Defective blood vessel development and pericyte/pvSMC distribution in \( \alpha 4 \) integrin-deficient mouse embryos

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Abstract

Blood vessel development is in part regulated by pericytes/presumptive vascular smooth muscle cells (PC/pvSMCs). Here, we demonstrate that interactions between PC/pvSMCs and extracellular matrix play a critical role in this event. We show that the cranial vessels in \( \alpha 4 \) integrin-deficient mouse embryos at the stage of vessel remodeling are increased in diameter. This defect is accompanied by a failure of PC/pvSMCs, which normally express \( \alpha 4 \beta 1 \) integrin, to spread uniformly along the vessels. We also find that fibronectin but not VCAM-1 is localized in the cranial vessels at this stage. Furthermore, cultured \( \alpha 4 \) integrin-null PC/pvSMCs plated on fibronectin display a delay in initiating migration, a reduction in migration speed, and a decrease in directional persistence in response to a polarized force of shear flow. These results suggest that specific motile activities of PC/pvSMCs regulated by mechanical signals imposed by the interstitial extracellular matrix may also be required in vivo for the distribution and function of the PC/pvSMCs during blood vessel development.

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Introduction

In a developing embryo, the vascular system is formed by two main events, vasculogenesis and angiogenesis. In vasculogenesis, endothelial precursor cells, angioblasts, form de novo vascular networks (Coffin and Poole, 1988; Pardanaud et al., 1987). During angiogenesis, the primitive vasculature is remodeled through the development of angiogenic vessels from the pre-established tubules, restructuring the existing network to form the more complex vasculature of the adult (Beck and D’Amore, 1997). The walls of mature vessels are composed of two distinct cell types: vascular endothelial cells which form a single cell sheath that lines the luminal surface of the vessel, and perivascular cells, including vascular smooth muscle cells (vSMCs) and pericytes, which encircle the outside of the vascular endothelium (Rhodin, 1968). vSMCs form a multilayered tissue that cover large vessels, whereas solitary pericytes are associated with small capillary vessels (Bergers and Song, 2005). vSMCs and pericytes have complex ontogeny (DeRuiter et al., 1997; Dettman et al., 1998; Hungerford et al., 1996; Le Lievre and Le Douarin, 1975) and it has been controversial as to whether or not they share the same progenitors in the developing vasculature (Bergers and Song, 2005). For this reason, in this article, the perivascular progenitors are named ‘pericytes/presumptive vSMCs (PC/pvSMCs)’. During vessel remodeling, PC/pvSMCs become associated with endothelial vessels and play a critical role in vessel maturation (Gerhardt and Betsholtz, 2003; Hungerford and Little, 1999).

The importance of PC/pvSMCs in blood vessel development has been demonstrated by studies on platelet-derived growth factor receptor-\( \beta \) (PDGFR-\( \beta \)) and its ligand PDGF-B. Knockout studies show that paracrine interactions between PDGFR-\( \beta \) on PC/pvSMCs and PDGF-B secreted by sprouting endothelial vessels play an essential role in the recruitment of PC/pvSMCs to the sprouting vessels (Hellstrom et al., 1999; Lindahl et al., 1997). In PDGFR-\( \beta \)- and PDGF-B-deficient mouse embryos, there is a severe PC/pvSMC deficit that leads to hemorrhage and perinatal lethality. In these mutants, angiogenic sprouting
proceeds relatively normally in the absence of PC/pvSMCs, but the diameter of PC/pvSMC-deficient vessels is abnormal and heterogeneous with increased or decreased vessel width, suggesting that one of the major functions of PC/pvSMCs is to shape the developing vessels (Hellstrom et al., 2001). It is hypothesized that PC/pvSMCs may regulate vessel diameter through their contractile activities; alternatively, PC/pvSMCs may secrete VEGF, angiopoietin and other factors that control the proliferation and migration of underlying endothelial cells (Reynolds et al., 2000; Uemura et al., 2002).

While there is an extensive literature on the role of paracrine signaling in angiogenesis, little is known about the role of cell contacts and extracellular matrix (ECM) in this process. Electron microscopic studies show that the vascular endothelium and PC/pvSMCs share a fibronectin-rich ECM interstitia, which is likely to play key regulatory roles in angiogenesis during development (Courtoy and Bohles, 1983). Fibronectin and other ECM proteins transduce signals into cells through integrins. One of the fibronectin receptor integrins, α4β1, has been implicated in PC/pvSMC function by its localization in vSMCs of the developing vasculature (Sheppard et al., 1994). α4β1 integrin binds to an alternatively spliced V25 (also called CS-1) region of fibronectin (Guan and Hynes, 1990; Wayner et al., 1989). Another major ligand for α4β1 is vascular adhesion molecule-1 (VCAM-1) (Osborn et al., 1998). The α4 subunit of this integrin also associates with β3; α4β1 integrin is a leukocyte-specific integrin (Hynes, 2002) that mediates leukocyte trafficking by binding to VCAM-1 and MadCAM-1 (Berlin et al., 1993; Ruegg et al., 1992). α4β1, on the other hand, is expressed in many cell types and has important functions during embryonic development. In addition to vSMCs, α4β1 is expressed in neural crest cells, hematopoietic cells and epicardial progenitor cells, and has been shown by knockdown and functional perturbation studies to play critical roles in the migration of these cells during development (Kil et al., 1998; Konstantopoulos et al., 1998; Pinco et al., 2001; Sengbusch et al., 2002; Yednock et al., 1992). Besides migratory cells, α4β1 is expressed in the chorion and the mature epicardium, where its binding to VCAM-1 is required for heterophilic cell–cell adhesion to form placental connections and maintain the epicardium of the heart, respectively (Gurtner et al., 1995; Kwee et al., 1995; Yang et al., 1995). α4β1 is also expressed in the peripheral nervous system and is involved in innervation of sympathetic neurons into the heart (Wingerd et al., 2004). In addition to the in vivo studies, a critical role of α4β1 in cell migration has also been demonstrated using tissue culture cells (Goldfinger et al., 2003; Kassner and Hemler, 1993; Nishiyama et al., 2005; Pinco et al., 2002).

In this paper, we explore novel functions of α4β1 in murine blood vessel development, using our previously described α4KI-lacZ mouse model (Sengbusch et al., 2002). By immunohistochemical and confocal microscopic studies on α4 integrin-deficient embryos and analyses of primary PC/pvSMCs isolated from these embryos, we provide evidence for an important role of α4β1 in regulating PC/pvSMC motility that might be critical for the distribution and function of PC/pvSMCs during blood vessels development.

### Materials and methods

#### Reagents, antibodies and mouse lines

Rat plasma fibronectin was purchased from Calbiochem. The sources of antibodies were: anti-NG2 and desmin from Chemicon, anti-BrdU from Sigma; anti-VCAM-1 (MK-2) from ATCC; anti-β-galactosidase from Eppendorf 5′-3′; anti-α4 (R1–2) and PECAM (MEC 13.3) from BD Pharmining. Anti-fibronectin antibody was a gift from Richard Hynes, MIT. The α4KO and α4KI-lacZ mouse lines have been reported elsewhere (Sengbusch et al., 2002; Yang et al., 1995).

### X-gal and antibody staining of mouse embryos

Whole mount X-gal staining was performed as described (Hogan et al., 1994). The X-gal-stained embryos were post-fixed in 4% paraformaldehyde in PBS overnight at 4°C, sectioned in paraffin and counterstained with eosin. Immunohistochemical staining for VCAM-1 and fibronectin was performed as described by Sengbusch et al. (2002). To stain whole mount embryos for PECAM, E11.5 embryos were fixed in 4% paraformaldehyde in PBS at 4°C for 5 h and washed twice with PBS. The subsequent steps were performed at room temperature. The embryos were dehydrated through a methanol series, quenched for 4 h with 1% H2O2 in methanol and DMSO mixed 4:1 (can be stored at −20°C), washed in TNT (150 mM NaCl, 10 mM Tris HCl, pH 7.8 and 0.1% triton X-100), incubated with the anti-PECAM antibody diluted 1: 500 in TNTM (5% non-fat dry milk, 2% normal goat serum in TNT) supplemented with 5% DMSO and 0.1% Na azide. After 48 h, the embryos were washed 6×, 1 h each in TNT, followed by incubation with an HRP-conjugated anti-rat antibody diluted in TNTM, for 36 h. The embryos were then washed in TBS 3× and incubated in TBS overnight, followed by incubation with 0.03% diaminobenzidine (Sigma) and 0.005% H2O2 in TBS to visualize the staining. Whole mount dual-color immunofluorescence for NG2 and PECAM was performed on whole mount embryos using the above procedures with the following modifications. The dehydrated embryos from −20°C were washed 4× in PBS, blocked with 0.5% Triton-X 100, 1% BSA in PBS overnight. The same blocking buffer was used to dilute the antibodies. All washes were performed with PBS. After washing off the secondary antibody, the head of each embryo was sliced sagittally through the midline and mounted for confocal microscopy. The stained whole mount embryos or embryo sections were photographed using Axioskop or Zeiss LSM 510 Meta microscopes (Carl Zeiss). The confocal images were processed and analyzed using ImageJ (NIH) and Velocity (Improvision) softwares.

#### Primary embryonic PC/pvSMC culture

Embryos were harvested and washed in PBS. For each embryo, the tail was removed and used for genotyping as described (Sengbusch et al., 2002), and the remaining part was treated with 1% Type I collagenase (Invitrogen) in PBS at 37°C for 1 h. The embryonic tissue was then passed through a 5-ml syringe with 20.5-gauge needle along with 1 ml of F-12 medium. Cells were plated on 10-cm tissue culture plates pre-coated with 0.1% gelatin (Sigma) and 5 μg/ml fibronectin and cultured in medium containing 40% DMEM low glucose, 40% F-12, 20% fetal bovine serum and antibiotics (DMEM-F-12 medium). After 3 days in culture, PC/pvSMCs were isolated from the embryonic cell culture by flow cytometric sorting using an anti-NG2 antibody and fluorescein di B-D-galactopyranosidase (FDG).

#### Flow cytometric sorting

To isolate PC/pvSMCs, the embryonic cells in culture with the same genotype were pooled. At least 1.2 × 10⁸ cells were stained with anti-NG2 using a standard staining protocol as described (Pinco et al., 2002), followed by FDG loading using a FlouReporter lacZ Flow Cytometry Kit (Molecular Probes). Flow cytometric sorting was then performed to collect NG2/FDG positive cells into 96-well plates pre-coated with 0.1% gelatin and 5 μg/ml fibronectin. PC/pvSMC culture was maintained in the DMEM-F-12 medium.
Transwell and BrdU incorporation assays

Transwell assays were performed as described elsewhere (Pinco et al., 2002). The assay was performed simultaneously for α4-positive and α4-null cells, each in triplicates. The number of cells that migrated through the pores was counted in each of 10 independent 10× microscopic fields from each membrane and mean and standard deviations were calculated.

BrdU incorporation assays were performed by treating the cells with 50 μM 5-bromo-2-deoxyuridine (BrdU) for 30 min at 37°C. The cells were washed in PBS, fixed in methanol and stained with an anti-BrdU antibody (Sigma-Aldrich). The percentage of cells with BrdU incorporation was counted and mean and standard deviation were calculated.

Shear flow migration assay

35 mm culture dishes were coated with 10 μg/ml fibronectin for 1 h at 37°C prior to plating 5 × 10³ cells at the center of the dish. After allowing the cells to adhere for 30 min at 37°C, the dish was assembled to the flow chamber and mounted onto the stage of an inverted microscope (Nikon TE300) equipped with phase contrast optics, and enclosed in a 37°C incubator (McCarty et al., 2000). Cells were subjected to a shear stress of 4 dyn/cm² by continuously perfusing Leibovitz’s L-15 CO₂-buffered medium (Invitrogen) supplemented with 10% FBS through the chamber for 10 h. Digital images of a single 10× field of view (1.16 mm²) were acquired every minute. For each experiment, the time-lapse images were compiled into a 20-s avi movie at 30 fps using a cinepak codec for compression. Antechinus Animator Professional (C Point) was used to compress the images into video format.

Detachment assay

After having allowed 10⁴ cells to adhere on a fibronectin-coated substrate (10 μg/ml) for 30 min at 37°C under static conditions, cells were subjected to a shear stress of 4 dyn/cm² for 10 min followed by an increase of the shear stress to 8 dyn/cm². Thereafter, the wall shear stress was increased by 8 dyn/cm² on 1-min intervals up to 96 dyn/cm². At the end of each shear stress increment, the percentage of cells that remained adherent on the fibronectin-coated substrate was determined.

Analysis of cell tracking data

The centroid position (x, y) of each cell migrating under shear was calculated at each timestep (τ = 10 min) for the entire duration of the experiment (up to 10 h) using ImageJ software (NIH). These coordinate data were used to calculate the individual time-averaged mean square displacement (MSD) in the 2-D space using the following equation (Kole et al., 2004; Tseng et al., 2002):

\[ \text{MSD} = \left( (x_{t+τ} - x_t)^2 + (y_{t+τ} - y_t)^2 \right) \]

where \( τ \) is the elapsed time, \( τ \) is the timestep. The distance that the centroid of the cell moved over each timestep in the 2-D space was used to calculate the cell migration speed during this time period. Migration speed data for each cell at all time-points were then compiled to obtain the root mean squared cell migration speed, \( S \), for the entire duration of the shear flow experiment using the following equation:

\[ S = \sqrt{\frac{(x^2 + y^2 + ... + z^2)}{n}} \]

Alternatively, the path-independent average migration speed was calculated using the final and initial position coordinate data divided by the entire duration of cell migration. The values of time-averaged MSD and \( S \) calculated for each cell were then employed to determine the persistence time, \( P \), which is the characteristic time in which cell movement persists in the same direction, using the following equation (Dickinson, 1993; Kole et al., 2004; Tseng et al., 2002):

\[ \text{MSD} = 2S^2P\left[ τ - P(1 - e^{-t/τ}) \right] \]

The value of \( P \) for each cell at each time-point was determined using the Solver function of Excel. Moreover, an average persistence time for each cell as well as an ensemble-average persistence time over the entire duration of cell migration under shear flow (10 h) were calculated.

Results

Blood vessel diameter is defective in α4-null embryos

To study the role of α4 integrin in blood vessel development, we analyzed mouse embryos deficient in the α4 integrin subunit. In these studies, we used two α4 integrin knockout alleles, a conventional knockout allele (Yang et al., 1995) referred to as α4KO and a lacZ knock-in allele (Sengbusch et al., 2002) referred to as α4KI-lacZ. α4KI-lacZ was generated by replacing the α4 integrin gene with a lacZ reporter, placing lacZ under the control of the endogenous α4 integrin promoter. Among the α4 integrin-deficient (α4-null) embryos homozygous or compound heterozygous for these alleles, about 50% had a defect in choorio-allantiosis fusion and died at E11; another 50% did not have this defect, but had defective hearts lacking the epicardium and died at E12—14 due to cardiac hemorrhage. Among the α4-null embryos with the epicardium defect, a majority did not show any apparent morphological defects before E12 (Sengbusch et al., 2002; Yang et al., 1995) when the development of the epicardium was not yet complete; these embryos are the focus of this paper.

We stained the apparently normal α4-null embryos at E9.5, E10.5 and E11.5 by whole-mount using an anti-PECAM antibody, a marker for vascular endothelial cells. For this analysis, we chose α4-null and α4-positive (α4KI-lacZ⁺) embryos of the same size and developmental stage (Figs. 1A–C). At E9.5, both α4-positive and α4-null embryos had formed a homogeneous network of primitive vessels, and there were no apparent vascular defects in the α4-null embryos (Figs. 1D and E). At E10.5 and E11.5, however, α4-null embryos had a vascular defect in the cranial mesenchyme surrounding the developing brain, featured by wider vessel diameter (Figs. 1F–M). Our measurements showed that cranial macrovessels (>25 μm in diameter) of α4-null embryos had a 25–40% increase in diameter compared to those of α4-positive embryos (Fig. 1N).

In the cranial mesenchyme, blood vessels undergo extensive remodeling. Many angiogenic microvessels extend out of the macrovessels and invade the primitive vasculature formed by vasculogenesis. To examine these vessels, E10.5 and E11.5 embryos stained with anti-PECAM antibody were examined respectively by stereo and confocal microscopy. The microvessels (<25 μm in diameter) appeared less homogeneous in some microvessels much wider than those in α4-positive embryos (Figs. 1H, I, L, M). Our measurements showed that the mean diameters of microvessels in the cranial regions of E11.5 α4-null embryos had a 44% increase compared to those in α4-positive embryos (Fig. 1N). This defect appeared to be restricted to cranial angiogenic microvessels. No apparent vessel diameter defect was observed in the caudal regions of α4-null embryos.
α4β1 is expressed in PC/pvSMCs but not in endothelial cells of the developing vasculature

To determine how α4β1 may contribute to blood vessel development, we examined the location of α4β1 integrin expression in vessels by whole-mount X-gal staining of α4KI-lacZ+/+ embryos at E9.5–12.5. We have previously shown that the knocked-in lacZ in α4KI-lacZ/+ embryos is faithfully expressed under the control of the α4 integrin promotor, and the blue lacZ-expressing cells stained by X-gal.

Fig. 2. PC/pvSMCs express α4β1 but does not require α4β1 for their association with the developing vessels. α4-positive (A–C, E, I, K, L) and α4-null (D, F, H, J) embryos at E9.5 (A, C, D), E10.5 (B, E, F), E11.5 (G, H) and E12.5 (I–L) were stained with X-gal (A–J), or immuno-stained with anti-β-galactosidase (in green) and PECAM (in red) (K) or anti-α4 integrin (in red) and NG2 (in green) (L). Panels A–F show regions of dorsal aorta, panels G and H show angiogenic microvessels indicated by asterisks in the vessel lumens. Note in panels A–F, the blue α4KI-lacZ-expressing cells (arrows) are clearly PC/pvSMCs that sit on the outer surface of the endothelial lining (pink, arrowheads); the number of blue α4KI-lacZ-expressing cells (arrows) increases in number at E10.5 in panel B in comparison to those at E9.5 in panel A; the number of blue α4KI-lacZ-expressing cells (arrows) increases in number at E10.5 in panel B in comparison to those at E9.5 in panel A; and became multiple-layered at E12.5 in panels I and J; the expression of α4 integrin or α4KI-lacZ overlaps with NG2 but not with PECAM. Also note that the PC/pvSMCs are present in both the major vessels and angiogenic microvessels in α4-null embryos. Scale bars, 0.1 mm.

Fig. 1. Cranial vascular defect in α4-null embryos. α4-positive and α4-null embryos at identical developmental stages (A–C) were compared for PECAM staining patterns. Cranial regions (boxed in panels A–C) of α4-positive (D, F, H, J, L) and α4-null (E, G, I, K, M) embryos at E9.5 (A, D and E), E10.5 (B, F–I) and E11.5 (C, J–M) are presented. Note that a majority of macrovessels (F, G, J, K) and microvessels (H, I, L, M) in the cranial mesenchyme of α4-null embryos at E10.5 and E11.5 were wider in diameter than those of α4-positive embryos. For quantification, the diameters of macrovessels were measured from the most prominent cranial vessel at two specific regions (marked as I and II in panels F, G, J, K), and those of microvessels were measured from distal cranial vessel branches (marked as III in panels J and K) where at least 11 measurements were taken and the mean calculated. The histograms in panel N represent the average diameters of macrovessels at regions I and II in E10.5 and E11.5 embryos (n = 9, P < 0.05), and the average mean diameter of microvessels at region III in E11.5 embryos (n = 8, P < 0.0001). Scale bars, panels A–C, 1 mm, panels D–K, 0.2 mm.
also express α4 integrin (Sengbusch et al., 2002). The X-gal-stained α4KI-lacZ/+ embryos were sectioned in paraffin and counterstained with eosin. We found that the endothelial cells lining the vessel lumen were pink and the PC/pvSMCs that sit on the outer surface of the endothelial lining were blue (Figs. 2A–C, E). Thus, α4 integrin was expressed in the PC/pvSMCs, but not in the endothelial cells. The α4 integrin-expressing PC/pvSMCs began to appear at the dorsal aorta and other major vessels at E9.5 (Figs. 2A and C) and increased in number in the major vessels at E10.5 (Fig. 2B). At E10.5 and E11.5, the α4 integrin-expressing-PC/pvSMCs were also found at angiogenic microvessels (Fig. 2G). As the vessels matured, PC/pvSMCs began to mature into a multilayered vSMC tissue that became prominent in vessels along angiogenic vessels. In these vessels, α4 integrin was expressed in the PC/pvSMCs, but not in the endothelial cells. The α4 integrin-expressing-PC/pvSMCs began to appear at the dorsal aorta and other major vessels at E9.5 (Figs. 2A and C) and increased in number in the major vessels at E10.5 (Fig. 2B). At E10.5 and E11.5, the α4 integrin-expressing-PC/pvSMCs were also found at angiogenic microvessels (Fig. 2G). As the vessels matured, PC/pvSMCs began to mature into a multilayered vSMC tissue that became prominent in vessels at E12.5 (Fig. 2I). In these vessels, α4KI-lacZ-encoded β-galactosidase and PECAM had non-overlapping localization (Fig. 2K), whereas α4 integrin and NG2, a marker for PC/pvSMCs (Ozerdem et al., 2001), colocalized (Fig. 2L), confirming that α4 integrin was expressed in PC/pvSMCs but not in endothelial cells.

Distribution of PC/pvSMCs in the cranial vasculature is compromised in α4-null embryos

To determine if α4β1 contributes to the association of PC/pvSMCs to vessels, we compared the X-gal staining pattern of α4KI-lacZ/α4KO (α4-null) embryos (Figs. 2D, F, H and J) with the α4KI-lacZ/+ (α4-positive) embryos (Figs. 2C, E, G and I). In the absence of α4β1, PC/pvSMCs were present at vessels as either a single cell layer (Figs. 2D, F, H) or multiple cell layers (Fig. 2I), with no apparent difference compared to those in α4-positive embryos. This result indicates that PC/pvSMCs are associated with the blood vessels in the absence of α4β1.

For more detailed analysis of the PC/pvSMCs on cranial microvessels, dual-color laser-scanning confocal microscopy was performed by staining E11.5 embryos whole-mount for NG2 and PECAM. The NG2-positive PC/pvSMCs of α4-positive embryos were evenly distributed along the length of the vessel (Figs. 3A, C, E). In contrast, those of α4-null embryo were clustered, mostly at branch points (Figs. 3B, D, F), and there was on average a 70% reduction in PC/pvSMC coverage compared to those in α4-positive embryos. When taking into consideration their relative difference in vessel diameter, the PC/pvSMC coverage in α4-null embryos was still significantly less (56% reduction) than that in α4-positive embryos (Fig. 3G).

Fibronectin but not VCAM-1 is expressed in the developing vasculature

Our analysis suggests that the vascular defect in α4-null embryos is likely due to a failure of PC/pvSMCs to migrate along angiogenic vessels. α4β1 is known to promote cell migration by binding to fibronectin, an ECM ligand, or VCAM-1, a counter-receptor; both are expressed in early embryonic stages. To determine if fibronectin and VCAM-1 are involved, we examined the expression of these two proteins in the vessels of E10.5 embryos by immunohistochemical analysis. It has been shown previously that VCAM-1 is expressed specifically in the myocardium of the heart but not in the mesenchyme at early embryonic stages (Gurtner et al., 1995). Consistent with these observations, our results showed that there was no apparent expression of VCAM-1 in the cranial vessels that expressed PECAM (Figs. 4A–D). On the other hand, fibronectin was strongly expressed in both cranial macro-
and microvessels (Figs. 4E, F), suggesting that fibronectin was the ligand of α4β1 in the cranial angiogenic vessels.

Primary α4-null PC/pvSMCs exhibit defects in their motile activities under polarized mechanical stimuli

To test the hypothesis that α4-null PC/pvSMCs have defects in their motile activities, we examined the migratory and adhesive properties of primary α4-null and α4-positive PC/pvSMCs isolated from E11.5 embryos. The PC/pvSMCs were isolated by dual-color flow cytometry and sorting using an antibody against NG2, and a fluorogenic β-galactosidase substrate, FDG, that marks the cells expressing α4KI-lacZ. This dual-color sorting strategy is based on previous studies showing that the only NG2-expressing cell type besides PC/pvSMCs in E11.5 embryos, the glial progenitors of the CNS (Levine and Card, 1987), does not express α4 integrin (Sheppard et al., 1994). As shown in Fig. 5A, the α4-positive (α4KI-lacZ/+) and α4-null (α4KI-lacZ/α4KO) pools had equivalent populations of positively double-labeled cells. In addition, both populations expressed desmin (Figs. 5B, C), another PC/pvSMC marker (Chan-Ling et al., 2004). The two populations also had the same proliferation rate as assessed by a BrdU incorporation assay (Fig. 5E) and the same expression levels of α5 and αV integrins (unpublished data).

The migratory activities of α4-positive and α4-null PC/pvSMCs were then compared in a transwell assay. Our analysis showed that the α4-null PC/pvSMCs migrated at a slower rate toward a fibronectin gradient than the α4-positive PC/pvSMCs (Fig. 5F). We hypothesize that fibronectin in the ECM interstitia may regulate PC/pvSMC distribution along the vessels by sending mechanical signals to the cells via α4β1 integrin. To test if α4β1 is required for cells to respond to mechanical signals, we compared migration of α4-positive and α4-null PC/pvSMCs on fibronectin under conditions of directional shear flow established by a parallel plate flow chamber system. This assay allowed quantitative assessment of the motile activities of a large number of cells that are spontaneously stimulated by a polarized mechanical force. PC/pvSMCs plated on fibronectin-coated coverslips were subjected to a shear flow of 4 dyn/cm², a force equivalent to the hemodynamic shear stress of blood.

Fig. 4. Fibronectin but not VCAM-1 was localized in blood vessels of cranial mesenchyme. Sections of wild-type embryos at E11.5 were stained for VCAM-1 (A and B), PECAM (C–F, in red in E and F) and fibronectin (E and F, in green). The section in panels C and D stained for PECAM was adjacent to that in panels A and B stained for VCAM-1 and was used to visualize the vessels. Panels B, D and F are a higher magnification of panels A, C and E, respectively. The enlarged area is marked with an asterisk (*) in panels A–D. The arrowheads in panels B and D point to blood vessels. Note that fibronectin but not VCAM-1 is localized in the blood vessels. A, atrium; V, ventricle. Scale bars, 0.1 mm.
vessels. Under this experimental setting, the cells were imaged
by time-lapse microscopy at 1-min intervals over a period of
10 h (Fig. 6A, also see movies in Supplemental Materials). In
the absence of shear flow, both α4-positive and α4-null PC/
pvSMCs protruded membrane extensions in random directions
with little locomotion. Under shear flow, both cell types
migrated in the direction of the flow. However, our data analysis
revealed that α4-null PC/pvSMCs responded to the shear flow
forces more slowly, requiring 94.4 ± 10.9 min before the
initiation of migration as opposed to 45.8 ± 4.7 min for the α4-

Fig. 5. Isolation and characterization of primary PC/pvSMCs. (A) Flow cytometry analysis showing the populations of α4-positive (right panel) and α4-null (left
panel) PC/pvSMCs respectively from α4KI-lacZ/α4KO and α4KI-lacZ/α4KO embryonic cultures by double-staining the cells with an antibody against NG2, and FDG, a
fluorogenic β-galactosidase substrate. Note that the percentages of double-positive cells from α4-positive and α4-null embryonic cultures are similar. (B–D) The
isolated α4-positive (B) and α4-null (C) primary cells were examined for expression of desmin, a PC/pvSMC marker. Primary mouse embryonic fibroblasts were used
as a negative control (D). Note that the staining of desmin in the primary PC/pvSMCs is much stronger than that in fibroblasts. Scale bar, 10 μm. (E) The primary PC/
pvSMCs were compared for cell proliferation by a BrdU assay. Note that there is no significant difference between the α4-positive and α4-null cells. (F) The primary
PC/pvSMCs were compared for cell migration on a fibronectin gradient by a transwell assay. Note that the migration of α4-null cells is significantly reduced compared
to α4-positive cells.
Fig. 6. Analyses of primary PC/pvSMCs migrating on fibronectin under shear flow. (A) Primary α4-positive (left panels) and α4-null (right panels) PC/pvSMCs migrating on fibronectin under shear flow were imaged at 1-min intervals for 10 h by time-lapse microscopy. For each cell type, five frames at designated time-points from a typical movie sequence are presented. In each movie sequence, four typical cells are highlighted. The position of the nucleus of each highlighted cell is marked and plotted to show the migration path (bottom panels). The arrows in the bottom panels point to the direction of the shear flow. Scale bar, 100 μm. (B) The time it takes for the PC/pvSMCs to initiate migration in response to shear flow. Note that it takes longer for the α4-null cells to initiate migration compared to α4-positive cells (n = 36, P < 0.0001). (C) Cell migration speed. The root mean squared (RMS) speed defined as the square root of the averaged square of cell speeds at each time-step and the path-independent (INDP) average speed calculated from the initial and final coordinate position of the cell were recorded for both the α4-positive and α4-null cells migrating over fibronectin under shear flow for up to 10 h. α4-positive (n = 36) and α4-null (n = 36) cells were tracked from two and three independent experiments, respectively. Note that with both calculations the migration speed of α4-null cells is significantly reduced (P < 0.01 and 0.00001, respectively). (D) Ratio of RMS speed to path-INDP average migration speed. The larger ratio for the α4-null cells versus the α4-positive cells (P = 0.033) indicates that the α4-null cells follow a more tortuous path in their migration than the α4-positive cells. (E) Ensemble-average cell persistence time. The ensemble-average cell persistence time indicates that the α4-positive cells have a more directionally persistent motion than the α4-null cells (n = 36, P < 0.001). (F) Detachment assay. There is no significant difference between α4-positive and α4-null cells in their ability to detach from fibronectin-coated surface under shear flow (P > 0.5).
positive cells (Fig. 6B). In addition, α4-null cells migrated at a substantially slower speed than the α4-positive cells (Fig. 6C). The slower migration speed of α4-null cells was revealed by two distinct calculations: (a) by assessing the root mean squared cell migration speed, $S$, defined as the square root of the averaged square of cell speeds at each time-step, $\tau$ (10 min), and (b) the path-independent average speed, $\bar{U}$, calculated using the initial and final coordinate position of the cell. The first calculation reflects the migration speed of each cell along the migration path, whereas the second calculation reflects the speed of the displacement of the cell from the start point to the end point. It is noteworthy that although these calculations yielded qualitatively similar results, the absolute values of $S$ were significantly higher than those of path-independent average speed, $\bar{U}$. This difference reflected the deviation of the actual migration path away from the total displacement between the start and end points of the cells, such that the actual travel distance was longer than the start-to-end displacement. Thus, a larger value of the ratio of $S$ to $\bar{U}$ corresponded to a more tortuous path of cell migration. Our data analysis indicated that this ratio was substantially larger for the α4-null cells than for the α4-positive cells (Fig. 6D), suggesting that the migration of α4-null cells was less directionally persistent than that of α4-positive cells. To assess this more rigorously, we estimated for both cell types the ensemble-average cell persistence time, $P$, which is the characteristic time in which cell movement persists in the same direction. Our quantitative analysis clearly indicated that the α4-null cells migrated with reduced directionality compared to α4-positive cells (Fig. 6E).

Adhesive interactions between cells and ECM are known to play critical roles in cell migration. The absence of α4β1 in the α4-null PC/pvSMCs may confer a change in adhesion that could potentially affect the migratory ability of the cells. To test this possibility, the adhesion strength of PC/pvSMCs on fibronectin under shear flow was determined by assessing the percentage of cells detaching from fibronectin-coated surfaces under increasing shear flow forces. Both α4-positive and α4-null cells began to detach under a shear stress of 96 dyn/cm² with no statistically significant difference in the number of cells detached (Fig. 6F), indicating that the defective motile activities of α4-null cells in a shear flow environment was not due to an alteration in their adhesive strength on fibronectin.

**Discussion**

**α4β1 integrin is required for PC/pvSMC distribution along developing blood vessels**

To investigate the function of α4β1 in blood vessel development, we have focused on the vasculature of cranial mesenchyme where extensive blood vessel remodeling can be readily observed. Our analyses of the cranial vessels in α4-null embryos reveal a defect in vessel diameter. The vessel defect in α4-null embryos is first observed at E10.5, a stage that marks the beginning of active angiogenesis in the cranial mesenchyme. At this stage, the epicardium of the heart begins to form, which is defective in α4-null embryos (Sengbusch et al., 2002). However, it is unlikely that the epicardial defect of the α4-null embryos at this stage affects blood circulation and vessel development. Since normally at this stage the epicardial cells have not yet completely encircled the heart, the epicardium has little impact on cardiac muscle contraction. In addition, the vessel defect in α4-null embryos appears restricted to angiogenic vessels, but not the vessels formed through vasculogenesis; there is no global vessel dilation in α4-null embryos that would be expected if cardiac malformation is the cause. Therefore, the timing and the type of vessels affected in the mutants argue against the possibility that the vessel diameter defect is a secondary effect of cardiac malfunction.

A direct role for α4β1 in blood vessel development is strongly supported by our findings that the PC/pvSMCs in α4-null embryos have a defect in their distribution along angiogenic vessels in vivo and a defect in their motile activities on fibronectin in vitro. In the absence of α4β1, PC/pvSMCs are present at the vessels, but display an uneven distribution along the vessels; the PC/pvSMCs tend to cluster at angiogenic branch points, and their coverage on angiogenic microvessels is reduced. Based on these observations, we propose that the association of PC/pvSMC with the developing vessels involves two steps. In the first step, the PC/pvSMCs become associated with vessel branch points. In the second step, the cells spread out along the angiogenic vessels to form a uniform tissue layer. These two steps involve distinct mechanisms with the latter dependant on interactions between α4β1 and fibronectin. The role of α4β1 in the spreading of PC/pvSMCs along angiogenic vessels is reminiscent of that observed during the formation of the epicardium of the heart. During this event, the pro-epicardial cell aggregates reach the heart. After their attachment to the myocardium, the pro-epicardial cells migrate out from the aggregates and spread to form the epicardium, a process also defective in α4-null embryos (Sengbusch et al., 2002). In contrast to angiogenic vessels, no apparent distribution defect of PC/pvSMCs is observed in the more mature vessels in which multiple vSMC layers appear to form normally (Fig. 2J). One explanation for this could be that α4β1 is responsible for the initial spreading along the angiogenic vessels but, as the vessels mature, a complementary mechanism takes over and allows the PC/pvSMCs to spread over the more mature vessel. Alternatively, the α4-null PC/pvSMCs might be capable of spreading over the vessels, but there is a time lag for this event due to a delayed response to migratory cues and slowed migration speed.

It is not clear if there is a causal relationship between reduced PC/pvSMC coverage and vessel dilation in the α4-null embryos. Since some vessels in α4-null embryos that have the diameter defect do have PC/pvSMCs present, these two defects may not be related. α4β1 may have two independent functions during vessel development, one function is to promote PC/pvSMC spreading along vessels, and the other function is to regulate PC/pvSMC functions in controlling vessel diameter. There are at least three possible mechanisms by which α4β1 may contribute to the regulation of vessel diameter. First, α4β1 may regulate contraction and relaxation of the PC/pvSMCs to affect vessel width by conferring a mechanical linkage between the PC/pvSMCs and the underlying ECM interstitia, as
proposed by Courtoy and Boyles when they observed abundant presence of fibronectin in the interstitia shared by the PC/pvSMCs and the underlying endothelium (1983). Secondly, α4β1 may be involved in intussusceptive angiogenesis, a process that split primitive vessels into smaller vessels (Burri et al., 2004). In the absence of α4β1, the vessels may fail to split, leading to vessels with wider diameter. It is not clear, however, how α4β1 might contribute to this process. Finally, α4β1-mediated signaling pathway may regulate the paracrine signals released by vascular endothelial cells that in turn regulates vessel diameter, as suggested by the studies on PDGFR-β- and PDGF-B-deficient mouse embryos (Hellstrom et al., 2001). It might be possible that there is cross-talk between signaling pathways mediated by α4β1 and PDGFR-β. However, although the vessel diameter and PC/pvSMC defects observed in α4-null embryos are very similar to the defects in PDGFB- and PDGFR-β-dependent mouse embryos (Hellstrom et al., 1999), they occur at a much earlier developmental stage. Therefore, it is likely that α4β1-mediated PC/pvSMC activities are independent of the PDGF signaling pathway.

α4 integrin is a marker for the PC/pvSMCs in developing blood vessels

The earliest marker for PC/pvSMCs that has been reported is NG2, which is expressed in PC/pvSMCs of the dorsal aorta and the microvasculature in E10 mouse embryos (Ozerdem et al., 2001). Using an α4KI-lacZ mouse that expresses lacZ under the control of the α4 integrin promoter (Sengbusch et al., 2002), we show in this paper that the α4 integrin gene is expressed in PC/pvSMCs as early as E9.5. We also show that α4 integrin is expressed in PC/pvSMCs of newly formed macro- and microvessels, and confirm a previous observation that α4 integrin is localized at later stages in multilayered vSMCs (Sheppard et al., 1994). In a recent report, Garmy-Susini et al. showed that α4β1 and VCAM-1 are expressed in proliferating endothelial cells of neovessels and their pericytes, respectively, in growth factor-stimulated chorioallantoic membranes of chicken embryos and cancer cells (Garmy-Susini et al., 2005). In contrast to these results, our data clearly show that, in mouse embryos, α4β1 is not expressed in vascular endothelial cells but in PC/pvSMCs before E12.5. In addition, VCAM-1 is not detected in the embryonic vasculature but in the myocardium of the heart before E11.5. This discrepancy suggests that angiogenesis during blood vessel development and neovascularization in malignant tumors does not use the same cell-ECM signaling strategy.

α4β1 regulates specific migratory behaviors of PC/pvSMCs on fibronectin in response to a polarized mechanical force in culture

In this paper, we show that the α4-null mutation leads to a series of abnormal motile activities of primary PC/pvSMCs under shear flow. The defective motile activities include a delay in migration initiation, diminished migration speed, and reduced directional persistence in response to forces of shear flow. Accordingly, these results suggest that α4β1 is involved in a mechanosensing mechanism that allows the cells to migrate in response to a polarized mechanical input. In vivo, hemodynamic forces from blood circulation may be transmitted to PC/pvSMCs through the ECM interstitia of vessel walls and affect the movement and maturation of these cells, as proposed by Hungerford (Hungerford and Little, 1999). Consistent with this hypothesis, it has been demonstrated that blood flow can generate a wall shear stress on vSMCs equivalent to that imposed on vascular endothelial cells (Wang and Tarbell, 1995). These forces may provide mechanical cues that stimulate α4β1-dependent movement and spreading of the PC/pvSMCs along the vessels. The idea that a mechanical cue from the ECM could regulate cell migration has been supported by classical studies demonstrating that cultured fibroblasts can migrate on substrates with a rigidity gradient (Pelham and Wang, 1997), and neuritis can grow in response to mechanical tension (Bray, 1984).

Our results support a role for α4β1 in regulating cell motility. This is consistent with earlier studies showing that α4β1 promotes the formation of lamellipodia (Pinco et al., 2002). α4β1 regulates the formation of lamellipodia by molecular interactions at the cytoplasmic tail of α4 integrin subunit. At the α4 tail, α4 integrin, paxillin, and GIT1, an ADP-ribosylation factor GTPase-activating protein, form a complex that inhibits activation of Rac (Nishiyama et al., 2005). The formation of this complex is inhibited by serine phosphorylation of the α4 tail (Han et al., 2001). Paxillin binding to the α4 tail negatively regulates the lamellipodia-promoting activity of α4β1 (Pinco et al., 2002), whereas serine phosphorylation of the α4 tail is required for this activity (Goldfinger et al., 2003). It might be possible that these molecular interactions are also involved in the motile activities of PC/pvSMCs.

In conclusion, our in vivo and in vitro studies have demonstrated that α4β1 integrin plays important roles in blood vessel development. α4β1 is required for PC/pvSMC spreading along angiogenic vessels, and also for specific motile activities of cultured PC/pvSMCs in response to polarized mechanical force. These findings have important implications in elucidating the mechanism by which integrin-mediated signaling pathways regulate cell migration and for the understanding of the signaling network that regulates PC/pvSMCs during blood vessel development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.01.026.

References


