β3-Integrin Mediates Smooth Muscle Cell Accumulation in Neointima After Carotid Ligation in Mice

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Background—Pharmacological blockade of β3-integrins inhibits neointimal lesion formation in nonmouse animal models of arterial injury. In contrast, β3-integrin-deficient (β3−/−) mice are not protected from neointimal lesion formation after arterial injury. We investigated this discrepancy in β3−/− and wild-type (β3+/+) mice using different models of injury.

Methods and Results—After disruption of the carotid with a transluminal probe, there was no significant difference in neointimal thickening between β3−/− and β3+/+ mice. However, after ligation of the carotid without medial disruption, there was reduced neointimal thickening in β3−/− mice compared with β3+/+ mice at intervals up to 3 months. Lesion reduction in β3−/− mice was associated with fewer intimal smooth muscle cells (SMCs) without a difference in SMC apoptosis or proliferation rate compared with β3+/+ mice, consistent with reduced SMC migration from the media into the intima of β3−/− mice. Moreover, combined eccentric medial disruption and ligation of the carotid in β3−/− mice resulted in reduced neointimal lesion formation after carotid ligation injury, confirming the importance of αβ3 and not αmβ3 in the attenuated response.

Conclusions—The αβ3-integrin mediates intimal SMC accumulation that contributes to neointimal thickening in the setting of arterial ligation. (Circulation. 2004;109:1564-1569.)

Key Words: restenosis / cell adhesion molecules / muscle, smooth / angioplasty

Percutaneous transluminal coronary angioplasty has become the most common interventional treatment of coronary artery disease, with >560,000 procedures in 2000 in the United States alone.1 Despite continuing advances in this technology, restenosis secondary to intimal hyperplasia is still a major limitation. This serious complication occurs, at least in part, as a result of smooth muscle cell (SMC) migration and proliferation within the neointima of coronary arteries. In 1994, we reported that blocking αβ3-integrin attenuated SMC accumulation in neointima after balloon angioplasty in animals.2 When a randomized human clinical trial (Evaluation of Platelet IIb/IIIa Inhibition for Prevention of Ischemic Complication [EPIC]) revealed that abciximab, a functional fragment of a blocking antibody to the β3-integrin subunit, improved clinical outcome after coronary balloon angioplasty, it was speculated that αβ3-integrin blockade might have contributed to the inhibition of intimal hyperplasia, leading to less luminal narrowing in these patients.3 Indeed, Blindt et al4 reported that abciximab is a potent inhibitor of human coronary SMC migration and invasion, which may account for the benefit observed after coronary intervention.

In contrast to reports of benefit from αβ3-integrin blockade in animal models, Smyth et al5 showed that β3-integrin-deficient (β3−/−) mice were not protected from developing neointimal lesions after guidewire-induced injury to the femoral artery. This discrepancy could be explained by data showing that β3-integrin deficiency is mechanistically distinct from β3-integrin blockade with antagonists.6,7 For instance, Stupack et al8 found that unligated or blocked integrins could act as negative regulators of endothelial cell survival, initiating a process referred to as “integrin-mediated death.” When they reduced or prevented expression of αβ3 on endothelial cells, there was prolonged cell survival. However, integrin-mediated SMC apoptosis alone does not explain the role of the αβ3-integrin in SMC migration seen in animal experiments. To investigate whether β3-integrin deficiency affects SMC migration in vitro and SMC accumulation in vivo in a manner similar to that of β3-integrin blockade, we designed a study to assess SMC migration, proliferation, and apoptosis in vitro and in vivo after different types of vascular injury in β3−/− mice.

Methods

Mouse Aortic Explant and SMC Preparation

All procedures conformed to the Guide for the Care and Use of Laboratory Animals (US Department of Health, Education, and

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Explant Migration and SMC Chemotactic Assay

Random SMC outgrowth was assessed as described previously. The number of SMCs migrated from the explants was assessed after 3 and 7 days. Chemotactic migration was measured with a modified Boyden chamber technique as previously described. Experiments were performed at least twice with the use of quadruplicate wells.

In Vitro Assay for Cell Proliferation, Viability, and Apoptosis

β3−/− and β1−/− SMCs were harvested and subcultured (passages 2 to 4). Cell proliferation rate was determined with use of the cell proliferation assay kit (Chemicon International, Inc). Cell viability was determined with use of the LIVE/DEAD viability kit (Molecular Probes). For apoptosis, the cells were fixed for 2 minutes in buffered formalin. The cells were rinsed twice in PBS and incubated in Hoechst stain (Molecular Probes) prepared according to the manufacturer’s suggestions for 5 minutes at room temperature. Four random fields were viewed at ×100 magnification for morphological signs of apoptotic cells, including compacted and condensed nuclei (pyknosis) and/or nuclear fragmentation (karyorrhexis).

Preparations of Carotid Arterial Injury

Only 8- to 12-week-old male β3−/− and β1−/− mice were used. The animals were anesthetized with sodium pentobarbital (40 to 50 mg/kg IP) and prepared for left common carotid arterial injury through a midline neck incision with use of a sterile technique. Three different carotid injury methods were used: (1) trans luminal arterial injury with a beaded probe (0.57-mm diameter epoxy resin bead on 0.30-mm guidewire); (2) arterial ligation injury, and (3) longitudinal arteriotomy and repair after arterial ligation (combined injury). The guidewire probe injury method is a modification of a method described previously, with insertion of the probe into the common carotid artery from the external carotid artery, which was ligated after the probe removal. The arterial ligation injury method was reported previously by Kumar and Lindner. The combined injury was created with medial disruption induced by longitudinal arteriotomy in the mid common carotid artery with a bevel blade, followed by repair of the arteriotomy with 9-0 Prolene suture and arterial ligation at the bifurcation.

After 1, 2, or 3 weeks or 3 months, the animals were anesthetized, and the carotid arteries were perfusion-fixed via the left ventricle at 100-mm Hg pressure with saline followed by Histochrome fixative. The left carotid arteries were excised and embedded in paraffin. Cross sections (5 μm) were taken at 0.5 mm from the carotid bifurcation for Verhoeff-van Gieson elastin staining. Total vessel (external elastic lamina), luminal, intimal, and medial areas were measured, and intimal and medial SMCs were counted with use of a Zeiss Axioskop microscope connected to a digital camera and an IBM computer containing Zeiss Image software.

Immunohistochemistry for Proliferation and Apoptosis

Immunohistochemical staining of the proliferating cell nuclear antigen (PCNA) (rabbit polyclonal antibody; 1:1000 dilution; Santa Cruz Biotech) and active caspase-3 (rabbit polyclonal antibody; 1:1000 dilution; Dr Don Nicholson, Merck, Quebec, Canada) were used to determine SMC proliferation and apoptosis in the neointima and media after ligation injury according to a previously described immunohistochemistry protocol.

Irradiation and Bone Marrow Transplantation

β3−/− and β1−/− mice underwent lethal gamma irradiation (~9.5 Gy) from a cesium source, followed 6 hours later by transplantation with femoral bone marrow cells obtained from 8- to 10-week-old β3+/+ or β1+/+ donors (5×10^6 cells per recipient; 0.3 to 0.5 mL by intravenous injection). Three groups were studied: (1) β3+/+ donor marrow transplanted to β3−/− recipients; (2) β1+/+ donor marrow transplanted to β1−/− recipients; and (3) β3+/+ donor marrow transplanted to β3−/− recipients. Mice were housed in a specific-pathogen-free barrier environment. All nontransplanted mice died 7 to 14 days after irradiation. Animals achieving successful engraftment underwent the carotid ligation injury 6 weeks after transplantation, and carotids were harvested 3 weeks later.

Statistical Analysis

Results are shown as mean ± SEM. An Excel 2000 statistical package was used for the quantitative analyses of parameters such as intima-medial lesion area, intimal-medial SMC number, and migration assays (ie, ANOVA and Student t test). A probability value <0.05 was considered significant.

Results

β3-Integrin Mediates SMC Migration In Vitro

SMC outgrowth was less with β3−/− aortic explants compared with β3+/+ aortic explants (Figure 1, A through C). To determine whether chemotactic migration was similarly affected, modified Boyden chambers were used with SMCs (passages 2 to 4) harvested from β3−/− and β3+/+ aortas. With platelet-derived growth factor-AB (PDGF-AB) (10 μg/mL)
stimulation in the lower chamber, directed SMC migration was reduced with β3−/− SMCs compared with β3+/+ SMCs (Figure 1D). To determine whether integrin-mediated cell death was present in β3−/− SMCs, we compared cell viability and apoptosis between β3−/− and β3+/+ SMCs for 24 hours. Hoechst staining demonstrated no differences in condensed nuclei (pyknosis) or nuclear fragmentation (karyorrhexis) between the 2 groups, suggesting that there was no difference in apoptosis (data not shown). Furthermore, there were no differences in cell viability or proliferation rate between β3−/− and β3+/+ SMCs (data not shown).

β3-Integrin Deficiency Reduces Neointimal Lesion Formation After Carotid Ligation but Not Guidewire Probe or Combined Injury

To determine whether the effect of β3-integrin deficiency on neointimal lesion formation in mice depends on the type of injury, we compared 3 methods of carotid arterial injury. After transluminal probe injury, which disrupts both the internal elastic lamina and the media, there were no differences in the neointimal lesion size between β3+/+ (0.029±0.008 mm²; n=8) and β3−/− (0.037±0.014 mm²; n=9; P=0.44) mice after 3 weeks (Figure 2, A through C). After ligation of the carotid artery, which induces minimal, focal mechanical trauma to the internal elastic lamina and media, neointimal formation was nearly abolished at 3 weeks in β3−/− mice (0.001±0.001 mm²; n=8) (Figure 2, D through F). In contrast, β3+/+ mice had significantly more neointimal lesions (0.011±0.005 mm²; n=8; P<0.01) than the β3−/− mice. After the combined injury, with disruption of the media eccentrically by arteriotomy and ligation of the artery at the bifurcation, there was neointimal thickening only in the area of medial disruption. No neointimal lesion was seen on the opposite, nondisrupted section in β3−/− mice (Figure 3). A similar pattern was observed in each of 5 β3−/− mice that underwent the combined injury.

To determine whether neointimal formation was reduced earlier and later than 3 weeks after carotid ligation in β3−/− mice, we also observed results at 1 and 2 weeks and 3 months. The neointimal lesion size was less in β3−/− mice at all intervals compared with β3+/+ mice, whereas there were no differences in the medial areas (Figure 4, A and B). To assess whether there were differences in the total number of SMCs in the neointima, we counted the SMCs in the neointima and media. At each interval up to 3 months after carotid ligation, the number of SMCs in the neointima was less in the β3−/− mice compared with β3+/+ mice, whereas there were no differences in the number of SMCs in the media (Figure 4, C and D).

Reduced SMC Accumulation After Carotid Ligation Injury in β3−/− Mice

To determine whether the inhibitory effect of β3-integrin deficiency on intimal SMC accumulation was due to a reduction in SMC proliferation, we examined the tissue by immunohistochemical staining for PCNA in the neointima and media at 1, 2, and 3 weeks and 3 months after carotid ligation injury (Figure 5, A and B). Because there were fewer SMCs present in the neointima of β3−/− mice compared with the number in β3+/+ mice at 1 and 2 weeks, the percentages of SMCs staining for PCNA could not be determined accurately for comparison at 1 and 2 weeks. However, there were no differences in the percentages at 3 weeks and 3 months, and the percentages of medial SMCs were not different between
and β3−/− mice at all time points (Figure 5D). In addition, caspase-3 immunostaining demonstrated no differences in the number of apoptotic cells between the 2 groups, similar to the PCNA analysis (Figure 5, E through H). Thus, β3−/− integrin deficiency was associated primarily with reduced SMC accumulation in the neointima without affecting SMC proliferation or apoptosis. Furthermore, β3−/− integrin deficiency had no effect on arterial remodeling, as determined by the lack of differences in total vessel areas (data not shown).

α,β3-Integrin Deficiency Is Responsible for Neointimal Lesion Reduction

To further elucidate the role of β3-integrins in intimal lesion formation, we studied the 2 β3-integrins found in the arterial vasculature, αmβ3 and αβ3. To separate the role of αmβ3 and αβ3-integrins in intimal formation, we performed total body irradiation of β3−/− mice (recipients) followed by bone marrow transplantation with bone marrow cells harvested from β3+/+ mice (donors). Three weeks after the bone marrow transplantation, the rescued animals underwent carotid ligation injury. After 3 weeks, only β3−/− recipient mice with reconstituted β3+/+ bone

Figure 3. Neointimal lesion development 3 weeks after eccentric medial disruption by arteriotomy and arterial ligation in β3−/− mouse carotid (×200). The lesion was confined to the area of disruption to the media, whereas no neointima formed on the opposite, nondisrupted section.

Figure 4. Time course of neointimal and medial areas and SMC number after ligation of a carotid artery in β3−/− and β3+/+ mice. A, β3−/− mice had fewer neointimal areas than β3+/+ mice at 1 and 3 weeks and 3 months (*P<0.05). B, No differences in medial areas between β3−/− and β3+/+ mice. C, Fewer SMCs in neointima in β3−/− mice than in β3+/+ mice (*P<0.05; **P<0.01). D, No differences in medial SMCs between β3−/− and β3+/+ mice.

marrow had significantly reduced neointimal lesions, whereas other groups had the expected neointimal lesions (n=7; P<0.01) (Figure 6).

**Discussion**

We investigated the functionality of β3-integrin deficiency on SMC migration and neointimal formation. We showed that β3−/− SMCs exhibited less motility than the wild-type SMCs in culture, without differences in proliferative and apoptotic rates. Smyth et al5 used a “combination of guidewire-induced endothelial denudation and arterial ligation” and demonstrated that β3-integrin deficiency did not have a role in neointimal lesion formation. We elected to simplify the injury methods by creating 3 distinct injury patterns that differed in the extent of medial injury induced: (1) guidewire probe–induced transmural injury with medial disruption; (2) nonmedial disruptive ligation injury; and (3) eccentric medial disruptive injury followed by arterial ligation. We believe that guidewire probe injury generates more transmural mechanical damage to the media over a longer segment of the vessel compared with the ligation injury, which generates a more modest, focal lesion with stagnant flow and thrombosis.

Indeed, we saw that the probe injury resulted in greater neointimal lesion formation associated with greater evidence of disruption in the medial layer. Some vessels had injury extending to the adventitial layer, with disruption of the external elastic lamina. With this disruptive injury to the media, we demonstrated that β3-integrin deficiency did not protect against neointimal lesion formation. In contrast, in the setting of arterial ligation injury, β3-integrin deficiency protected against neointimal lesion formation at 1, 2, and 3 weeks and 3 months after injury. Because the endothelia remain intact after ligation, at least in this setting, neointimal reduction appears to be independent of the effect β3 integrin has on endothelial regrowth. When the combination of medial disruption and arterial ligation was used in β3−/− mice, there was eccentric neointimal lesion formation only at the site of disruption (Figure 4). The lack of neointimal lesion formation on the opposite, nondisrupted section is consistent with the dependence of neointimal formation on the mechanical disruption of the internal elastic lamina and media as described by others.15,16

The mechanism accounting for less neointima formed after arterial ligation compared with transmural injury may relate to the process by which cells accumulate in the neointima. With the disruption of the elastic lamellae, including the external elastic lamina, adventitial fibroblasts may play a more significant role in the intimal lesion formation, which may explain why lack of β3 integrins had no effect. Although all intimal cells stained positively for smooth muscle–specific α-actin after arterial injury, recent data have shown that some neointimal cells may be redifferentiated fibroblasts derived from the adventitia.17 One satisfactory explanation for these discrepancies is that different models and/or methodologies accentuate the various, distinct functions of β3 integrins. For instance, Carmeliet et al18 compared mechanical injury–induced intima formation in plasminogen activator inhibitor 1 (PAI-1)−/− and wild-type mice and demonstrated that PAI-1 blocks intimal thickening by inhibiting the migration of SMCs. In contrast, Peng et al19 demonstrated that when ligation-induced intima formation was examined in PAI-1+/+ and PAI-1−/− mice, PAI-1 promoted neointimal thickening. One can conclude that these injury models emphasize a contrasting cascade of events despite the apparently simple injuries.

To delineate further which of the 2 β3-integrins (αβ3 or αvβ3) was responsible for neointimal lesion development after arterial ligation injury, we performed total body irradiation and bone marrow transplantation experiments. Platelets and leukocytes are present in the arterial lumen after injury and have a major role in the development of intimal hyperplasia.5,20 Of these bone marrow–derived cells, only the platelets express the αvβ3-integrin.14 Resident cells (eg, SMCs, endothelial cells) of the arterial wall and inflammatory cells (eg, macrophages, neutrophils) express αvβ3 but not αvβ3.21,22 Therefore, to determine whether αvβ3 is required for SMC migration and neointimal hyperplastic lesion development, β3-integrin–deficient mice were subjected to total body irradiation and bone marrow “rescue” with wild-type bone marrow, followed by carotid ligation. The result was nearly complete inhibition of neointimal formation in β3−/− mice rescued with β3+/+ bone marrow. In contrast, when β3+/+ mice underwent irradiation and transplantation with β3−/− or β3−/− bone marrow, typical amounts of neointima were observed (Figure 6). Furthermore, Smyth and colleagues5 observed luminal platelet deposition after endothelial denudation in both β3−/− and β3+/+ mice, and we found thrombus formation in β3−/− and β3+/+ mice at all levels of histological analyses (data not shown). Therefore, we conclude that αvβ3 does not play a significant role in neointimal formation in this model of ligation injury. This finding is consistent with previous results showing that only αvβ3-integrin blockade and not αvβ3-integrin blockade was effective in reducing SMC migration.23,24

In summary, our data support the conclusion that αvβ3 plays a critical role in SMC migration and neointimal lesion formation. The effectiveness of β3-integrin deficiency is dependent on the severity of vascular injury, with benefit exhibited after nondisruptive arterial wall injury but not with
more severe transmural injury. The benefit of \( \beta_3 \)-integrin deficiency results from reduction in SMC accumulation and does not depend on changes in proliferation of cells or integrin-mediated cell death. Therefore, although \( \beta_3 \)-integrin blockade effectively reduces neointimal hyperplasia in animal models, this blockade may not be effective for prevention of neointimal lesion formation in the less defined, more disruptive injury induced by percutaneous transluminal coronary angioplasty in human coronary arteries. Indeed, the ERASER trial demonstrated that potent \( \beta_3 \)-integrin blockade effectively reduces neointimal hyperplasia in patients with sepsis, shock, and multiple organ dysfunction. Crit Care Med. 1999;27:1230–1251.

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