Overexpression of p27^{Kip1} by doxycycline-regulated adenoviral vectors inhibits endothelial cell proliferation and migration and impairs angiogenesis

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ABSTRACT Formation of new blood vessels in the adult animal (i.e., angiogenesis) is an important event for tissue repair and for tumor growth and metastasis. Angiogenesis involves the migration and proliferation of endothelial cells. We have investigated the role of the growth suppressor p27^{Kip1} (p27) on endothelial cell function in vitro and angiogenesis in vivo. We have generated Ad-TetON, a replication-deficient adenovirus that constitutively expresses the reverse tet-responsive transcriptional activator, and Ad-TRE-p27, which drives expression of p27 under the control of the tet response element. Western blot analysis demonstrated doxycycline-dependent overexpression of p27 in human umbilical vein endothelial cells (HUVECs) coinfecting with Ad-TetON and Ad-TRE-p27, which resulted in a marked inhibition of DNA replication and cell migration in vitro. Inducible overexpression of p27 in cultured HUVECs inhibited the formation of tubelike structures and, when applied in a murine model of hind limb ischemia, reduced hind limb blood flow recovery and capillary density. These findings thus underscore a novel role of p27 in regulating endothelial cell migration in vitro and angiogenesis in vivo, suggesting a novel anti-angiogenic therapy based on inducible p27 overexpression.—Goukassian, D., Díez-Juan, A., Asahara, T., Schratzberger, P., Silver, M., Murayama, T., Isner, J. M., Andrés, V. Overexpression of p27^{Kip1} by doxycycline-regulated adenoviral vectors inhibits endothelial cell proliferation and migration and impairs angiogenesis. *FASEB J.* 15, 1877–1885 (2001)

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THE DEVELOPMENT OF blood vessels is an important event for embryonic organogenesis and tissue repair during postnatal development (i.e., collateral vessel development after a heart attack) (1). Similarly, the requirement for a tumor to elaborate a vascular infrastructure in order to grow and metastasize is well documented (2, 3). Therefore, a better understanding of the molecular mechanisms that control blood vessel

formation is important for the development of novel therapies to augment or inhibit postnatal angiogenesis.

Angiogenesis involves the remodeling of the surrounding extracellular matrix and the migration of endothelial cells along a chemotactic gradient. Moreover, endothelial cells must leave their normal quiescent state and reenter the cell cycle during angiogenesis. Progression through the mammalian cell cycle requires the activation of cyclin-dependent kinases (CDKs) through their association with regulatory subunits called cyclins (4, 5). Different CDK/cyclin holoenzymes are orderly activated at specific phases of the cell cycle. Active CDK/cyclin complexes phosphorylate the retinoblastoma gene product (pRb) and the related pocket proteins p107 and p130 from mid-G1 to mitosis, which in turn regulate the activity of members of the E2F family of transcription factors (6, 7).

CDK activity is negatively regulated by specific cell cycle regulatory proteins, termed CDK inhibitors (CKIs), which associate with and inhibit the activity of CDKs (4, 8). CKIs of the CIP/KIP family (p21, p27, and p57) bind to and inactivate both the cyclin D-CDK4 and cyclin E-CDK2 complexes, whereas members of the INK4 family (p15, p16, p18, p19) are specific for CDK4- and CDK6-containing holoenzymes. We and others have suggested that up-regulation of p27 may limit the growth of vascular smooth muscle cells (VSMCs) at late time points after balloon angioplasty (9, 10). Consistent with this possibility, overexpression of p27 efficiently blocked mitogen- and c-fos-dependent induction of cyclin A promoter activity in cultured VSMCs (9, 11), and adenovirus-mediated overexpression of p27 attenuated neointimal thickening in balloon-injured arteries (9, 12). In contrast, recent studies using p27 knockout mice have demonstrated that the loss of p27 results in prolonged proliferation of cardiac myocytes (13) and enhanced atherosclerosis in hypercholesterolemic apo-

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lipoprotein E-deficient mice (14). p27 has also been implicated in the regulation of cardiomyocyte hypertrophy (13) and angiotensin II-stimulated VSMC hypertrophy (15, 16).

The studies cited above identify p27 as an important regulator of the phenotypic response of cardiac and vascular myocytes to hyperplastic and hypertrophic stimuli in vitro and in vivo. It has also been suggested that alterations in p27 expression regulate the proliferation of cultured human umbilical vein endothelial cells (HUVECs) and bovine aorta endothelial cells during the transition from contact inhibition to the proliferative state (17). However, little is known about the role of p27 on endothelial cell migration and angiogenesis. In the present study, we describe the generation of an adenoviral system that allows doxycycline-dependent overexpression of p27. Using this inducible system, we investigated the effect of forced overexpression of p27 on proliferation and migration of HUVECs in vitro and on angiogenesis in a murine model of surgically induced hind limb ischemia.

MATERIALS AND METHODS

Generation of adenoviral vectors

Replication-defective recombinant adenoviral constructs were produced according to standard techniques (18). pACCMV-TetON was generated by subcloning into the multiple cloning site of pACCMVpLpA (19) an *EcoRI/BamHI* restriction fragment isolated from pTetON (Clontech, Palo Alto, CA) that contained the rtTA cDNA downstream from the CMV promoter. To generate p Δ E1Sp1A-TRE-p27, a *XhoI/HindIII* p27 expression cassette was subcloned into p Δ E1Sp1A (Microbix Biosystems, Ontario, Canada) digested with *XhoI* and *HindIII*. The ~1.5 kb p27 expression cassette contained a FLAG epitope-tagged human p27 cDNA downstream from the TRE and CMV minimal promoter. Recombinant adenovirus Ad-TetON and Ad-TRE-p27 were generated by homologous recombination in human embryo kidney 293 cells (20) cotransfected with pJM17 (21) and pACCMV-TetON or p Δ E1Sp1A-TRE-p27, respectively. Viral stock titers were determined by plaque assay. For the hind limb ischemia studies (see below), viruses were purified by CsCl density gradient centrifugation.

In vitro assays with adenovirus

HUVECs were isolated from human umbilical cords as described previously (22). Cells were grown onto 1.5% gelatin-coated plates (Sigma, St. Louis, MO) in medium M199 supplemented with 20% fetal bovine serum (FBS) (both from BioWhittaker, Walkersville, MD), 0.4% bovine brain extract (Clonetics, San Diego, CA), 0.5% penicillin/streptomycin, and 0.5% Fungizone. HUVECs were used between passages 3 and 8. Cells were maintained at 37°C in a humidified atmosphere of air with 5% CO₂.

To assess the level of p27 overexpression using the adenoviral system, HUVECs were seeded in 60 mm culture dishes, grown to ~80% confluency, and incubated for 24 h with Ad-TetON and Ad-TRE-p27 with or without doxycycline (1 μ g/ml, Sigma) in the culture medium. Preparation of whole cell lysates and Western blot analysis was as described previously (9). Rabbit polyclonal anti-p27 antibodies (1/250, Ab-1, Oncogen Research Products, Cambridge, MA) were used to

detect adenovirus-encoded p27, which displayed slower electrophoretic mobility than endogenous p27 due to the presence of an amino-terminal FLAG tag. Recombinant p27 expression could not be detected using monoclonal anti-FLAG antibody.

HUVECs for flow cytometry were seeded in 6-well dishes and serum-starved in medium containing 2% FBS (without bovine brain extract). After 2 days of serum starvation, 6×10^7 pfu of each Ad-TetON and Ad-TRE-p27 were added for an additional 16 h with or without doxycycline. Infected cells were washed twice with PBS, fed fresh medium containing 20% FBS with or without doxycycline, and harvested 12 h later for flow cytometry as described previously (11).

To assess the effect of doxycycline on cell proliferation, asynchronously growing HUVECs exposed to different concentrations of doxycycline were incubated for 5 h with ³H-thymidine (1 μ Ci/ml, Amersham, United Kingdom), after which cells were washed with PBS and incubated for 1 h with ice-cold 5% trichloroacetic acid. Labeled DNA was extracted with 0.5N NaOH and counted in a scintillation counter (Wallac, Turku, Finland).

Migration assays were performed using a 48-well chemotaxis chamber (Neuroprobe, Bethesda, MD). After exposing HUVECs to Ad-TRE-p27 and Ad-TetON (multiplicity of infection of 30 and 15, respectively) for 16 h, cells were trypsinized and resuspended at 3×10^5 /ml in chemotaxis medium (M199 supplemented with 0.5% bovine serum albumin) before being placed in the upper wells of a chemotaxis chamber. The lower wells contained either control medium or PDGF-BB (10 ng/ml) (Sigma). Upper and lower wells were separated by a 8 μ m pore polyvinylpyrrolidone-free polycarbonate filter (Poretics, Livermore, CA) precoated with fibronectin (20 μ g/ml, Sigma). Chemotaxis chambers were incubated for 4 h at 37°C in a humidified atmosphere of air with 5% CO₂. Thereafter, the filters were fixed and stained with Diff Quick (DADE, Aguada, Puerto Rico). Cells that had not migrated were removed from the upper surface. After air drying, the coverslips were mounted onto glass slides and migration was quantified by counting the migrated cells in 20 randomly selected high power fields (200 $\times$).

To assess the effect of doxycycline on cell migration, HUVECs labeled with the fluorescent dye Calcein-AM were placed in FALCON HTS FluoroBlock inserts (8.0 μ m pore size, 2×10^4 cells/insert) (Becton Dickinson, Bedford, MA) in serum-free medium. To induce migration, the lower chamber contained complete medium. When indicated, doxycycline (1 μ g/ml) was added to both chambers. Chemotaxis was assessed by detecting the fluorescence of cells migrating to the lower chamber using a Victor 4120 multilabel counter (Wallac).

To examine the formation of tubelike structures, HUVECs were seeded on 4-well chamber slides (5×10^4 cells/well) coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) and incubated for 24 h in medium containing 5% FBS. Control cells were not infected with adenovirus. When indicated, cells were coinfecting with Ad-TetON (7.5×10^5 pfu) and Ad-TRE-p27 (1.5×10^6 pfu) with (1 μ g/ml) or without doxycycline in the medium.

In vitro assays with retrovirus

Recombinant retrovirus were generated using the retroviral vectors pBabePuro-p27^{wt} and pBabePuro-p27^{ck-} (gift of B. Amati), which encode for wild-type p27 and a mutated p27 protein that is unable to interact with CDKs and cyclins (23), respectively. Infection of asynchronously growing HUVECs was performed as suggested by the supplier of the PT67 packaging cells (Clontech). Uninfected HUVECs were used for control. Cells were trypsinized, counted with a hemocytometer, and plated on 12-well culture dishes at a density of 3×10^3 cells/well.

Quantification of ^3H -thymidine incorporation into DNA and p27 Western blot analysis were performed as indicated above for the studies with adenoviral vectors.

Hind limb ischemia studies

Unilateral hind limb ischemia in male C57/BL6J mice was produced by occlusion of the femoral artery as described previously (24). One week before surgery, mice were randomly divided into - doxycycline and + doxycycline group (at 1 mg/ml, in drinking water containing 2.5% sucrose). Immediately after surgery, animals received in the left hind limb an i.m. injection consisting of 60 μl of each Ad-TetON (1×10^8 pfu/ml) and Ad-TRE-p27 (1×10^9 pfu/ml). Western blot analysis was performed to assess the expression of endogenous and adenovirally encoded p27 in cell extracts prepared from ischemic muscle specimens pooled from three mice retrieved 3 and 28 days after surgery. Serial assessment of hind limb blood flow was performed using a PIM 2.0 laser Doppler perfusion imager (Lisca, Mahwah, NJ) as described (24, 25). Calculated perfusion was expressed as a ratio of left (ischemic) to right (control) limb.

To determine capillary density, whole nonischemic and ischemic limbs of two mice from each group were immediately fixed in methanol overnight. Histological sections (5 μm thick) were prepared from paraffin-embedded tissue samples. Endothelial cells were identified by histochemical staining with biotinylated Griffonia simplicifolia lectin I isolectin B4 (Vector, Burlingame, CA), followed by peroxidase-conjugated ultrastreptavidin (Signet, Dedham, MA) and DAB substrate (Vector). Sections were counterstained with Gill's hematoxylin solution (Sigma). Six microscopic fields containing cross sections of muscle fibers were randomly selected for each animal (at a magnification of 66 $\times$). Areas of 0.06 mm 2 were photographed and printed on high quality photographic paper. Capillaries were identified as structures with

positive staining for isolectin B4 and appropriate morphology. Neither vessel lumen nor red blood cells were used to define a microvessel. The number of capillaries per muscle fiber was counted in each photograph, and the counts of the six chosen areas were averaged for each mouse. To eliminate interobserver variations, a single investigator, who was blinded to the treatment regimens, performed all counts.

RESULTS

Overexpression of p27 by a doxycycline-dependent adenovirus-mediated system

Figure 1A schematizes the adenoviral system generated to achieve inducible p27 expression. Ad-TetON directs expression of the rtTA regulatory protein from the CMV promoter. rtTA is a fusion of amino acids 1–207 of the tet repressor (rTetR) and the carboxyl-terminal activation domain (130 amino acids) of the VP16 protein of herpes simplex virus. A second adenoviral vector, Ad-TRE-p27, directs expression of human p27 from the tet response element (TRE) and a minimal CMV promoter (P_{minCMV}). Under basal conditions, transcription from Ad-TRE-p27 is minimal because rtTA is in its inactive conformation. Inducible transcription of the p27 cDNA is achieved via activation of rTetR-VP16 by exposure to tetracycline or its derivative doxycycline. We tested this system by Western blot analysis of control and infected HUVECs (Fig. 1B). Whereas endogenous p27 was detected at similar levels in all conditions tested, adenovirally encoded p27 was

Figure 1. Doxycycline-dependent adenovirus-mediated overexpression of p27 in HUVECs. A) Schematic showing the adenoviral system used to achieve doxycycline-dependent overexpression of p27. Addition of doxycycline activates DNA binding activity of the fusion protein rTetR/VP16 encoded by Ad-TetON. 'Active' rTetR/VP16 turns on transcription of the p27 cDNA encoded by Ad-TRE-p27 (see text for details). B) Western blot analysis using anti-p27 antibody and 50 μg of protein from whole cell extracts of HUVECs coinfecting with the indicated amounts of Ad-TetON and Ad-TRE-p27, with (+) or without (-) doxycycline in the culture medium (at 1 $\mu\text{g}/\text{ml}$). The first lane corresponds to uninfected cells. The position of molecular weight markers is shown in the right. Arrowheads on the left indicate the position of adenovirally encoded p27 (exo) and endogenous p27 (endo).

Figure 2. Doxycycline-dependent adenovirus-mediated overexpression of p27 inhibits proliferation of HUVECs. *A*) Starvation-synchronized HUVECs were incubated for 16 h with 6×10^7 pfu of each Ad-TetON and Ad-TRE-p27 with (1 µg/ml) or without doxycycline, after which cultures were fed fresh medium containing 20% FBS with or without doxycycline and harvested 12 h later for flow cytometric analysis. First bar: serum-starved cells. Data represent the mean of $n = 3$ experiments $\pm$ SE. Differences were evaluated by ANOVA and Fisher's PLSD post hoc test. Comparisons between serum-restimulated cells: * $P < 0.001$; ** $P < 0.0001$. Comparisons between serum-starved and serum-restimulated cells: † $P < 0.005$; †† $P < 0.0001$. *B*) Uninfected HUVECs were maintained in the presence of 20% FBS and the indicated concentrations of doxycycline. [³H]-thymidine was added during the last 5 h; incorporation of radioactivity into DNA was determined in triplicate samples. ANOVA and Fisher's PLSD post hoc test revealed no statistically significant differences among groups ($P > 0.05$).

detected only in cells coinfecting with Ad-TetON and Ad-TRE-p27 when doxycycline was added to the culture medium. Moreover, the level of ectopically expressed p27 increased gradually as the amount of Ad-TRE-p27 augmented from 1.5×10^5 to 4.5×10^6 pfu.

Serum-inducible proliferation of HUVECs is inhibited by overexpression of p27

To test this system functionally, we examined the cell cycle kinetics of HUVECs by flow cytometry (**Fig. 2A**). In the absence of doxycycline, cultures coinfecting with Ad-TetON and Ad-TRE-p27 entered the cell cycle after serum restimulation when compared with starvation-synchronized HUVECs, as indicated by a significant reduction in the proportion of cells in G0/G1 and increased S-phase entry. In marked contrast, infected cells exposed to doxycycline at 1 µg/ml failed to progress through the cell cycle despite serum refeeding. Control experiments showed that the addition of doxycycline up to 100 µg/ml fails to inhibit de novo DNA synthesis in serum-stimulated cultures of HUVECs (**Fig. 2B**). These results demonstrate that doxycycline-dependent overexpression of p27 can block cell cycle progression of serum-stimulated HUVECs.

Additional control experiments were performed to assess whether the inhibitory effect of ectopically expressed p27 might be due to nonspecific interference with basic cellular functions resulting from massive overexpression. Cultures of asynchronously growing HUVECs were infected with retroviral vectors encoding for wild-type p27 (p27^{wt}) or a double mutant that had lost the ability to form stable complexes with cyclins and CDKs (p27^{ck-}) and fails to inhibit CDK2 activity (23). Consistent with our data from adenoviral vectors (**Fig. 2A**), retrovirus-mediated overexpression of p27^{wt} greatly inhibited DNA synthesis compared with uninfected cells (**Fig. 3A**). In marked contrast, retrovirally

Figure 3. Overexpression of an inactivated mutant of p27 does not inhibit proliferation of HUVECs. Asynchronously growing HUVECs were infected with retroviral vectors encoding for wild-type p27 (p27^{wt}) or a mutated version that abrogates its interaction with CDKs and cyclins (p27^{ck-}). Uninfected HUVECs were used for control. *A*) Cells were incubated with [³H]-thymidine to assess the effect of p27^{wt} and p27^{ck-} overexpression on DNA synthesis. Results are expressed relative to control (=1) and represent the mean $\pm$ SE of the indicated number of assays. Differences were evaluated by ANOVA and Fisher's PLSD post hoc test. Comparisons vs. control: * $P < 0.001$; ** $P < 0.0001$. Comparisons vs. p27^{ck-}: † $P < 0.0001$. *B*) Western blot analysis using anti-p27 and anti-tubulin antibodies. Note that infected cells overexpress recombinant p27 compared with the level of endogenous p27 in control cultures.

Figure 4. Doxycycline-dependent adenovirus-mediated overexpression of p27 inhibits migration of HUVECs. *A*) After exposing HUVECs to Ad-TRE-p27 and Ad-TetON for 16 h, cells were trypsinized and placed in the upper wells of a modified Boyden chamber. The lower wells contained either control medium (first bar) or PDGF-BB (10 ng/ml). When indicated, doxycycline was added at 1 μ g/ml. Migration was quantified by counting the migrated cells in 20 randomly selected high power fields (HPF). Data represent the mean of $n = 8$ assays $\pm$ SE. Differences were evaluated by ANOVA and Fisher's PLSD post hoc test. Comparisons between unstimulated and PDGF-BB-stimulated cells: * $P < 0.0001$. Comparisons between PDGF-BB-stimulated cells: † $P < 0.0001$. *B*) HUVECs labeled with the fluorescent dye Calcein-AM were placed in FALCON HTS FluoroBlock inserts in serum-free medium with (1 μ g/ml) or without doxycycline. Chemotaxis was assessed by detecting the fluorescence of cells migrating to the lower chamber (which contained complete medium). Data represents the mean $\pm$ SE of 2 assays. For each condition, results are expressed relative to baseline fluorescence at 0 h (=1). Two-tail unpaired Student's t test at each time point only revealed increased migration in the doxycycline group at 1 h (* $P < 0.05$).

encoded p27^{ck-} slightly enhanced DNA synthesis. These differences occurred despite the similar level of overexpression of p27^{wt} and p27^{ck-} (Fig. 3B).

Migration of HUVECs and tubulogenesis is inhibited by overexpression of p27

We next used two well-established assays to assess the potential regulatory role of p27 on cell motility. First, the effect of p27 overexpression on cell migration was tested in a modified Boyden chamber (Fig. 4A). Addition of PDGF-BB to HUVECs coinfecting with Ad-TetON and Ad-TRE-p27 and maintained in the absence of doxycycline significantly increased the number of migrated cells over basal migration in unstimulated cells. However, addition of doxycycline at 1 μ g/ml significantly reduced both basal and PDGF-BB-induced migration of infected cells. Control experiments demonstrated that doxycycline by itself, at the concentration used in these studies, does not inhibit migration of HUVECs (Fig. 4B).

Endothelial cells plated on a Matrigel substrate attach and migrate to form tubelike structures (26, 27). Therefore, we also investigated the effect of p27 on HUVEC motility in this in vitro model system. Cultures of HUVECs were uninfected or coinfecting with Ad-TetON and Ad-TRE-p27 and seeded on Matrigel-coated culture dishes. As shown in Fig. 5, addition of doxycycline to infected HUVECs reduced tubulogenesis relative to both uninfected cultures and infected cells in the absence of doxycycline.

Overexpression of p27 inhibited blood flow recovery and reduced capillary density in ischemic limbs

The in vitro results presented thus far demonstrate that ectopic overexpression of p27 can inhibit endothelial cell functions essential for angiogenesis (i.e., cell proliferation and migration). To examine whether induc-

Figure 5. Doxycycline-dependent overexpression of p27 inhibits HUVEC tubulogenesis. HUVECs were seeded at 5×10^4 cells/well on Matrigel-coated 4-well chamber slides and incubated for 24 h in medium containing 5% FBS. Control cells were not infected with adenovirus. When indicated, cells were coinfecting with Ad-TetON (7.5×10^5 pfu) and Ad-TRE-p27 (1.5×10^6 pfu) with or without doxycycline in the medium. All assays were done in quadruplicate. Two representative photomicrographs of each condition are shown.

Figure 6. Doxycycline-dependent overexpression of p27 impairs blood flow recovery and reduces capillary density after acute ischemia. Mice were randomly divided into two groups: - doxycycline and + doxycycline 1 wk before inducing ischemia in the left hind limb. All mice received in the ischemic leg an i.m. injection containing Ad-TetON (6×10^6 pfu) and Ad-TRE-p27 (6×10^7 pfu) immediately after surgery. **A)** Western blot analysis was performed to assess doxycycline-dependent overexpression of adenovirally encoded p27 [p27 (exo)] in cell extracts from ischemic muscle specimens retrieved 3 and 28 days after surgery. p27 (endo): endogenous p27. **B)** Hind limb blood flow recovery was assessed by Doppler analysis at different time points after surgery. Results at each time point represent the mean $\pm$ SE of 8–10 mice. Differences were evaluated by ANOVA and Fisher's PLSD post hoc test. Comparisons between groups at each time point: * $P < 0.025$; ** $P < 0.004$; *** $P < 0.0001$. Comparisons postsurgery vs. day 0 (within each group): † $P < 0.00025$; †† $P < 0.0001$. Analysis of all postsurgery time points vs. 'before surgery' disclosed $P < 0.0001$. **C)** Relative capillary density at postoperative day 35 in nonischemic (-surgery) and ischemic (+surgery) hind limbs is expressed as number of capillaries per muscle fiber. Results represent the mean $\pm$ SE of 12 randomly chosen areas of 0.06 mm^2 of muscle fiber from 2 mice. See Material and Methods for details.

ible overexpression of p27 can indeed impair de novo blood vessel formation, we performed gene transfer experiments in a murine hind limb model of acute ischemia. Immediately after surgery, all mice received Ad-TetON and Ad-TRE-p27 via an i.m. injection in the injured leg. Western blot analysis demonstrated doxycycline-dependent overexpression of adenovirally encoded p27 in ischemic muscle specimens retrieved 3 and 28 days after surgery (Fig. 6A). Angiogenesis was quantified by laser Doppler perfusion imaging and measurement of capillary density. As revealed by Doppler analysis of Fig. 6B, hind limb blood flow in the - doxycycline group of adenovirally infected mice was precipitously reduced after surgery (day 0), increased steadily between postsurgery days 7, 14, and 21, and remained elevated by day 28. A similar precipitous reduction in hind limb blood flow occurred in mice receiving adenovirus and doxycycline; however, in contrast to - doxycycline group, flow recovery was negligible up to postoperative day 28 (Fig. 6B). This corresponded to a significant reduction in capillary density

in the ischemic hind limb at day 35 after surgery in mice receiving adenovirus and doxycycline (Fig. 6C). Control experiments with uninfected animals demonstrated that doxycycline by itself, at the concentration used in these studies, does not inhibit blood flow recovery (Fig. 7). Collectively, these results demonstrate that doxycycline-dependent overexpression of p27 can impair blood flow recovery and reduce capillary density in a murine model of hind limb ischemia.

DISCUSSION

Four major conclusions arise as a result of our studies: 1) an adenoviral system has been developed that allows doxycycline-dependent overexpression of p27 in cultured cells and in experimental animals; 2) inducible overexpression of p27 in HUVECs blocked DNA replication; 3) inducible overexpression of p27 in HUVECs inhibited cellular migration and tubulogenesis in vitro, and 4) inducible overexpression of p27 impaired angiogenesis in a murine model of hind limb ischemia. In addition to the expected inhibitory effect of p27 on cell growth, these findings underscore a novel role for p27 in the regulation of endothelial cell migration in vitro and on angiogenesis in vivo. Our in vitro results extend previous studies reporting the ability of the CKIs p16 and p21 to inhibit cell spreading and migration when overexpressed in a variety of cell types, including HUVECs, VSMCs, and CS-1 $\beta 3$ melanoma cells (28, 29). Together, these studies suggest that members of the

Figure 7. Doxycycline treatment does not affect blood flow recovery after acute ischemia. Mice were treated as described in Fig. 6, except that animals did not receive the injection of adenovirus. Hind limb blood flow recovery was assessed by Doppler analysis at different time points after surgery. Results at each time point represent the mean $\pm$ SE of 10 mice. Differences were evaluated by ANOVA and Fisher's PLSD post hoc test. Analysis at each time point revealed no differences between - doxycycline and + doxycycline groups ($P > 0.05$). Comparisons between day 0 and all postsurgery time points disclosed $P < 0.0001$ in both groups. Analysis of all postsurgery time points vs. 'before surgery' disclosed $P < 0.0001$, except day 21 in the + doxycycline group ($P < 0.005$).

CKI family of growth suppressors are important regulators of cell migration.

Consistent with previous *in vitro* studies (30), incubation with doxycycline up to 100 μ M did not significantly inhibit the growth of HUVECs. Similarly, we have shown that doxycycline at 1 μ M did not inhibit the migration of HUVECs nor did concentrations up to 10.4 μ M impair the formation of tubelike structures (30). It is also noteworthy that tetracycline analogs, including doxycycline, have been shown to inhibit the activity of several metalloproteinases *in vitro* and *in vivo* (31–36). This inhibitory effect of doxycycline could impair matrix degradation and therefore limit angiogenesis in the murine hind limb model of acute ischemia. However, at the concentrations used in our studies, treatment with doxycycline did not affect blood flow recovery.

Cellular migration and proliferation are important events during angiogenesis (1, 2). Thus, it is reasonable to presume that the ability of p27 to inhibit cellular growth and migration contributes to the anti-angiogenic effect of p27 overexpression reported here. Of further interest is the wealth of evidence indicating that angiogenesis is required for tumor growth and metastasis (2, 3). Indeed, several anti-angiogenic therapies have proved efficient at inhibiting tumor progression in animal models of cancer (3, 37–39). Moreover, it is appropriate to consider previous studies showing that adenovirus-mediated overexpression of the growth suppressors p53 (40) and p16 (41) inhibited angiogenesis induced *in vivo* by human cancer cells. Schreiber et al. (42) compared the efficacy of different CKIs at inhibiting tumor formation in a polyomavirus middle T antigen model of murine breast carcinoma. These authors demonstrated that adenovirus-mediated overexpression of p16, p18, p21, and p27 results in a significant delay in tumor progression, varying from 5 wk for p21 to more than 25 wk for p27. Similarly, *in vitro* experiments with human breast cancer cells MDA-MB-231 and MCF-7 infected with recombinant adenovirus expressing p27 and p21 showed a markedly more (up to 56-fold) potent growth inhibitory effect of p27 (43). Thus, p27 may be a better candidate than other CKIs for gene therapy of tumors. p27-deficient mice are predisposed to tumorigenesis in multiple tissues when challenged with γ irradiation or a chemical carcinogen; p27 heterozygous mice were also more susceptible than wild-type animals, but at intermediate rates (44). The sensitivity to p27 dosage suggests that reduced p27 expression might be a common event during tumor growth. Indeed, low expression of p27 has frequently been found in several human cancers, and this is highly predictive of tumor progression and patient mortality (45–52). Conversely, acquired expression of p27 might be a favorable independent prognostic indicator in patients with hepatocellular carcinoma (53). It is also noteworthy that p27 expression appears to correlate inversely with vascular cell proliferation in human atherosclerotic tissue (10, 54) and that p27 inactivation accelerates the development of atherosclerotic lesions

in hypercholesterolemic apolipoprotein E-deficient mice (14). Collectively, these studies suggest that restoration of p27 function might be effective for the treatment of cancer and atherosclerosis.

Although available scientific evidence supports the feasibility of human gene therapy, major benefit is expected from the perfection of current vectors and/or the development of new gene delivery strategies (55). As indicated by numerous animal studies, a major limitation of current adenoviral vectors is the substantial drop in gene expression normally seen shortly after delivery (typically 1–2 wk). Our study shows sustained doxycycline-dependent overexpression of adenovirally encoded p27 up to 28 days postinjection into the hind limb. Similarly, Harding and co-workers reported persistent doxycycline-regulated adenovirus-mediated gene expression up to 3 months after local transfer into neuronal cells *in vivo* (56). Thus, the long-term expression achieved with doxycycline-regulatable adenoviral system may be considered for future experimental and clinical applications. This inducible system may provide additional advantages over conventional gene therapy strategies. For example, an inescapable concern when overexpressing a therapeutic gene is the possibility of undesirable systemic effects, especially when a potentially harmful gene is being delivered. Thus, using an inducible strategy to maintain gene expression for a defined length of time seems sure to mitigate inherent potential risks, particularly in diseases for which a relatively short period of treatment might be effective (i.e., postangioplasty restenosis). The combined use of constitutive and inducible expression systems may be desirable in cases where the susceptibility of target cells to a given therapeutic agent depends on exposure to a 'priming' agent. Last, future anti-cancer therapies that rely on the combination of gene therapy and conventional strategies (i.e., chemotherapy, radiotherapy, immunotherapy) may also benefit from inducible gene expression. **[F]**

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