Integrin $\beta 1$ is required for maintenance of vascular tone in postnatal mice

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Abstract Connective tissue is required for maintaining the integrity of tissues. Integrins are the cell surface receptors responsible for cell attachment to extracellular matrix; however, their tissue-specific role in this process is poorly understood. Here, we test whether integrin $\beta 1$ is required for blood vessel maintenance and integrity in adult mice. We show that adult mice containing a fibroblast/smooth muscle cell-specific deletion of integrin $\beta 1$ exhibit impaired bleeding time and maintenance of vessel architecture, including progressively reduced levels of extracellular matrix (ECM). Vessels also possessed diminished levels of α -smooth muscle actin (α -SMA), and cells derived from vessels showed reduced production of mRNAs encoding ECM and α -SMA as well as reduced α -SMA protein and stress fibers and ECM contraction. Integrin $\beta 1$ in adult fibroblasts/smooth muscle cells/pericytes is required for vasoconstriction and vascular maintenance.

Keywords α -SMA · Matrix contraction · Integrins

Introduction

Connective tissue, comprised largely of extracellular matrix (ECM), is required for the structure and integrity of tissues. For connective tissue to appropriately function, cells attach to ECM via specialized cell surface structures termed focal adhesions (FAs) which mediate,

through integrin cell surface receptors, adhesion between the ECM and the actin cytoskeleton (BurrIDGE and Chrzanowska-Wodnicka 1996; Zaidel-Bar et al. 2007). The mesenchymal cells responsible for this phenomenon include vascular smooth muscle cells (VSMCs), which surround large vessels, and pericytes (PCs), which surround smaller vessels.

Ablation of integrin genes leads to various phenotypes during mouse development, ranging from apparently normal mice to early lethality (Bouvard et al. 2001). For example, disruption of the gene encoding the ubiquitously expressed integrin $\beta 1$ protein leads to the loss of at least 12 different integrin receptors and results in peri-implantation lethality (Fässler and Meyer 1995; Stephens et al. 1995). Integrin $\beta 1$, a common mediator of mesenchymal cell attachment to ECM components such as collagen type I and fibronectin (Lafrenie and Yamada 1996), suggesting that integrin $\beta 1$ expression by VSMCs and PCs may play a key role in maintenance of blood vessel integrity. Integrin $\beta 1$ knockout mice die in utero (Fässler and Meyer 1995; Stephens et al. 1995), thus conditional knockout mouse models are necessary to investigate this issue. Mice harboring the integrin $\beta 1$ allele flanked by loxP sites have been generated (Raghavan et al. 2000). These mice have been used to delete integrin $\beta 1$ *in vivo* using mice expressing cre recombinase under the control of a tissue-specific promoter (Piwko-Czuchra et al. 2009; Bauer et al. 2009; Abraham et al. 2008). A conditional knockout in which integrin $\beta 1$ is deleted in VSMCs and PCs, resulting in loss integrin $\beta 1$ protein expression in embryogenesis, has revealed that integrin $\beta 1$ is required for angiogenesis and vasculogenesis during development (Abraham et al. 2008). However, whether integrin $\beta 1$ is required postnatally for blood vessel maintenance is unknown.

Previously, we have employed a strategy using a tamoxifen-dependent cre recombinase expressed under the control of a type I collagen-specific promoter to test the effects of loss of integrin $\beta 1$ deletion in adult skin fibroblasts

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(Liu et al. 2009). This promoter is expressed in fibroblasts and type I collagen-producing cells such as VSMCs and PCs (Ponticos et al. 2004), and thus can be used to test the effect of integrin β 1 expression in fibroblasts, VSMCs and PCs on blood vessel integrity in adult mice. Our data provide valuable insights into the role of integrin β 1 and cell attachment to ECM to maintaining vascular structure postnatally.

Materials and methods

Generation of integrin β 1 conditional knockout mice

Integrin β 1 conditional knockout mice were generated as described (Liu et al. 2009). Briefly, mice possessing a tamoxifen-dependent Cre-recombinase under the control of a fibroblast-specific regulatory sequence from the pro α 2(I) collagen gene (Zheng et al. 2002) were crossed with mice homozygous for a conditional integrin β 1 allele (Raghavan et al. 2000; Jackson Laboratories) to generate Cre/integrin β 1 heterozygote mice which were mated to generate Cre/integrin β 1 homozygote mice. Animals used for experiments were genotyped by polymerase chain reaction (Raghavan et al. 2000; Zheng et al. 2002). To delete integrin β 1, 3 week-old mice were given intraperitoneal injections of tamoxifen suspension (0.1 ml of 10 mg/ml 4-hydroxytamoxifen, Sigma, St. Louis, MO) over 10 days. Deletion of integrin β 1 was tested by PCR genotyping. Procedures were conducted under approved animal protocols were approved university protocols and US National Institutes of Health guidelines.

Tail bleeding time

Tail bleeding was measured as described by Severin et al. (2007). Briefly, mice were anesthetized by an i.p. injection of a mixture of ketamine (25 mg/kg) and xylazine (10 mg/kg). A 3-mm tail-tip transection was cut and a stopwatch was started immediately upon transection to determine the time required for the bleeding to stop. Blood drops were removed every 30 s with the use of a paper filter. If bleeding did not recur within 30 s of cessation, it was considered stopped.

Histology and immunofluorescence staining

Aortas, skin and kidney from adult mice were fixed in 4% paraformaldehyde at 4°C overnight, and embedded in paraffin for sectioning. Tissue sections (0.5 μ m) were cut using a microtome (Leica, Richmond Hill, ON, Canada) and collected on Superfrost Plus slides (Fisher Scientific, Ottawa, ON, Canada). Sections were then de-waxed in xylene and rehydrated by successive immersion in descending concentrations of alcohol. H&E staining was used to assess the

structure and amounts of extracellular matrix of artery. To assess the effects of integrin β 1 deletion on collagen synthesis, trichrome collagen staining was used.

The sections were also subjected for immunofluorescence staining of integrin β 1 and α -SMA. Briefly, tissue sections were blocked by incubated with 5% donkey serum for 1 h and washed with phosphate-buffered saline (PBS). Sections were then incubated with anti-integrin β 1 (1:100 dilution, R&D Systems, Minneapolis, MN) and anti- α -smooth muscle actin (Anti- α -SMA, 1:500 dilution, Sigma, St Louis, MO, USA) for 1 h at room temperature under humidified conditions. Sections were then washed with PBS and incubated with Texas Red- label anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Sections were then washed with PBS and mounted using 4',6-diamidino-2-phenylindole (DAPI) and photographed using a Zeiss fluorescence microscope and Northern Eclipse software (Empix, Mississauga, ON, Canada). For double staining of blood vessel in skin and kidney, mouse Anti- α -SMA (1:500, Sigma, St Louis, MO, USA) and rabbit anti-PECAM-1 (1:100, Santa Cruz) were used. Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA).

Smooth muscle cell culture and staining

Aortas from adult integrin β 1 conditional and knockout mice were isolated and placed in DMEM. The isolation, culture, verification of smooth muscle cells was performed as described by Golovina and Blaustein (2006). For immunofluorescence staining, cells were grown on glass coverslips coated with fibronectin (Sigma, 10 μ g/ml in PBS). Cells were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 for 5 min, washed, blocked with 5% donkey serum in PBS for 1 h, followed by primary antibody incubation for 1 h. Primary antibodies were goat anti-integrin β 1 (1:100 dilution, R&D Systems, Minneapolis, MN) and mouse anti- α -SMA, (1:500 dilution, Sigma, St Louis, MO, USA). After washing with PBS, specimens were incubated with an appropriate secondary antibody (Jackson ImmunoResearch) at RT for 1 h and then washed with PBS and mounted using mounting medium with 4',6-diamidino-2-phenylindole (DAPI) and photographed using a Zeiss fluorescence microscope and Northern Eclipse software (Empix, Mississauga, ON, Canada).

Western blot analysis

Equal amounts of protein (20 μ g) were subjected to SDS-PAGE. Gels were electrophoretically transferred to nitrocellulose (Invitrogen). Membrane was blocked with 5% nonfat dry milk in Tris-buffered saline, 0.1% Tween 20 (Sigma, St. Louis, MO), and immunoblotting was performed using goat

Fig. 1 Generation of mice deleted for integrin $\beta 1$ in fibroblasts/smooth muscle cells/pericytes. Mice deleted for the integrin $\beta 1$ gene (K/K) and control mice (C/C) that were otherwise genetically identical were generated and genotyped as described previously (Liu et al. 2009) and in methods. **a** Sections of blood vessels were fixed and stained with anti-integrin $\beta 1$ antibody or IgG control, as indicated, followed by FITC-conjugated secondary antibody (green) and DAPI to detect nuclei (blue). **b** Fibroblasts/smooth muscle cells/pericytes from integrin $\beta 1$ (K/K) and control mice (C/C) were cultured and stained with anti-integrin $\beta 1$ antibody followed by Texas red-conjugated secondary antibody (red) and DAPI to detect nuclei (blue). Cell extracts were subjected to SDS-PAGE and Western blot analysis with anti-integrin $\beta 1$ or β -actin antibodies. Six mice per group were analyzed. Data represent average protein expression, as detected by densitometry, and standard deviation from all these mice (*, $p < 0.05$, Students t test)

anti-integrin $\beta 1$ (1:1000 dilution, R&D Systems, Minneapolis, MN) and mouse anti- α -SMA, (1:5000 dilution, Sigma, St Louis, MO, USA). Blots were then developed by incubation with peroxidase-labeled anti-goat or anti-mouse antibodies (1:1000; Vector Laboratories, Burlingame, CA) as secondary antibodies, followed by incubation with ABC reagent (Vector Laboratories, Burlingame, CA). Signal was detected using a luminescence kit (ECL kit; Amersham, Little Chalfont, United Kingdom) and x-ray film.

Real time polymerase chain reaction (RT-PCR)

RT-PCR was performed essentially as described (Kennedy et al. 2007, 2008; Pala et al. 2008). A total of 10 control and knockout mice were analyzed independently for each data

point (averages $\pm$ standard deviation are shown represent averages from these 4 independent animals; Student's paired t test). Cells were cultured until 80% confluence, serum starved for 24 h, and total RNA isolated (Qiagen). Integrity of the RNA was verified by gel electrophoresis. Total RNA (25 ng) was reverse transcribed and amplified (TaqMan Assays on Demand; Applied Biosystems) as described (One-step Mastermix; Applied Biosystems) using the ABI Prism 7900 HT sequence detector (Perkin-Elmer-Cetus, Vaudreuil, QC). Triplicates of each samples were run, and expression values were standardized to values obtained with control 18 S rRNA primers using the delta delta ct method.

Collagen measurement

Salt-soluble collagen of artery was analysed by SIRCOL assay. Briefly, blood vessels were homogenized in RIPA buffer and centrifuged at 15,000 g for 15 min. Collagen content in the supernatant was analysed by the Sircol collagen assay (Biocolor Ltd., Belfast, United Kingdom) following the manufacturer's instructions.

Collagen gel contraction assay

Experiments were performed essentially as described (Shi-wen et al. 2004). For a floating gel assay, 24-well tissue culture plates were pre-coated with BSA. Cells were used at passage 3. Trypsinized fibroblasts were suspended in MCDB medium (Sigma) and mixed with collagen solution (one part of 0.2 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], pH8.0; four parts collagen [Nutragen, Inamed, 3 mg/ml] and five parts of MCDB X 2) for a final concentration of 80,000 cells per ml in 1.2 mg/ml collagen. Collagen/cell suspension (1 ml) was then added to each well to polymerize. Gels were then detached from wells by adding 1 ml of MCDB medium. Gel contraction was quantified by measuring changes in diameter using image analysis software (Empix).

Fig. 2 Loss of integrin $\beta 1$ results in delayed bleeding time. Bleeding time was calculated as described in methods. Four mice per group (K/K or C/C) were analyzed. Data represent average values and standard deviation from all these mice (*, $p < 0.05$, Students t test, C/C versus K/K)

Fig. 3 Conditional deletion of integrin β 1 results in impaired vessel structure. **a** Vessels of control (C/C) or knockout (K/K) mice 4 and 8 weeks post-tamoxifen injection were sectioned and stained as described in Methods using hematoxylin and eosin (H and E). Four mice per group were analyzed. Representative sections are shown. **b** Vessels

of control (C/C) or knockout (K/K) mice 4 and 8 weeks post-tamoxifen injection were sectioned and stained as described in Methods using trichrome stain or collagen ELISA to detect collagen. For all assays, 4 mice per group were analyzed. (*, $p < 0.05$, Students t test)

Results

Deletion of integrin β 1 causes leaky blood vessels

To test if integrin β 1-deficient mice showed impaired blood vessel integrity, we examined 8 week old mice homozygous for deletions in the integrin β 1 gene (which received prior injections with tamoxifen), or genetically identical littermate counterparts (which did not receive tamoxifen). Loss of β 1 integrin was shown by immunofluorescence analysis of tissue section of skin from control (C/C) and integrin β 1-deficient (K/K) animals (Fig. 1a) and immunofluorescence and Western blot analyses of SMC isolated from control (C/C) and integrin β 1-deficient (K/K) animals (Fig. 1b). Compared to wounded control animals (C/C), integrin β 1-deficient (K/K) animals showed elevated rates of bleeding time (that is, impaired vasoconstriction) (Fig. 2). Studies using wild-

type mice treated with or without tamoxifen revealed that tamoxifen alone did not affect bleeding (not shown).

Deletion of integrin β 1 causes impaired vascular integrity

To further study the effects of loss of integrin β 1 to the vasculature of mice, we examined the blood vessels of tissue sections of skin using H and E analysis and trichrome staining. Time points 4 weeks and 8 weeks post-cessation of tamoxifen injection were examined (Fig. 3a, b). Moreover, collagen content was assessed (Fig. 3b). Integrin β 1-deficient animals displayed impaired vessel architecture, diminished cellularity, reduced collagen production and less granulation tissue (Fig. 3). Moreover, immunofluorescence and Western blot analyses of tissue revealed that integrin β 1-deficient mice possessed reduced α -SMA expression

Fig. 4 Loss of integrin β 1 results in a lack of maintenance of vascular smooth muscle cells in vivo. Vessels of control (C/C) or knockout (K/K) mice 4 and 8 weeks post-tamoxifen injection were sectioned. For all assays, 4 mice per group were analyzed. (*, $p < 0.05$, Students t test). **a**

Tissue was fixed and stained with anti- α -SMA antibody and DAPI. Representative sections are shown. **b** Tissue was subjected to Western blot analysis with an anti- α -SMA antibody. Data represent averages and standard deviation from all these mice (*, $p < 0.05$, Students t test)

Fig. 5 Loss of integrin $\beta 1$ results in a lack of maintenance of vascular smooth muscle cells: Real-time PCR analysis of cultured cells. Messenger RNA harvested from control (C/C) or integrin $\beta 1$ knockout (K/K) cells (4 mice total per group, each assay performed in triplicate, average $\pm$ standard deviation is shown) was subjected to real-time PCR analysis to detect the mRNAs (*, $p < 0.05$, Students t test)

(Fig. 4), suggesting that the function of smooth muscle cells/pericytes surrounding the vessels are impaired. PCNA

Deletion of integrin $\beta 1$ causes α -SMA expression and ECM contraction

In order further study the effect of loss of integrin $\beta 1$ on smooth muscle cell function, we cultured primary smooth

muscle cells from explants of animals deleted for integrin $\beta 1$ (K/K) or not (C/C). Real time PCR analysis revealed that integrin $\beta 1$ -deficient cells showed reduced integrin $\beta 1$, collagen 1A2 and α -SMA mRNA expression (Fig. 5). Integrin $\beta 1$ -deficient cells also contained reduced α -SMA-containing stress fibers (Fig. 6a). Consistent with this observation, integrin $\beta 1$ -deficient cells possessed reduced α -SMA protein expression (Fig. 6a). Finally, integrin $\beta 1$ -deficient cells were less able to generate contractile forces to contract free-floating collagen gel matrices (Fig. 6b). These data suggest that integrin $\beta 1$ -deficiency results in a dramatic failure of vascular cells to remodel ECM.

Discussion

Integrins mediate cellular attachment to ECM. The ECM itself has been shown to contribute to vascular function. Laminin and type I or type IV collagens, but not fibronectin, play essential roles in maintaining a differentiated, contractile phenotype of cultured SMCs (Hedin et al., 1988; Yamamoto et al. 1993; Thyberg and Hultgardh-Nilsson 1994). Integrin $\beta 1$, which plays a role in cell attachment to ECM, is essential for embryonic development (Fässler and Meyer 1995; Stephens et al. 1995). To further assess the role of integrin $\beta 1$, cell type-specific deletions of the integrin $\beta 1$ gene have been used. These studies have shown integrin $\beta 1$ is important in the orientation of the mitotic spindle (Lechler

Fig. 6 Loss of integrin $\beta 1$ results in a lack of maintenance of vascular smooth muscle cell phenotype: Expression of α -SMA protein and ECM contraction. Cells were isolated by explant culture from vessels from mice containing the integrin $\beta 1$ gene (C/C) or not (K/K). **a** Cells were fixed and stained with α -SMA antibody or subjected to Western blot analysis with α -SMA or β -actin antibodies. **b** Loss of integrin $\beta 1$ results in a reduced ability of fibroblasts/smooth muscle cells/pericytes to contract a collagen gel matrix. The effect of loss of integrin $\beta 1$

expression on ECM contraction generated by fibroblasts within in a floating collagen gel matrix was assessed over a 24 h period. Contraction was assessed photographically and by measuring the weight of contracted gels (Fibroblasts from three separate animals were used, and experiments were performed in triplicate; Average $\pm$ standard deviation is indicated). Wild-type (C/C) cells better contracted a collagen gel matrix (Student's t test*, $p < 0.05$) compared to integrin $\beta 1$ -deficient cells (K/K)

and Fuchs 2005) the attachment of basal keratinocytes to the basement membrane, (Raghavan et al. 2000) and for bleomycin-induced skin fibrosis (Liu et al. 2009). Recently, it was shown that integrin $\beta 1$ was required for angiogenesis and vasculogenesis during development (Bouvard et al. 2001; Abraham et al. 2008).

In this report, we extend these studies and show that integrin $\beta 1$ is required for blood vessel maintenance and for SMC function in adult mice. Integrin $\beta 1$, a mediator of fibroblast adhesion to type I collagen and fibronectin (Lafrenie and Yamada 1996), was required for maintaining vascular architecture, collagen production and maintenance of SMC differentiation, as revealed by α -SMA expression in vivo. Mice containing a deletion for integrin $\beta 1$ showed elevated bleeding time post-injury, suggesting that vasoconstriction was impaired. Consistent with these results, cultured SMC derived from integrin $\beta 1$ -deficient mice possessed decreased α -SMA mRNA and protein expression, reduced α -SMA stress fiber formation and reduced type I collagen and collagen mRNA expression. Moreover, integrin $\beta 1$ was required for maximal ECM contraction.

Our results demonstrate for the first time that integrin $\beta 1$ expression by SMC is required for the maintenance of vessel architecture in postnatally. In the future, modulating integrin $\beta 1$ activity may prove to be useful for controlling vascular repair and regeneration in adults.

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