p21<sup>Cip1</sup> Levels Differentially Regulate Turnover of Mature Endothelial Cells, Endothelial Progenitor Cells, and In Vivo Neovascularization

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Abstract—p21<sup>Cip1</sup> (p21) controls cell cycle progression and apoptosis in mature endothelial cells (ECs) and regulates size and cycling of the hematopoietic progenitor cell pool. Because circulating endothelial progenitor cells (EPCs) contribute to postnatal neovascularization in addition to mature ECs, we investigated the regulation of ECs and EPCs in p21-deficient mice. Mature aortic EC proliferation was increased in homozygous p21<sup>−/−</sup> and heterozygous p21<sup>+/−</sup> mice, in which p21 protein levels are reduced to one third of wild-type (WT). In contrast, apoptosis sensitivity was increased by 3.5-fold only in p21<sup>−/−</sup>, but not in p21<sup>+/−</sup> mice. Consistently, in vivo apoptosis of ECs within areas of neovascularization was elevated in p21<sup>−/−</sup> but not in p21<sup>+/−</sup> mice. EPC numbers were elevated 2-fold in p21<sup>−/−</sup> mice compared with WT (<i>P</i>&lt;0.001), and clonal expansion capacity of EPCs was increased from 25±4 (WT) to 57±8 colony-forming units in p21<sup>−/−</sup> mice (<i>P</i>&lt;0.005). EPC numbers and expansion were likewise increased in p21<sup>+/−</sup> mice. As the integrative endpoint, in vivo neovascularization reflecting all p21-affect ed parameters was increased over WT only in p21<sup>−/−</sup> (<i>P</i>&lt;0.001), but not in p21<sup>+/−</sup> mice. In conclusion, reduced p21 protein levels of mice lacking one p21 allele are associated with increased proliferation of ECs and EPCs, whereas survival of ECs to apoptotic stimuli in vitro and in vivo is not impaired. Under these conditions, neovascularization was increased. In contrast, complete p21 deficiency did not result in an increased neovascularization despite increased mature EC and EPC proliferation. This may be due to the sensitization of ECs against apoptosis. (Circ Res. 2004;94:686-692.)

Key Words: angiogenesis ■ vasculogenesis ■ endothelial cells ■ progenitor cells ■ apoptosis

Angiogenesis and vasculogenesis contribute as two essentially different mechanisms to blood vessel formation in the adult organism. Angiogenesis is understood as capillary sprouting from preexisting blood vessels, which relies on the proliferation, survival, and migration of mature endothelial cells (ECs; see reviews<sup>1,2</sup>). In contrast, vasculogenesis is confined to new vessel development arising from circulating endothelial progenitor cells (EPCs), which are released from the bone marrow into the circulation and home to sites of neovascularization.<sup>3-6</sup>

The cell cycle protein p21<sup>Cip1</sup> (p21) regulates cell cycle progression<sup>2</sup> and inhibits apoptosis<sup>8</sup> of mature ECs, suggesting a potential role for angiogenesis signaling.<sup>9</sup> Likewise, p21 maintains the quiescence of hematopoietic stem cells,<sup>10</sup> which may serve as common precursors for vascular progenitor cells. Therefore, p21 could also regulate vasculogenesis. p21 regulates the cell cycle concentration-dependently in an ambiguous manner.<sup>11</sup> At low levels, p21 is sequestered to cyclin D during mitotic stimulation facilitating the activation of cyclin D–dependent kinases 4 and 6 and, thus, cell cycle progression,<sup>12</sup> whereas at high concentrations, p21 robustly inhibits cyclin-E–dependent kinase 2, leading to cell cycle arrest.<sup>13-15</sup>

p21-deficient mice develop autoimmune disease<sup>16,17</sup> and are characterized by enhanced tumor susceptibility.<sup>18</sup> Interestingly, the latter includes in particular the formation of benign hemangiomas,<sup>18</sup> which may highlight the dependency of endothelial cell cycle regulation on p21. Besides angiomma formation, however, no apparent vascular phenotype of p21<sup>−/−</sup> mice has been reported. We, therefore, investigated the native adult blood vessel formation by the disc neovascularization model in p21-deficient mice. To understand the cellular mechanisms regulating angiogenesis and vasculogenesis in these animals, we examined the effect of p21 deficiency on both, mature EC growth and survival as well as EPC number and function. Because of the concentration-dependency of p21 effects, we studied heterozygous p21<sup>+/−</sup> mice in addition to p21<sup>−/−</sup> mice.
Materials and Methods

Animals

Heterozygous p21+/− and homozygous p21−/− mice were developed in a C57/BL6 background strain of mice and kindly provided by S.J. Elledge.18a Age-matched littermate wild-type C57/BL6 mice were used as controls. The hetero- or homozygotic absence of one or both p21 alleles from the mouse genome, respectively, was confirmed by PCR using the following primers: 5′-ctctggtgtctccagtgc-3′ (forward p21); 5′-ccctttgcctcctcctag-3′ (reverse p21); 5′-gagggagctctgctg-3′ (neo- mycin cassette forward); 5′-ctcctttggccctgagt-3′ (neomycin cassette reverse). The present study was performed with permission of the State of Hesse, Regierungspräsidium Darmstadt, according to section 8 of the German Law for the Protection of Animals and conforms to the Guide for the Care and Use of Laboratory Animals.

Isolation of Mature Aortic ECs

Outgrowth of ECs from aortic tissue was performed as previously described.19,20 In brief, aortas from female or male mice were cleaned, longitudinally opened, and placed with the intiminal side downward onto a collagen matrix in a medium supplemented with 10% FCS and 100 μg/mL endothelial cell growth supplement (Calbiochem) for 6 days. After digestion of the matrix, the remaining cells were pelleted, seeded on fibronectin-coated 24-well plates, and grown to confluence in endothelial basal medium (EBM; Cell Systems) supplemented with 10% FCS and 100 μg/mL endothelial cell growth supplement (Calbiochem) for 6 days. After digestion of the matrix, the remaining cells were pelleted, seeded on fibronectin-coated 24-well plates, and grown to confluence in endothelial basal medium (EBM; Cell Systems). To identify mature ECs from aortic outgrowth, cells were trypsinized, stained with a phycoerythrin (PE)-labeled anti–Flk-1 antibody (BD Pharmingen) or with an FITC-labeled antibody against CD146 (S-Endo-1, MUC-18), and analyzed by fluorescence-activated cell sorting (FACS Calibur, BD Biosciences).

Measurement of Proliferation

For proliferation analysis, cells were, in addition to PE-labeled anti–Flk-1, incubated with bromodeoxyuridine (BrdU) and subsequently stained with a FITC-labeled annexin V, and 7-AAD. Flk-1–positive cells were selectively assessed for cell cycle phases using 3-channel flow cytometry. These data were confirmed by the quantitative measurement of BrdU incorporation –tagged p21 plasmid encoding wild-type full-length p21 under Transfection reagent (Qiagen).

Detection of Caspase-3 Activation

Mouse aortic endothelial cells were fixed and permeabilized before the addition of a rabbit affinity-purified polyclonal antibody raised against amino acid 163 to 175 of murine caspase-3 (Cell Technology). which is specifically present on the p17 subunit of cleaved caspase-3. A secondary PE-labeled goat anti-rabbit antibody and subsequent flow cytometric analysis was used to quantify caspase-3 activation.

Isolation and Characterization of Progenitor Cells

Total mononuclear cells (MNCs) were isolated from homogenized murine splenic tissue by density gradient centrifugation with Biocoll separating solution (density 1.077; Biochrom AG). MNCs (4×10^5) were plated on fibronectin-coated 24-well plates in 0.5 mL endothelial basal medium (EBM; Cell Systems) supplemented with 1 μg/mL hydrocortisone, 3 μg/mL bovine brain extract, 30 μg/mL gentamicin, 50 μg/mL amphotericin B, 10 μg/mL EGF, 20 ng/mL vascular endothelial growth factor (VEGF), and 20% fetal calf serum (FCS; Gibco). At day 4, adherent cells were washed with medium and incubated with 2.4 μg/mL 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acetylated LDL (DiLDL; Harbor Bio-Products) for 1 hour. Cells were fixed in 4% paraformaldehyde and counterstained with FITC-labeled lectin (Sigma R848). Two independent investigators counted the number of EPCs in three randomly selected high-power fields.

Progenitor cell function was quantitatively assessed by their ex vivo clonal expansion capacity. For this purpose, 1×10^5 MNC isolated from splenic tissue were kept in 1.5 mL methylcellulose agar (Methocult GF M3434, Cell Systems), which discriminates between cells with true progenitor potency giving rise to cell colonies.21 In order to induce clonal expansion, the agar was supplemented with granulocyte-monocyte colony-stimulating factor (GM-CSF; 20 ng/mL) and with murine VEGF to induce EC formation (100 ng/mL; both from Cell Concepts). After 2 weeks of incubation, formed colonies were microscopically evaluated with regard to their morphology. Endothelial colonies were representative confirmed by DiLDL and lectin double-staining.

Human Umbilical Vein Endothelial Cell Culture

Pooled human umbilical vein endothelial cells (HUVECs; Cell Systems/Clonetics) were cultured in EBM supplemented with hydrocortisone (1 μg/mL), bovine brain extract (5 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 μg/mL), epidermal growth factor (10 μg/mL), and 10% FCS until the third passage. After detachment with trypsin, cells (3.5×10^5 cells) were grown on 60-mm culture dishes for at least 18 hours.

For small interference RNA (siRNA) oligonucleotide transfections, scrambled siRNA (5′-UCGACUGUACUGUCUCAU-3′) or siRNA (5′-GAUGACGAUGACCGUACGA-3′; each 360 pmol) corresponding to the human p21 mRNA sequence were transfected into HUVECs (3.5×10^5 cells per 6-cm well) using GeneTrans II (Mobitec) according to the instructions of the manufacturer.

For overexpression of p21, aortic ECs from wild-type or p21−/− mice were transiently transfected with a c-myc–tagged p21 plasmid encoding wild-type full-length p21 under Superfect transfection reagent (Qiagen).

Western Blot Analysis

Cell were lysed with 200 μL buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 10 mM Na,VO₃, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 15 minutes on ice. After centrifugation for 15 minutes at 20 000g (4°C), protein content was determined according to the Bradford method. Homogenates (40 μg per lane) were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes, which were then incubated with monoclonal antibodies to p21 (BD Pharmingen) or tubulin (NeoMarkers). Then, films were scanned and semiquantitatively analyzed, and the protein ratio of p21 versus tubulin was calculated.

Disc Neovascularization Model

According to the disc angiogenesis system introduced by Fajardo et al.,22 polyvinyl alcohol sponge discs (11 mm in diameter and 1 mm in thickness; Rippey) covered at each side with nitrocellulose cell-impermeable filters (Millipore) were subcutaneously implanted into the back of 3-month-old anesthetized C57/BL6 wild-type, p21−/−, or p21−/− mice. Two weeks later, space-filling fluorescent microspheres (0.2 μm; Molecular Probes Inc) were injected into the left ventricle to assess functional perfusion of neovascularization of the discs before discs were explanted.23 After explantation, both the vascularized area of the disc invested by vascular ingrowth and the vessel density were quantified by fluorescence microscopy.24

In order to determine EC apoptosis during adult vessel formation, we implanted additional discs into wild-type, p21−/−, or p21−/− mice for 10 days, whereupon discs were explanted, paraffin-embedded, sectioned, and stained against the endothelial-specific epitope.
confirmed the absence of p21 protein from ECs derived from (Figure 1A). Western blot analysis of whole cell lysates of p21 protein in mature ECs from p21+/−, p21+/-, and p21−/− mice. Mature ECs were isolated from aortic tissue by outgrowth into a matrix. A, Flow cytometric analysis of trypsinized cells after staining with an FITC-labeled antibody against CD146 (top), or with a phycocerythrin (PE)-labeled antibody against murine Flk-1 (bottom). B, Western blot analysis of whole-cell extracts with an antibody against p21 (top) and tubulin (bottom; for normalization of the amount of protein loaded into the lanes). Top, Densitometric analysis of p21 protein levels. Bars indicate mean ± SEM or as stated otherwise. Two statistics 3B). Likewise, both heterozygous p21+/- and homozygous p21−/− cells showed an increase in CFUs (Figure 3B). We then analyzed the proliferation of mature ECs by BrdU/MECA32 (BD Pharmingen), and apoptosis was visualized by DNA nick-end labeling according to the TUNEL procedure (Roche Diagnostics Inc).

Statistics
Data are expressed as mean ± SEM or as stated otherwise. Two treatment groups were compared by the independent sample t test, and three or more groups by one-way ANOVA followed by post hoc analysis adjusted with an LSD correction for multiple comparisons (SPSS). Results were considered statistically significant when P<0.05.

Results
In order to assess the effect of p21 on mature EC proliferation and apoptosis, ECs were cultivated out of aortic tissue as previously described.19,20 After 10 days in culture over 2 to 3 passages, 65±3% or 56±8% of the cells were positive for the EC-specific surface markers Flk-1/KDR/VEGF-R2 receptor tyrosine kinase or CD146 (S-Endo-1; MUC-18), respectively (Figure 1A). Western blot analysis of whole cell lysates confirmed the absence of p21 protein from ECs derived from homozygous p21−/− mice, whereas p21 protein levels were significantly reduced in cells from heterozygous p21+/- mice (Figure 1B).

Mature EC Proliferation and Apoptosis
We then analyzed the proliferation of mature ECs by BrdU/7AAD staining followed by flow cytometric measurement. Only Flk-1-positive cells were evaluated for cell cycle progression. The percentage of cells in S-phase was increased in both heterozygous p21+/- as well as in homozygous p21−/− mice when compared with wild-type animals (Figure 2A). In parallel, cells from both heterozygous p21+/- and homozygous p21−/− mice revealed significantly higher BrdU incorporation than ECs from wild-type mice as assessed by ELISA (Figure 2B). In contrast, apoptosis sensitivity toward serum/growth factor withdrawal determined by flow cytometric analysis of annexin binding to Flk-1-positive cells was enhanced only in cells derived from homozygous p21−/− mice, whereas apoptosis induction was not significantly different between mature ECs from heterozygous p21+/- and wild-type mice (Figure 2C). In order to assess apoptosis induction by an additional, specific parameter of apoptosis, we measured caspase-3 activity by flow cytometric detection of active caspase-3. In parallel with the enhanced increase in annexin V binding to p21+/- cells following serum starvation, the percentage of cells with activated caspase-3 was higher in ECs from p21+/- mice compared with both wild-type and p21+/- cells (Figure 2D). Likewise, depletion of p21 in cultured human ECs by siRNA oligonucleotide transfection induced a 2-fold increase in basal apoptosis when compared with control oligonucleotides (Figure 2E). In order to investigate, whether the increased apoptosis susceptibility of p21−/− cells is specifically due to the lack in p21, we overexpressed p21 by transient transfection of a plasmid encoding wild-type p21 (Figure 2F, inset). When compared with control, transfection of p21 completely abrogated the increased sensitivity toward apoptosis induction after serum starvation for 18 hours in ECs from p21−/− mice. (Figure 2F). These data suggest that the enhanced apoptosis sensitivity of ECs from p21−/− mice can be rescued by p21 overexpression and, thus, is indeed specifically attributable to the deficiency in p21.

Endothelial Progenitor Cells
To determine the number of EPCs, mononuclear cells were isolated from splenic tissue and characterized by dual positivity for DiI-DLDL uptake and lectin staining as previously described.25 The number of EPCs was significantly increased in both heterozygous p21+/- as well as homozygous p21−/− mice compared with wild-type mice (Figure 3A). We confirmed these data by a methylcellulose colony-forming assay. Endothelial cell–specific colony-forming units (CFUs) were increased in both heterozygous p21+/- and homozygous p21−/− mice in comparison with wild-type animals (Figure 3B). Likewise, both heterozygous p21+/- and homozygous p21−/− mice both showed an increase in CFUs (Figure 3B).

Neovascularization and EC Apoptosis In Vivo
We then assessed the in vivo relevance of p21 protein concentration for neovascularization using the disc angiogen-
esis model. Native neovascularization was assessed by quantification of the vascularized area and by measurement of caliber and density of the vascular network. The vascularized area was increased only in heterozygous p21<sup>H11001/H11002</sup> mice, whereas it was not significantly different between homozygous p21<sup>H11002/H11002</sup> and wild-type mice (Figure 4). Likewise, in heterozygous p21<sup>H11001/H11002</sup> mice, the fluorescence intensity reflecting caliber and density of the vascular network was increased by about 3.5-fold, whereas in homozygous p21<sup>H11002/H11002</sup> mice the vessel density showed a nonsignificant 2-fold increase compared with WT mice (Figure 4).

Because ECs from p21<sup>H11002/H11002</sup> mice were significantly more sensitive to ex vivo apoptosis induction, we investigated whether the lack of a significant neovascularization increase

Figure 2. Regulation of mature EC cycling and apoptosis in p21<sup>H11001/H11002</sup>, p21<sup>H11001</sup>/<sup>H11002</sup>, and p21<sup>H11002</sup> mice. A, Percentage of cells in S-phase out of all Flk-1–positive cells determined by flow cytometric cell cycle analysis. Bars indicate mean±SEM. *P<0.05 vs wild-type. B, BrdU incorporation of ECs assessed by ELISA, given as percent of BrdU incorporation of wild-type cells. Bars indicate mean±SEM. *P<0.05 vs wild-type. C, Flow cytometric analysis of apoptosis induction by serum and growth factor withdrawal for 18 and 24 hours measured by annexin binding of Flk-1–positive cells, given as percent of baseline apoptosis in ECs of the respective genotype under serum-containing conditions. Bars indicate mean±SEM. *P<0.01 vs wild-type and vs p21<sup>H11001/H11002</sup>; **P<0.05 vs wild-type and vs p21<sup>H11001/H11002</sup>; n.s. indicates no significant differences between wild-type and p21<sup>H11001/H11002</sup>. D, Induction of caspase-3 activation after 18-hour serum withdrawal, *P<0.005 vs wild-type and <i>P</i><0.05 vs and p21<sup>H11001/H11002</sup>; n.s. indicates no significant differences between wild-type and p21<sup>H11001/H11002</sup>. E, Apoptosis rates of HUVECs evaluated by fluoromicroscopic assessment of nuclear morphology after serum withdrawal for 12 hours and subsequent staining with the nuclear dye DAPI in cells transfected with siRNA oligonucleotides against human p21 mRNA. Bars indicate mean±SEM. *P<0.05 vs scrambled. Bottom, Analysis of p21 protein and tubulin levels to demonstrate equal loading of the gel. F, Apoptosis measured by flow cytometric detection of annexin V binding in aortic ECs from wild-type or p21<sup>H11002/H11002</sup> mice transfected with c-myc–tagged p21 at the indicated plasmid dose after subsequent serum starvation for 18 hours. Inset, Western blot analysis against p21 to confirm the expression of c-myc–tagged p21.
indeed increased EC apoptosis in p21 knockout mice. 17,18 The mechanism, by which p21 protects ECs against apoptosis, is still unclear. The apoptosis-suppressive capabilities of p21 were hypothesized to be linked to the induction of growth arrest by p21.26 However, because the unchanged protection against apoptosis observed in heterozygous p21−/− mice coincides with accelerated cell cycle progression of mature ECs, growth arrest at p21-sensitive cell cycle checkpoints is likely not to be attributed to the prosurvival role of p21.26 The antiapoptotic effect of p21 was shown to occur independent of cdk inhibition in cardiac myocytes.27 Beyond cell cycle regulation, p21 may also affect the mitochondrial pathway of apoptosis in colon cancer cells28 and inactivate caspase-3 by complex formation in hepatoma cells.29 In addition to the

in p21−/− mice is associated with increased EC apoptosis in vivo. Therefore, we determined EC apoptosis rates of newly formed vessels within discs. In order to monitor apoptosis selectively in ECs, we performed a counterstaining with the endothelial marker MECA32. Homozygous p21−/− but not p21−/+ mice displayed a significantly elevated percentage of apoptotic ECs in disc vessels (Figure 5), indicating that indeed increased EC apoptosis in p21−/− mice is associated with reduced neovascularization in p21−/− mice compared with p21+/+ mice.

Discussion

In this study, we investigated the concentration-dependent effects of the cell cycle protein p21 on growth and survival of mature ECs as well as on the number and function of EPCs. In addition, we determined the concentration-dependent effects of p21 on adult neovascularization as an associated biological function in a mouse model of p21 deficiency. Our data indicate that loss of one p21 allele causes decreased p21 protein levels in aortic mature ECs, increased proliferative capacity of mature ECs, and maintained protection of these cells against apoptosis in vitro and in vivo. In addition, the number and clonal expansion capacity of EPCs is significantly augmented, and adult blood vessel formation is significantly increased. In contrast, lack of both alleles of p21, and thus, complete deficiency in p21 protein, is associated with increased apoptosis susceptibility of mature aortic ECs in vitro and increased apoptosis rates during neovascularization in vivo. Although the proliferative capacity of both mature

ECs and EPCs is increased, p21−/− did not show increased neovascularization.

Low levels of p21 in mice lacking one p21 allele were still sufficient to prevent EC apoptosis, whereas ECs from mice with a homozygous p21 deficiency displayed increased apoptosis in vitro and in vivo, consistent with data on increased T-cell apoptosis in p21−/− mice.37,38 The mechanism, by which p21 protects ECs against apoptosis, is still unclear. The apoptosis-suppressive capabilities of p21 were hypothesized to be linked to the induction of growth arrest by p21.26 However, because the unchanged protection against apoptosis observed in heterozygous p21−/− mice coincides with accelerated cell cycle progression of mature ECs, growth arrest at p21-sensitive cell cycle checkpoints is likely not to be attributed to the prosurvival role of p21.26 In accordance, the antiapoptotic effect of p21 was shown to occur independent of cdk inhibition in cardiac myocytes.27 Beyond cell cycle regulation, p21 may also affect the mitochondrial pathway of apoptosis in colon cancer cells28 and inactivate caspase-3 by complex formation in hepatoma cells.29 In addition to the

Figure 3. Number and colony-forming capacity of EPCs from p21+/+, p21−/−, and p21−/− mice. A, EPCs were isolated by 4-day ex vivo growing of MNC from splenic tissue on fibronectin-coated dishes in the presence of VEGF and 20% FCS. Bars indicate a mean±SEM of n=10 animals of each genotype. *P<0.05 vs wild-type. B, Functional assessment of the clonal expansion capacity of EPCs using a methylcellulose colony-forming assay. Open bars indicate total number of progenitor cells given by the total number of formed colonies; filled bars, EPCs given by the number of colonies with an endothelial-specific morphology. Bars indicate mean±SEM, n=12. *P<0.05, #P=0.062 vs wild-type.

Figure 4. Neovascularization in p21+/+, p21−/−, and p21−/− mice. Spongiform discs were subcutaneously implanted into the backs of 3-month-old mice, and 2 weeks later the functional vascularization of the cross-sectional area of the discs visualized by preexplantation perfusion with fluorescent microspheres was quantified. A, Vascularized area illustrated by representative images. B, Quantification of the vascularized area given as medians with box plots representing 25th and 75th percentiles as boxes and range as thin lines; n=6. *P<0.001 vs wild-type; n.s. indicates nonsignificant vs wild-type. C, Vessel caliber and density of the network shown by microscopic analysis at ×10 magnification.
In conclusion, we show that decreased p21 protein levels in mice lacking one p21 allele increases the proliferative capacity of aortic mature ECs, whereas protection of these cells against apoptotic stimuli is still maintained. In addition, haploinsufficiency of p21 enhances number and clonal expansion capacity of EPCs and augments adult blood vessel formation in vivo. In contrast, complete p21 protein deficiency in mice lacking both alleles of p21 sensitizes ECs to apoptosis induction in vitro and results in increased EC apoptosis during neovascularization, which is not enhanced in p21<sup>-/-</sup> animals despite increased proliferation of both mature ECs and EPCs. Our data therefore suggest that the protection against apoptosis is essential to ensure that an increase in neovascularization results from accelerated cycling of mature and/or endothelial progenitor cells.

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