemoral arteries immediately after injury results in recombinant gene expression in ~25–30% of intimal cells; gene expression in porcine arteries peaks at 7 days, is diminished at 14 days, and is absent at 28 days (17). Recombinant p21 expression was analyzed 7 days after injury and gene transfer, and the effects of p21 expression on intimal cell proliferation and growth were measured 7 and 21 days after gene transfer. In vivo gene transfer of ADV-p21 was demonstrated in injured porcine arteries 7 days after infection by RT-PCR analysis. p21 RNA was detected by RT-PCR in infected left and right iliofemoral arteries (Fig. 6, lanes 8 and 10) but not in a noninfected carotid artery from the same animal (lane 6) or in ADV-ΔE1 noninfected and infected arteries (lanes 2 and 4).

The effect of p21 expression on intimal cell growth in vivo was next assessed by two methods, quantitating incorporation of BrdC into intimal cells 7 days after gene transfer and measuring 1/M area ratios at 21 days. A 35% reduction in intimal BrdC incorporation was observed in ADV-p21 infected arteries, compared with ADV-ΔE1 arteries, 7 days after gene transfer (5.3 ± 0.9% versus 8.1 ± 0.4%, p = 0.035). These BrdC labeled intimal cells costained with a monoclonal antibody to smooth muscle α-actin (data not shown), suggesting that intimal VSMC proliferation was inhibited by ADV-p21 in vivo. A significant reduction in 1/M area ratio was observed in ADV-p21 infected arteries, compared with ADV-ΔE1 infected and noninfected arteries (0.37 ± 0.06 versus 0.59 ± 0.06 and 0.57 ± 0.05, p < 0.05, ANOVA with Dunnett t test) (Fig. 7). However, there was no significant difference in procollagen synthesis in the intima between the three groups at 3 weeks (percent positive intimal procollagen cells, ADV-p21 41.5 ± 8.6%, ADV-ΔE1 43.0 ± 4.3%, noninfected 48.5 ± 8.6%, p = not significant). In addition, the endothelial surface was found to regenerate at 3 weeks, evidenced by von Willebrand factor immunostaining. These results suggest that infection of arteries with ADV-p21 at the time of balloon injury inhibits proliferation of intimal VSMC and significantly limits the development of a neointima.

**DISCUSSION**

We have analyzed the role of the p21 cyclin-dependent kinase inhibitor on vascular cell growth in vitro and in vivo. Interestingly, endogenous p21 was expressed at increased levels 7–21 days after arterial injury and was associated with a decline in intimal cell proliferation. Recombinant p21 gene expression was sufficient to inhibit EC and VSMC growth in vitro. Expression of this CDK-inhibitor by adenoviral gene transfer...
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(7 ± 10^3 pfu) or were not infected (n = 8 arteries).
Twenty-one days later, the cross-sectional areas of the intima and media were quanti-
tated (nontransfected: I = 1.5 ± 0.2 mm², M = 2.6 ± 0.2 mm²; ADV-ΔE1: I = 1.6 ± 0.3 mm², M = 2.7 ± 0.2 mm²; p21: I = 1.1 ± 0.1 mm², M = 2.9 ± 0.2 mm²), and I/M area ratios were calculated. +, p < 0.05, ADV-p21 versus ADV-ΔE1 arteries and nontransfected arteries, ANOVA with Dunnett t test. Representative cross-sections from iliofemoral arteries of pigs infected with ADV-ΔE1 (B) and ADV-p21 (C) are shown.

in a porcine model of arterial injury also reduced the extent of intimal hyperplasia in vivo, as recently shown in injured rat carotid arteries (10). These effects of p21 on vascular cell growth appear to be due to the ability of p21 to block cell cycle progression and to promote growth arrest in proliferating vascular cells. Taken together, these findings suggest that p21 and cell cycle regulatory proteins may normally play a role in limiting the response to vascular injury.

p21 is induced by p53 and has thus been implicated as a downstream effector of p53, which may serve to inhibit uncontrolled cell growth in normal and malignant cells (18). Tissue remodeling in injured arteries is characterized by intimal cell proliferation, followed by extracellular matrix synthesis and a decline in cell proliferation (19). Cell prolif-
eration requires the action of cyclins which in turn activate their CDKs. D- and E-cyclins have been implicated in controlling passage through the G1 checkpoint, after which cells become committed to a round of cell division (reviewed in refs. 1–3). Equally important in arterial injury is arrest of cell growth after the initial proliferative response. The control of replicating VSMC may be mediated by multiple mechanisms, including cell cycle arrest through binding of a retinoblastoma gene product to cellular transcription factors (20, 21) and/or inactivation of CDKs (22). One possible mediator of such negative control is p21, which inhibits G1 cyclin complexes containing CDK2, CDK4, and CDK6. The observation that p21 is an inducible cell cycle inhibitor raised the possibility that it could function to regulate cell proliferation associated with arterial wound healing.

We first assessed the ability of p21 to induce growth arrest in vascular cells. Infection of porcine EC and VSMC with a ADV-p21 vector resulted in arrest of a majority of these cells in G₀/G₁ phase of the cell cycle, demonstrating a role for p21-induced growth arrest in these nonmalignant, proliferating cells. We did not detect a bystander effect with p21 on neighboring, proliferating cells (11). Thus, p21 functions in vascular cells in vitro to block cell cycle progression and to promote growth arrest. These studies are consistent with a recent report in which expression of p21 in rat smooth muscle cells inhibits cell cycle progression through its ability to inhibit CDK-mediated phosphorylation of Rb (10).

Knowledge of the timing and location of p21 expression in injured arteries also provides evidence that p21 participates in growth arrest of neointima in vivo. In normal, uninjured porcine arteries, p21 expression was not detected by immuno-
staining or Western blot analysis. One day after injury, a few (<5%) intimal VSMC expressed p21. By 7 days, shortly after the peak of cell proliferation, expression of p21 increased in intimal VSMC, and at 21 days, when cells in the largely nonproliferating, p21 expression was present in the majority of VSMC in the lower area of the intima. This region, adjacent to the intimal elastic lamina, does not contain detectable proliferating cells. Interestingly, these regions of the well-formed intima are associated with transforming growth factor (TGF)-β1 expression and procollagen synthesis (23). These observations raise the hypothesis that after arterial injury, p21 is an inducible growth inhibitor and may act in concert with other cyclin inhibitors induced by TGF-β, such as p27 and p16, which inhibits cell growth and stimulates matrix synthesis. For example, p27 (24) and p15INK4B (25) block cell cycle in response to TGF-β, and thus may mediate TGF-β effects in tissue remodeling. This region of the intima expressing p21 has been associated with extracellular matrix synthesis and decreased cell proliferation in other animal models of arterial injury, such as the injured rat carotid artery (26).

p21 protein has been detected biochemically in different adult tissues (27), but it has not been observed by immuno-
staining in quiescent tissues. Our data would suggest that p21 is expressed at increased levels after arterial injury (7–21 days). Furthermore, the polyclonal anti-human antibodies bind porcine p21 specifically, as binding to porcine p21 was abolished by incubation of antisera with extracts from p21 expressing, but not control, cells.

p21 may have additional yet unexplored functions in vascular EC and VSMC. We have recently described the ability of p21 to facilitate transcriptional activation by NF-κB (N.D.P., L.K. Felzien, J. C. Betts, K. Leung, D. H. Beach, and G.J.N., unpublished data), providing a mechanism whereby p21 can also alter the expression of genes involved in cellular adhesion molecules and differentiation. The suppression of vascular cell growth may result from this altered pattern of gene expression, leading to growth arrest. p21 also functions as a growth inhibitor during terminal differentiation, for example, in skel-
etal muscle (28), but whether this occurs in developing smooth muscle is unknown. The degree of inhibition of cell proliferation and intimal hyperplasia achieved by ADV-p21 gene transfer in this study is comparable to that achieved with overexpression of a transdominant mutant retinoblastoma gene product (20), and thymidine kinase gene transfer with systemic ganciclovir therapy (13, 29, 30). Its efficacy in a porcine balloon injury model, a preferred animal model for human cardiovascular disease (31), suggests that it may have application to the treatment of vascular proliferative diseases.

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