Loss of p53 Compensates for αv-Integrin Function in Retinal Neovascularization*

Received for publication, January 23, 2002, and in revised form, February 19, 2002
Published, JBC Papers in Press, February 20, 2002, DOI 10.1074/jbc.C200044200

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αv-Integrin antagonists block neovascularization in various species, whereas 20% of αv-integrin null mice are born with many normal looking blood vessels. Given that blockade of αv-integrins during angiogenesis induces p53 activity, we utilized p53 null mice to elucidate whether loss of p53 can compensate for αv-integrin function in neovascularization of the retina. Murine retinal neovascularization was inhibited by systemic administration of an αv-integrin antagonist. In contrast, mice lacking p53 were refractory to this treatment, indicating that neovascularization in normal mice depends on αv-integrin-mediated suppression of p53. Blockade of αv-integrins during neovascularization resulted in an induction of p21CIP1 in wild type and, surprisingly, in p53 null retinas, indicating that αv-integrin ligation regulates p21CIP1 levels in a p53-independent manner. In conclusion, we demonstrate for the first time an in vivo intracellular mechanism for compensation of integrin function and that p53 and αv-integrins act in concert during retinal neovascularization.

We previously found that integrin αvβ3 is preferentially expressed on newly forming blood vessels and is functionally involved in controlling angiogenesis stimulated by basic fibroblast growth factor or tumor necrosis factor-α, whereas another αv-integrin, αvβ5, is functional in vessel formation induced by vascular endothelial growth factor or transforming growth factor-α (1, 2). Importantly, antagonists of αv-integrins block neovascularization in various animal models with or without exogenous angiogenic stimulation, including chick chorioallantois, in mouse retina, and in human skin transplants in SCID mice, causing apoptosis of proliferating, angiogenic cellular cells (3-5). This suggests that during vessel formation, αv-integrins promote signaling events ultimately promoting vascular cell survival, thereby facilitating neovascularization. However, whereas 80% of αv-integrin null mice die in mid-gestation, 20% of these mice survive until 1 day after birth (9). Similarly, combinatorial gene knockout of integrin β3 and β5 subunits in mice results in enhanced angiogenesis under certain conditions (10). This indicates either that mice lacking integrins αvβ3 and αvβ5 could compensate for the function of αv-integrins in blood vessel formation or that a function of αv-integrins, once expressed but blocked or unligated, is to inhibit neovascularization. However, at present it is not known whether possible compensatory or redundant mechanisms can mediate blood vessel formation in the absence of functional αv-integrins.

**EXPERIMENTAL PROCEDURES**

p53+/− mice (11) were used to set up p53+/− × p53+/− breeding pairs. Littermates from such matings were used in the neovascularization assay, and genomic DNA from mouse tails was genotyped for p53 by a 3-primer assay as described previously (12). Newborn mice were injected subcutaneously twice daily, starting within 8 h after birth with 40 μg of cyclo-RGDfV (αv antagonist peptide 66203) or cyclo-RADV (control peptide 69601) (lowercase denotes d-amino acids) dissolved in phosphate-buffered saline, pH 7.4. The cyclo-RGDfV peptide binds specifically with high affinity to αvβ3 and αvβ5-integrins and blocks their function both in vitro and in vivo, whereas the cyclo-RADV peptide is non-functional (3, 7, 8, 13). After 2–3 days, the eye globes were taken out, fixed in cold methanol for 10 min followed by 6 min in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, dissected, stained for collagen type IV, and photographed as previously described (7). The distance from the head of the optic nerve to the edge of the retinal vasculature at 6–8 different representative points was measured for each retina on the photographs, and the mean vascular radius was calculated. The mean vascular area between the two retinas in each animal was then calculated, assuming a circular shape of the vasculature. Without treatment, no difference in vascular areas was observed between p53 heterozygous and p53 null mice, and therefore, to standardize and compare the results from different litters, the mean retinal vascular area in p53 null mice in each litter was considered to represent a fully developed vasculature. All p53 genotyping and measurements of the retinal vasculature were performed in a double-blind fashion to avoid any bias. For measurements of wild type retinas, the mean vascular area of control treated retinas was considered as fully developed and compared with anti-αv-treated retinas within the same litters. For Western blot analysis, retinas were fixed only in cold methanol and dissected. Retinas were then lysed in a modified radioimmune precipitation buffer and analyzed by Western blot as previously described (5) using 1 μg/ml anti-p21CIP1 (UPF13C1 and 6A6, monoclonal antibodies (Chemicon), or anti-actin monoclonal antibody JLA20 (Developmental Studies Hybridoma Bank, University of Iowa).

**RESULTS**

Interestingly, inhibition of angiogenesis by blockade of αv-integrins is accompanied by an induction of endothelial cell p53 activity (5). Based on this, we hypothesized that loss of p53 might compensate for αv-integrin function during neovascularization. To examine this possibility, we analyzed p53 null mice, where the effect of a specific αv-integrin antagonist was studied on retinal neovascularization. Mouse retinal neovascularization occurs during the first days after birth, and therefore, newborn mice were treated with an αv-integrin antagonist as described (7). The retinal vasculature in wild type newborn
mice treated for 3 days with the αv-antagonist was significantly less developed than retinas from control treated mice (Table I).

When accounting for the retinal vascular area already existing when the treatment started, the inhibition was close to 100%, in accordance with our previous observations after 4 days of treatment (7). Targeted p53 null males were then mated with p53 heterozygous females. This type of mating was not only necessary for sufficient embryonic survival but also allowed for a comparison of p53 null mice with heterozygous mice within the same litter in a double-blind fashion, including animals of exactly the same age receiving identical treatment. No difference in retinal vascularization could be observed between p53 null and heterozygous mice during the first 3 days (data not shown). In addition, no difference in degree of neovascularization between p53 null and heterozygous mice could be observed after control treatment (Fig. 1B and Table I). This indicates that p53 does not influence normal vascularization of the retina. We then treated entire litters of newborn mice of a mixed genotype (see above) with the αv-integrin antagonist. Interestingly, p53 heterozygous animals had a markedly less developed retinal vasculature compared with p53 null mice when treated with the αv-antagonist (Fig. 1 and Table I). Statistical analysis of measurements performed on the vascular area of these retinas revealed that the vascular development in anti-αv-treated p53 heterozygous was suppressed, a suppression that was found to be statistically significant (p = 5.7 × 10⁻⁷) when compared with p53 null animals in the same litters receiving identical treatment (Table I). This result closely resembled the difference seen between retinas from antagonist versus control treated wild type mice (Table I). Taken together, these findings indicate that although p53 expression does not influence normal neovascularization, loss of p53 compensates for the function of αv-integrins in neovascularization.

A possible explanation for the lack of response to the αv-integrin antagonist in p53 null mice could be deficient retinal integrin αv expression. To test this possibility, retinal lysates were analyzed for αv protein levels by Western blot analysis. As shown in Fig. 2A, αv-integrin levels in p53 null retinas do not differ from that in p53 heterozygous mice. Another possibility for a lack of response to the αv antagonist in p53 null mice could be that cells lacking p53 are insensitive to this treatment because of alterations in αv-integrin function at the cell surface.

To examine this possibility, we examined αv-integrin function of mouse embryonic fibroblasts lacking p53, including their responsiveness to the antagonist in inhibiting αv-integrin attachment to vitronectin was virtually identical in p53 null fibroblasts and the mouse fibroblast cell line NIH 3T3, demonstrating that lack of p53 does not cause a general insensitivity to αv-integrin antagonists. These findings reveal that loss of p53 does not affect expression levels or general function of αv-integrins. Instead, we conclude that intracellular events involving p53 mediate the inhibition of neovascularization by αv antagonists, events that may be related to the activation of endothelial cell p53 that we previously observed upon αv-integrin blockade during angiogenesis (5).

p53 is a known activator of the cell cycle suppressor p21CIP1. In fact, in addition to regulating p53, ligation of integrin αvβ3 in endothelial cells also suppresses p21CIP1 protein levels during angiogenesis (5). In UV-irradiated fibroblasts, p53 exerts its functional effect on cell cycle arrest by transcriptional activation of the Cdk inhibitor p21CIP1 (14). As shown in Fig. 2C,
blockade of α₅-integrin during neovascularization induces p21CIP₁ levels in wild type and in p53 heterozygous retinas. Surprisingly, whereas untreated p53 null mice display no detectable p21CIP₁, the numeric increase in p21CIP₁ levels by anti-α₅ treatment of these mice is similar to what is observed in wild type mice, resulting in a higher relative increase. This indicates that the regulation of p21CIP₁ by integrin α₅ during neovascularization is independent of p53. Furthermore, the fact that p21CIP₁ is induced in p53 null retinas upon blockade of α₅-integrins while neovascularization is still active suggests that this induction of p21CIP₁ is not sufficient to block neovascularization, although we cannot exclude that the somewhat higher total levels of p21CIP₁ in heterozygous animals might contribute to this blockade.

**DISCUSSION**

Studies using targeted gene knockout mice have in some cases revealed surprising results in that expected phenotypes were not found. This is particularly surprising for molecules found to play a role in certain *in vivo* events by previous loss of function studies, including for integrin α₅ and the capacity for at least 20% of α₅-integrin null mice to form blood vessels (9) and for the capacity of integrin β₃ and β₅ subunit combinatorial gene knockouts to support enhanced pathological angiogenesis (10). In some cases, combinatorial knockout of two or three related genes has demonstrated compensatory mechanisms by displaying phenotypes missing in single gene knockout mice. However, it is unclear as to how the functions of α₅-integrins can be compensated for. To this end, although it does not represent the only possible mechanism of α₅-integrin compensation, our finding that p53 null mice form blood vessels in the absence of functional α₅-integrins that are critical in wild type mice reveals the first *in vivo* example of an intracellular mechanism that is able to compensate for loss of integrin function.

Alternatively, the function of α₅-integrins in neovascularization in wild type animals may be to negatively regulate and balance vessel formation in an unfavorable extracellular matrix environment in order to prevent angiogenesis in inappropriate locations. Such a function of α₅-integrins would then lead to enhanced angiogenesis when α₅ integrins are blocked in wild type animals (middle) and thereby prevented from forming clusters, endothelial cell p53 activity is induced, and the vascular cells undergo apoptosis leading to a block of blood vessel formation (5). However, when p53 is absent during vascularization, inhibition of α₅-integrins does not affect the formation of viable vessels (right). Taken together, this suggests that p53 and α₅-integrins are linked into the same pathway in the control of blood vessel formation. VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; ECM, extracellular matrix.

**Fig. 3. Hypothetical model for role of p53 in compensating for α₅-integrin function during blood vessel formation.** In neovascularization of wild type animals (left), α₅β₅- and/or α₅β₁-integrins are activated, (1, 2). The α₅-integrins are then allowed to ligate to their provisional matrix, a ligation that is necessary to keep endothelial cell p53 inactive and cells surviving (vascularization is facilitated (3, 5)). When α₅-integrins are blocked in wild type animals (middle) and thereby prevented from forming clusters, endothelial cell p53 activity is induced, and the vascular cells undergo apoptosis leading to a block of blood vessel formation (5). However, when p53 is absent during vascularization, inhibition of α₅-integrins does not affect the formation of viable vessels (right). Taken together, this suggests that p53 and α₅-integrins are linked into the same pathway in the control of blood vessel formation. VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; ECM, extracellular matrix.
lization (7, 8). This suggests that αv-integrin antagonists constitute a potential therapy for ocular diseases and cancer. Our findings indicate that the molecular mechanism for this potential anti-angiogenic treatment actively involves p53, similar to what was recently indicated for angiostatin and TNP-470 (18–20).

We were unable to detect apoptosis in the retinal vasculature because of an obscuring background with a large number of apoptotic cells in the whole mounts of developing retinas with no apparent differences between the groups (data not shown). However, previous studies in other models clearly demonstrate that blocking of αv-integrins during neovascularization leads to cellular apoptosis (3, 5). This suggests that the inhibition of vessel formation by αv antagonists may be caused by induction of apoptosis of the forming vascular cells, and the fact that vascular formation in p53 null mice is refractory to αv antagonist treatment suggests that these vessels do not undergo apoptosis (Fig. 3). Whereas p53 may mediate αv-integrin-regulated apoptosis in vascular cells, induction of p53 by loss of integrin signaling during neovascularization (7, 8). This suggests that αv-integrin antagonists constitute a potential therapy for ocular diseases and cancer. Our findings indicate that the molecular mechanism for this potential anti-angiogenic treatment actively involves p53, similar to what was recently indicated for angiostatin and TNP-470 (18–20).

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In conclusion, we demonstrate that loss of p53 compensates for the function of αv-integrins in retinal neovascularization, possibly by interfering with αv-integrin regulation of vascular cell apoptosis. This indicates a critical function for αv-integrin ligation during neovascularization in suppressing p53 and that p53 constitutes an important part of the control of neovascularization.

Acknowledgment—We thank Dr. Klas Wiman for providing p53 null mouse embryonic fibroblasts.

REFERENCES

1 The abbreviation used is: ERK, extracellular signal-regulated kinase.
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doi: 10.1074/jbc.C200044200 originally published online February 20, 2002

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