Impaired Integrin β3 Delays Endothelial Cell Regeneration and Contributes to Arteriovenous Graft Failure in Mice

Ming Liang, Yun Wang, Anlin Liang, Jin-Fei Dong, Jie Du, Jizhong Cheng

Objective—Neointima formation is associated with stenosis and subsequent thrombosis in arteriovenous grafts (AVGs). A role of integrin β3 in the neointima formation of AVGs remains poorly understood.

Approach and Results—In integrin β3−/− mice, we found significantly accelerated occlusion of AVGs compared with the wild-type mice. This is caused by the development of neointima and lack of endothelial regeneration. The latter is a direct consequence of impaired functions of circulating angiogenic cells (CACs) and platelets in integrin β3−/− mice. Evidence suggests the involvement of platelet regulating CAC homing to and differentiation at graft sites via transforming growth factor-β1 and Notch signaling pathway. First, CACs deficient of integrin β3 impaired adhesion activity toward exposed subendothelium. Second, platelets from integrin β3−/− mice failed to sufficiently stimulate CACs to differentiate into mature endothelial cells. Finally, we found that transforming growth factor-β1 level was increased in platelets from integrin β3−/− mice and resulted in enhanced Notch1 activation in CACs in AVGs. These results demonstrate that integrin β3 is critical for endothelial cell homing and differentiation. The increased transforming growth factor-β1 and Notch1 signaling mediates integrin β3−/−-induced AVG occlusion. This accelerated occlusion of AVGs was reversed in integrin β3−/− mice transplanted with the bone marrow from wild-type mice.

Conclusions—Our results suggest that boosting integrin β3 function in the endothelial cells and platelets could prevent neointima and thrombosis in AVGs. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.305089.)

Key Words: bone marrow • endothelial progenitor cells

The creation of functional arteriovenous grafts (AVGs) for patients who undergo coronary artery bypass or hemodialysis is a frequent and expensive surgical procedure. Even when initial surgery is successful, an AVG frequently fails. The primary patency rates of graft failure are 42% and 40% to 60% for coronary bypass surgery and hemodialysis, respectively, primarily because of neointima formation and AVG thrombosis. The integrin β3 family consists of αvβ3 and αIIbβ3. The former is expressed in endothelial cells and selective inflammatory cells, while the latter is primarily on platelets. Platelets are involved in the recruitment and differentiation of bone marrow-derived terminal progenitor cells, mediated through the adhesive receptor integrin αIIbβ3. Recent reports suggest that integrin β3 expressed on endothelial cell and platelets influences the function of bone marrow-derived progenitor cells. Platelets from integrin β3−/− mice are also reported to secrete more chemokines and cytokines compared with the wild-type (WT) mice, including transforming growth factor (TGF)-β1. In a mouse AVG model, there was a massive loss of the endothelium 3 days after surgery. The subsequent endothelial regeneration plays a key role for vascular remodeling and maintaining a patent AVG. Platelets influence the homing and differentiation of circulating angiogenic cells (CACs) into endothelial cells at sites of vascular injury by serving as carriers and by secreting SDF-1α. Here, we tested hypotheses that integrin β3 is critical for neointima formation and endothelial cell regeneration at sites of AVGs by studying the adhesion and differentiation of CACs in vitro and vascular repairs in a mouse AVG model. We demonstrate that CACs from integrin β3−/− mice homed poorly because of significant reduction in CAC adhesion and differentiation. The latter is caused by an altered TGF-β1-Notch1 signaling mediated by β3-deficient platelets.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.
Results

AVG Occlusion Accelerated in Integrin β3−/− Mice

Integrin β3 is expressed in artery, vein, and heart tissues; there was no such expression in integrin β3−/− mice (Figure 1A). In our model of AVGs in WT mice, we used an enface analysis to study the endothelium of the vein of the AVG and a normal vena cava. Integrin β3 was expressed in endothelial cells in a clustered pattern (Figure 1B). In a failed AVG from patients, integrin β3 expression was located in the endothelium and the neointima (Figure 1C). At 1 month after surgery, similar results were found in AVGs created in WT mice (Figure 1D). Compared with results in a control vena cava, there was reduced integrin β3 expression in the endothelium of the AVG (Figure 1D). Newly formed endothelial cells expressed integrin β3 on the basal side (Figure 1D). In AVGs, there was marked thinning and attenuation of the endothelium compared with findings in normal vena cava.

Integrin β3−/− and WT mice were subjected to AVG and evaluated 1 month after surgery. The rate of graft failure because of occlusion was 86.7% for β3−/− mice as compared with 5% for WT mice (Figure 2A and 2B). An intact vascular lining of CD31+ endothelial cells was found in AVGs of WT mice, but not in integrin β3−/− mice (Figure 2C). SMA-α was strongly expressed in neointima cells in WT AVGs but not in integrin β3−/− mice (Figure 2C). When stained for markers for thrombosis (von Willebrand factor, CD41, and CD42), we found much more expression of thrombotic positive markers in AVGs placed in integrin β3 knockout mice versus the expression in WT mice (Figure 2D and 2E), suggesting that the integrin β3 deficiency prevents the formation of an intact endothelium and causes thrombosis, which could be the potential factor influencing the AVG patency.

Integrin β3−/− Delays Endothelial Regeneration in AVGs

Endothelial cells became disorganized 3 hours after AVG surgery, and most were lost by 24 hours in WT and integrin β3−/− mice (Figure 3A and 3C). These results could not be claimed on surgeries as a cross section of AVG just after surgery revealed that the endothelium was intact (Figure 3B). However, endothelial cells regenerated to form the new endothelium around 7 to 14 days after surgery (Figure 3A and 3C). In contrast, endothelial cells were scarcely present at an AVG site of integrin β3−/− mouse during the same period (Figure 3D). This result was further verified by enface analysis, which detected significantly less CD31 signals in AVGs from integrin β3−/− mice as compared with that from WT mice (Figure 3E).

Integrin β3−/− Suppresses Bone Marrow–Derived CAC Adhesion In Vitro

Alternatively, the reduced endothelial regeneration could be because of the impaired CAC homing capability. Reduced CAC homing could be caused by the lack of integrin β3-mediated interaction between CACs and the subendothelial matrix. Consistent with the notion, CACs from integrin β3−/− mice adhered poorly to vitronectin, the ligand for integrin β3, while the adherence of integrin β3-deficiency CACs to laminin and collagen I was not affected (Figure 4A and 4B). These results are further confirmed with experiments testing lineage-negative/c-Kit/Sca I-positive CACs isolated from the
bone marrow of WT and β3−/− mice (Figure 4C). The absence of integrin β3 suppressed CAC adhesion to extracellular matrix. Notably, the extracellular matrix tested included vitronectin and fibronectin which are interacting with integrin β3 (Figure 4C). Together, these results suggest that β3-deficiency CACs adhere poorly to the subendothelium exposed at sites of AVGs.

This reduced CAC homing is not caused by a reduced number of CACs because integrin β3−/− mice had a comparable number of CACs in the bone marrow of WT mice (Figure 4D and 4E). Moreover, the lineage-negative CACs (lineage−/c-kit+/Sca I+ cells) were also purified and analyzed. The results indicate that β3-deficiency CACs did not affect CAC numbers but impaired their adhesion capability to vitronectin (Figure 4F and 4G). Similar results were found from circulating Sca I+/c-Kit+ cells (data not shown).

Platelets From Integrin β3−/− Mice Delay Homing and Differentiation of Bone Marrow–Derived CACs Platelets are known to play a critical role in CAC homing.10 We next examine whether integrin β3 deficiency in platelets contributes to the poor regeneration of endothelial cells at an AVG site. We found that there was significantly less Sca1+ CACs adhering to AVGs at day 5 in integrin β3−/− mice compared with controls (Figure 5A and 5B). Moreover, CACs have less potential to differentiate into mature endothelial cells when cocultured with platelets from integrin β3−/− mice compared with those on WT platelets (Figure 5C). Furthermore, these β3-deficient platelets expressed a 2-fold higher level of TGF-β1 (Figure 5D). Using ELISA assay, we found that thrombin (0.125 U/mL) or collagen I (5 μg/mL) treatments stimulated more TGF-β1 release from platelets isolated from integrin β3 knockout mice compared with that of WT mice (Figure 5E). In AVGs, we found that TGF-β1 colocalized with platelets, and more TGF-β1 level was detected in integrin β3 knockout mice versus that in WT mice (Figure 5F).

To assess the impact of TGF-β1, we treated WT CACs with TGF-β1 and found impaired CAC differentiation into endothelial cells (Figure 5G). Thus, high TGF-β1 in platelets from integrin β3 knockout mice suppresses CAC differentiation into mature endothelial cells and then reduced the recovery of the endothelium after endothelial cell losses in an AVG.

Increased TGF-β1 in β3-Null Platelets Inhibits CAC Differentiation by Stimulating Notch Signaling Results presented to date have suggested that poor endothelial regeneration in integrin β3 knockout mice at an AVG site may be caused by (1) lack of interaction between β3−/− CACs

Figure 2. Integrin β3 deficiency accelerates arteriovenous graft (AVG) failure. A, Hematoxylin and eosin staining of AVGs in wild-type (WT) and integrin β3−/− mice shows marked differences in patency. B, The ratio of failed to total AVGs was calculated. Total 15 AVGs were created in integrin β3−/− mice, and 9 in WT mice. C and D, The difference in AVGs of WT or integrin β3−/− mice is revealed by immunostaining with the endothelial marker, CD31 (C), smooth muscle marker SMA-α (C), and platelet markers (D). E, The density analysis of the expression of platelet markers in WT and integrin β3−/− mice (n=4). VWF indicates von Willebrand factor.
Bone Marrow Cells From WT Mice Rescue AVG Failure in Integrin β3−/− Mice

The results to date have suggested 2 potential pathways through which integrin β3 deficiency results in poor homing of CACs and regeneration of the endothelium (differentiation) at a site of AVG: decreased interaction between CACs and the subendothelium (directly mediated through platelets) and enhanced Notch signaling in CACs mediated by platelet-derived TGF-β1. These possibilities were further investigated by the transplantation of bone marrow from a WT mouse donor to an integrin β3−/− recipient. There was significant improvement in AVG patency in integrin β3−/− mice after transplantation of WT bone marrow cells versus results from integrin β3−/− mice (Figure 7A). In a reciprocal experiment, AVGs in WT mice transplanted with bone marrow cells from integrin β3−/− mice were clogged (Figure 7A). The AVG from transplanted integrin β3−/− mice also had an intact vascular wall lined with mature CD31+ endothelial cells (Figure 7B and 7C). There was >75% of AVGs in the mice with the bone marrow transplantation had open lumens versus the AVGs generated in WT mice with bone marrow from integrin β3−/− mice (Figure 7D). To further compare the CAC homing capability, a mixture of bone marrow cells from WT (GFP+) and integrin β3−/− mice was transplanted into the lumen area of the AVGs (Figure 7E), indicating that deficiency of integrin β3−/− limits CAC-mediated endothelium regeneration.

Discussion

In our current study, we found that integrin β3−/− causes AVG occlusion. Several lines of evidence support that the dysfunctions of platelets and CACs in integrin β3−/− mice are the potential reasons for this failure. First, deficiency of integrin β3 in CACs impairs their adhesion to the exposed extracellular matrix. Integrin β3−/− platelets are deficient in mediating CAC homing and prevent CAC differentiation into mature endothelial cells because of an increased production of platelet-derived TGF-β1, which activates the Notch signaling pathway (Figure I in the online-only Data Supplement).

AVG failure is a consequence of 3 related processes including early thrombosis formation, neointima formation, and graft atherosclerosis. About 40% of AVGs experience such a failure within 18 months of the operation. The failure of AVGs is attributable to adverse vascular remodeling, which remains poorly understood. Multiple events and cell types, including endothelial denude, platelet aggregation, smooth muscle cell proliferation, activated thrombocytes, and infiltration of monocytes, contribute to AVG failure. In mouse model of AVG, we found increased thrombosis and smooth muscle cell hyperplasia in AVGs at 2 to 4 weeks, and this process is accelerated in β3-integrin knockout mice. Deficiency of integrin β3 delayed the endothelial cell regeneration in AVG in early time point, and led to AVG failure, which related with thrombosis. These findings are consistent with previous reports. The initial loss of venous endothelium after AVG is followed by platelet accumulation and the recruitment of CACs at a denuded AVG. CACs are bone

and components of subendothelial matrix and (2) increased TGF-β1 in integrin β3−/− platelets. To explore the second possibility, we focus on Notch signaling in endothelial cells because TGF-β1 activates Notch by upregulating the Notch ligand Jagged 1. This Notch signaling has also been linked to endothelial cell regeneration. Representative data were from 2 mice.
marrow–derived cells that are mobilized to the systemic circulation in response to tissue injury and incorporate into sites of neovascularization. After vascular injury, CACs have been shown to mobilize and home to the subendothelium at the site of vessel injury. Peripheral blood CACs transplanted into balloon-injured arteries rapidly endothelialize the denuded vessels. In AVGs, the endothelium is denuded 72 hours after surgery followed by on-site platelet aggregation and endothelial cell regeneration. There is ≈30% of bone marrow–derived CAC incorporation into newly formed endothelium. An integrin β3 deficiency could potentially interfere with vascular repairs at an AVG site because the disruption of the integrin signal could result in a poor maturation of vessels. Moreover, the neovascularization was restored in a DiYF β3-integrin mutant mouse by transplantation with WT bone marrow.

Our data suggest an underlying mechanism for how integrin β3−/− could impair CAC homing and differentiation. First, integrin β3-deficient CACs failed to efficiently adhere to the denuded AVGs and to the subendothelial matrix proteins, vitronectin, and fibronectin (Figure 3). There are no differences in a total number of lineage-negative c-Kit/Sca I double-positive CACs (Figure 4G), suggesting that this loss-of-function phenotype is not caused by changes in the number of circulating CACs. This finding is consistent with a previous observation that the loss function of integrin β3 did not change the numbers of Sca I+ cells.20

The activated platelets could be another factor that involved in CAC homing in AVGs. After vascular insult, platelets first tether and adhere to subendothelium exposed by injury to not only seal the wound but also promote P-selectin-mediated CAC homing as previously demonstrated. We have recently shown that platelets are involved in trapping and recruiting bone marrow–derived fibroblast-specific protein 1-positive cells. In the current study, we further show that platelet aggregation was decreased in AVGs from integrin β3−/− mice, independent of platelet counts, which are normal in β3-null mice.24 The data are supported by previous finding that the integrin β3 partner, αIIb integrin, is required for the recruitment of bone marrow-derived CD34+ and Sca-1+ progenitor cells into injured vessel walls. More evidence showed that
microparticles from platelets promote CAC function and tube formation through integrin \( \beta_3 \).

Second, \( \beta_3 \) deficiency may also result in defective differentiation of CACs at sites of AVGs in a platelet-dependent manner. Human CD34+ CACs cocultured with immobilized platelets form colonies and are capable of differentiating into mature endothelial cells.21,26 This regulatory activity of platelets toward CAC differentiation is likely mediated by platelet-derived growth factors (eg, TGF-\( \beta_1 \), platelet-derived growth factor, vascular endothelial growth factor, epidermal growth factor, and insulin-like growth factor).27,28 Among these factors, the TGF-\( \beta_1 \) suppresses reprogramming of other type of cells into endothelial cells.29 A higher level of TGF-\( \beta_1 \) from integrin \( \beta_3 \)-deficient platelets adversely affected CAC regeneration (Figure 5B). The result is consistent with our recent report that integrin \( \beta_3 \) deficiency increases TGF-\( \beta_1 \) expression in muscle cells, impairing their capacity for muscle regeneration.30

Because integrin regulates outside-in signal through cytoskeleton, and a recent report showed that Wiskott–Aldrich syndrome protein, an actin accessory protein, through CDC42 regulates TGF-\( \beta_1 \) release.31 So the integrin \( \beta_3 \)-induced TGF-\( \beta_1 \) release could be associated with cytoskeleton changes.

We further show that the TGF-\( \beta_1 \)-induced activation of Notch signaling may play a critical role in accelerated AVG occlusion (Figure 6A–6C). Because Notch signal maintains multipotential progenitor cells in an undifferentiated32 and slows the differentiation of bone marrow-derived CAC.33,34 Increased Notch signaling in endothelial cells upregulates mesenchymal marker expression leading to endothelial barrier dysfunction in arteriovenous fistula.35 TGF-\( \beta_1 \) is significantly increased in \( \beta_3 \)-null platelets and could result in enhanced activation of Notch signaling through induction of Notch ligand Jagged 1 (Figure 6D). This finding was supported by an early report that endothelial repair is accelerated

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**Figure 5.** Integrin \( \beta_3^{−/−} \) affects circulating angiogenic cell (CAC) homing and differentiation. **A**, Detection of CD41+ platelets and Sca I+ CACs in arteriovenous grafts (AVGs) in wild-type (WT) and integrin \( \beta_3 \) knockout mice. **B**, Statistical analysis of ScaI+ cell accumulation in AVGs. n=4 mice from both WT and integrin \( \beta_3 \) knockout mice. **C**, Platelets isolated from WT and integrin \( \beta_3 \) KO mice were cocultured with CACs for 7 days. Endothelial markers (real-time reverse transcription–polymerase chain reaction [RT-PCR]) revealed that platelets promote CAC differentiation. This was more prominent with WT platelets. **D**, Integrin \( \beta_3 \)-null platelets express excess transforming growth factor (TGF)-\( \beta_1 \) detected by Western blot. **E**, Washed platelets isolated from WT and integrin \( \beta_3 \) knockout mice were stimulated with 0.125 U/mL thrombin or 5 \( \mu \)g/mL collagen at 37°C for 5 minutes and centrifuged at 14,000 g for 20 minutes. The TGF-\( \beta_1 \) level in the platelets and releasates was measured by ELISA after acidification (n=3). **F**, In vivo, prominent TGF-\( \beta_1 \) expression in AVGs from integrin \( \beta_3 \) knockout mice colocalized with platelets. **G**, CACs isolated from bone marrow and cultured in EGM2 with or without incubation with TGF-\( \beta_1 \) (3 ng/mL) for 7 days. Excess TGF-\( \beta_1 \) inhibits mature endothelial markers were detected by real-time RT-PCR. Representative data are present from 3 independent experiments. *P<0.05 compared with WT. DAPI indicates 4',6-diamidino-2-phenylindole; EPC, XXX; and VE, XXX.
after arterial injury in B6 mice transplanted with the bone marrow cells from Notch 1 knockdown mice. We also found that platelet-mediated CAC differentiation was suppressed by the excess presence of TGF-β1, leading to delayed endothelium regeneration at sites of AVGs (Figure 5D).

Finally, the influence of integrin β3 was demonstrated by the drastic reduction in the rate of AVG failure caused by the transplantation of WT bone marrow cells into integrin β3−/− mice (Figure 7). Our results differ from those found in models of arterial injury, where the inhibition of integrin β3 reduces or blocks the neointima formation of vascular smooth muscle cells. We found that deficiency of integrin β3 delayed CAC regeneration, leading to thrombosis and adversely affecting AVG function. Our results suggest that antiplatelet therapy in human should be carefully considerate. For AVG, the αIIβ3-based antiplatelet therapy could worsen the thrombosis because of the delayed endothelium repairmen, like the case in current animal model; in this case P2Y12-based antiplatelet therapy may be evaluated and considered. For example, a report showed that a delayed antiplatelet therapy with clopidogrel and everolimus prevents progression of transplant atherosclerosis in murine aortic allografts.
It is known that in addition to a negative role of TGF-β signaling in CAC differentiation, other factors also regulate CAC adhesion and migration through β3-integrin. Puri et al. found that proangiogenic factor thyminidine phosphorylase–induced CAC migration can be blocked by RGD peptides and inhibitory antibodies to integrin αVβ3. Without integrin β3 also impairs its interaction with VEGFR2 and hypoxia-induced angiogenesis. These results indicate that multiple factors could be involved in regulating integrin β3-impaired CAC function or differentiation.

In summary, we have uncovered a novel mechanism for causing AVG occlusion and failure. In addition to poor adhesion of β3-null endothelial progenitor cells to the subendothelium, β3 deficiency in platelet contributes to AVG occlusion (1) by defective as a carrier for CAC homing and (2) activating Notch signaling to suppress endothelial cell differentiation. Our results, therefore, suggest that strategies that block notch signaling or reduce TGF-β1 could improve the patency of an AVG.

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Disclosures
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References
3. Nachman RL, Rafii S. Platelets, petechiae, and preserv.
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Significance

Neointima formation is associated with stenosis and subsequent thrombosis in arteriovenous grafts (AVGs). Integrin β3 has been reported to regulate neointima formation in artery injury model. In a mouse model of AVG, we found that deficiency of integrin β3 suppresses homing and differentiation of endothelial progenitor cells into mature endothelial cells in AVGs, and delays endothelial regeneration. These responses result in thrombosis and failure of AVG. The increased transforming growth factor-β1 in platelets and Notch 1 signaling are the underlying molecular mechanisms that cause accelerated failure of the grafts in integrin β3-deficient mice. Wild-type bone marrow transplantation can rescue the AVG patency in integrin β3-deficient mice. Our results suggest that boosting integrin β3 function in the endothelial cells and platelets could prevent neointima and thrombosis in AVGs.

References


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Materials and Methods

Reagents: Penicillin, streptomycin, DMEM, and FBS were obtained from Invitrogen Life Technologies (Carlsbad, CA). A protein assay kit was purchased from Bio-Rad (Hercules, CA). Integrin β3 antibodies were from Abcam (Cambridge, MA); antibodies against SMA-α-FITC, recombinant collagen I, vitronectin, and fibronectin were from Sigma-Aldrich (Louis, MO); anti-integrin β3 and N1ICD (Cleaved Notch1) antibodies were from Cell Signaling (Danvers, MA); Fluorescence-700 or -800 secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Anti-CD42b, anti-CD41a, Gr-1-PE-Cy7, CD11b-APC, mouse hematopoietic lineage eFluor 450, and CD45-PerCP-Cy5.5 antibodies were purchased from eBioscience (San Diego, CA). The CD31 antibody, Sca I-FITC, CD41-FITC, C-Kit-APC, and CD144-APC were purchased from BD Pharmingen (San Jose, CA). Antibodies against Notch1, Jagged 1, and Hes1, Hes5, and αIIb integrin were from Santa Cruz Biotechnology (Santa Cruz, CA). An RBP-Jκ antibody was obtained from Millipore Corp (Billerica, MT); the adenovirus of soluble Jagged 1 was obtained from Dr. Post (Maastricht University, Netherlands). The recombinant TGF-β1, and γ-secretase inhibitor (DAPT) were purchased from R & D (Minneapolis, MN).

Mouse model of Venous Graft: All animal protocols were approved by IACUC of Baylor College of Medicine. Wild type C57/B6 and integrin β3−/− mice were purchased from Jackson Laboratory (Bar Harbor, Maine). AVG was performed as previously described. In brief, the right common carotid artery of a male mouse was surgically exposed to place a cuff on both ends of the artery. The ends were then everted over the cuff and ligated with an 8.0 silk suture. Vena cava from donor mice was grafted by “sleeving” ends of the vein over the artery cuff and secured with 8.0 silk sutures. After 4 weeks, the AVG was dissected and vessel wall thickness measured as the area of the vessel minus that of the lumen using NIS-Elements BR 3.0 program.

CAC isolation and characterization by flow cytometry. Bone marrow cells were isolated from wild type or integrin β3−/− mice. Sca I/c-Kit double-positive CACs and Sca I/c-Kit double-positive/lineage negative CACs, which are capable of differentiating into endothelial cells, were purified by fluorescence-activated cell sorter with a purity of >90%. The enriched CACs were cultured on fibronectin-coated dishes in the EGM-2 medium supplemented with EGM SingleQuots (Cambrex, Inc., East Rutherford, NJ) for 1 week. Attached CACs were rinsed with a Hank's buffered saline solution, fixed with 4% paraformaldehyde for 10 min at 37°C, and stained for VE-cadherin or PECAM. These cells were visualized under a fluorescence microscope using FITC or rhodamine excitation/emission filters. The Di-LDL uptake was performed as described to sure that cultured cells were enrich in functional CACs.

Blood collection and platelet preparation. Blood was collected from wild type and integrin β3−/− mice via interior vena cava and processed for platelets as described. Briefly, platelet-rich plasma was obtained by centrifuging whole blood (anti-coagulated with 0.38% of sodium citrate) at 150 x g for 15 min at 24 °C. To obtain washed platelets, platelet-rich plasma was obtained from whole blood using acid-citrate dextrose as the anticoagulant (Cytosol Laboratories Inc., Braintree, MA) and centrifuged at 900 x g for
10 min. Platelet pellets were washed once with a CGS buffer (13 mM sodium citrate, 30 mM glucose and 120 mM sodium chloride, pH 7.0), supplemented with 1 µM PGE1 (Santa Cruz), and centrifuged at 900 x g for 10 min. The washed platelets were re-suspended in Ca²⁺, Mg²⁺ Tyrode's buffer.

**Platelet TGF-β1 detection:** The immunologic enzyme-linked immunosorbent assay (ELISA) used an immobilized mAb specific for the active form of TGF-β1 (R & D systems). Active TGF-β1 was measured directly in untreated platelet, whereas total TGF-β1 (active + latent) was measured after pretreating the samples with 0.2 volume of 1N HCl for 20 min at room temperature to convert latent TGF-β1 to active TGF-β1. The platelets were stimulated with thrombin (0.125 U/ml) (Sigma Aldrich) or fibrillar type I collagen (5 µg/ml) (Sigma Aldrich) for 5 min at 37°C. Platelet-free platelet releasates were prepared by centrifuging at 14 000 g for 20 min at 4°C and the total TGF-β1 was determined.

**Co-culture of platelets on CAC differentiation.** Multiple-well glass slides were coated with 2 µg of fibrillar Type I collagen for 3 hrs at room temperature. Platelets isolated from wild type and integrin β3⁻/⁻ mice were seeded onto collagen-coated coverslips. Isolated CACs were plated on top of the platelets and grown in EGM2 medium. CAC colonies were counted 7 days after plating. To detect CAC differentiation, randomly selected colonies were stained for the endothelial cell markers CD31 and VE-cadherin. CACs were also collected for real time RT-PCR.

**Immunohistochemistry:** AVgs were perfused through the left ventricle as described¹¹. A graft was obtained by cutting the transplanted segment from the native vessel at the cuff end, fixed in 4% phosphate-buffered formaldehyde at 4 °C for 24 h, and processed for 5 µm sections as described.¹ Sections were blocked with 10% goat serum (Vector Laboratories, Burlingame, CA) for 30 min and then incubated with primary antibodies. They were then washed in 0.5% Tween 20 in PBS (PBST) and incubated with a biotinylated secondary antibody (Vector lab) at room temperature for 2 hrs. After washing with PBST, these sections were incubated with an Elite® ABC reagent (Vector Laboratories) according to the manufacturer’s protocol and counterstained with hematoxylin. For double immunofluorescent staining of samples, fluorescent secondary antibodies were applied to sections; DAPI was used as a counter stain. Pictures were recorded using a Nikon Eclipse 80i fluorescence microscope (Melville, NY).

**Real-time RT-PCR:** Total RNA from freshly removed vena cava and AVGs was isolated using an RNeasy kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed using an Opticon real-time RT-PCR instrument (MJ Research, Waltham, MA). The specificity of this real-time RT-PCR was confirmed by agarose gel electrophoresis and melting-curve analysis with GAPDH as the internal standard. Primers used for the amplification reaction were summarized in Supplemental Table 1.

**Western Blot Analysis:** Cells or AVgs were lysed in a RIPA buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and ~30 µg of
proteins were separated by SDS-PAGE. After being transferred to a PVDF membrane, antibodies were used to probe molecules specified in the result section.

**Bone marrow transplantation and flow cytometry analysis:** The bone marrow (BM) transplant was performed as described. Briefly, BM cells were harvested by flushing femurs and tibias of a donor mouse and infused (5 x 10^6 cells/mouse) into the lateral tail vein of a lethally irradiated (1100 rads) recipient mouse. For flow cytometry analysis, bone marrow and peripheral mononuclear cells from wild type and integrin β3-/- mice were isolated, incubated with antibodies, and analyzed by flow cytometry to identify neutrophils (Gr-1-PECy7), macrophages (CD11b-APC), monocytes (CD45-PerCP-Cy5.5) and stem cells (CD117-APC and Sca I-FITC; hematopoietic lineage eFluor 450).

Statistical analyses. All data are presented as the mean ± standard error of the mean. Comparison between groups was made using one-way ANOVA followed by pairwise comparisons with p value adjustment; P < 0.05 was considered to be statistically significant.

References:

Supplement Material

Supplemental figure I. A hypothetical model to explain the role of integrin β3 in endothelial regeneration in AVGs. Platelet aggregation in AVGs after surgery was decreased in integrin β3 mice, and less CACs were recruited in the denuded subendothelial matrices in integrin β3 mice. The higher level of TGF-β1 secreted from integrin β3 platelets delayed the CAC differentiation into mature endothelial cells, resulting in AVG failure.
Normal Vein

Arteriovenous Graft (1-3 days)
EC denudation and platelets deposition

Arteriovenous Grafts (5-10 days)
CAC homing and regeneration
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