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An essential requirement for β1 integrin in the assembly of extracellular matrix proteins within the vascular wall

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ABSTRACT

β1 integrin has been shown to contribute to vascular smooth muscle cell differentiation, adhesion and mechanosensation *in vitro*. Here we showed that deletion of β1 integrin at the onset of smooth muscle differentiation resulted in interrupted aortic arch, aneurysms and failure to assemble extracellular matrix proteins. These defects result in lethality prior to birth. Our data indicates that β1 integrin is not required for the acquisition, but it is essential for the maintenance of the smooth muscle cell phenotype, as levels of critical smooth muscle proteins are gradually reduced in mutant mice. Furthermore, while deposition of extracellular matrix was not affected, its structure was disrupted. Interestingly, defects in extracellular matrix and vascular wall assembly, were restricted to the aortic arch and its branches, compromising the brachiocephalic and carotid arteries and to the exclusion of the descending aorta. Additional analysis of β1 integrin in the pharyngeal arch smooth muscle progenitors was performed using wnt1Cre. Neural crest cells deleted for β1 integrin were able to migrate to the pharyngeal arches and associate with endothelial lined arteries; but exhibited vascular remodeling defects and early lethality. This work demonstrates that β1 integrin is dispensable for migration and initiation of the smooth muscle differentiation program, however, it is essential for remodeling of the pharyngeal arch arteries and for the assembly of the vessel wall of their derivatives. It further establishes a critical role of β1 integrin in the protection against aneurysms that is particularly confined to the ascending aorta and its branches.

Introduction

The assembly of the vascular wall during development occurs through the rapid investment of endothelial tubes by mesenchymal cells and their progressive differentiation into smooth muscle cells (Drake et al., 1998; Hungerford and Little, 1999; Owens, 1995). During this process, vascular smooth muscle cells (vSMCs) secrete and organize layers of extracellular matrix proteins that quickly intertwine with smooth muscle cells to generate a highly integrated tissue able to respond to and regulate intravascular pressure (Gasser et al., 2006; Jones et al., 1979; Li et al., 2003; Wagenseil and Mecham, 2009). The various extracellular matrix (ECM) components are critical to the integrity of the wall, as the smooth muscle cells themselves are not sufficient to comply with the constant mechanical stress imposed by the pulsatile blood flow (Wagenseil and Mecham, 2009). Human mutations in fibrillin 1 (*FBN1*) or type III α1 collagen (*COL3A1*) genes cause Marfan and Ehlers-Danlos syndromes respectively and result in aortic aneurysms, an abnormal enlargement of the aorta caused by thinning of the vessel wall (Dietz et al., 1991; Dietz and Pyeritz, 1995; Pope et al., 1975). Furthermore, mouse models that lack *Fbn1*, fibulin 4 (*FBLN4*) and biglycan (*BGN*) also led to the development of aneurysms in the aorta (Heegaard et al., 2007; Maki et al., 2002; McLaughlin et al., 2006; Pereira et al., 1997). Likewise, genetic mutations in smooth muscle contractile proteins were noticed to be responsible for hereditary vascular anomalies (Guo et al., 2007; Pannu et al., 2007; Zhu et al., 2006). For example, missense mutations in α actin (ACTA2) were found to be associated with 14% of inherited aortic dissections (Guo et al., 2007). More recently, heterozygous mutations in smooth muscle myosin heavy chain (*MYH11*) were identified in kindreds with a wide variety of vascular anomalies, including patent ductus arteriosus, thoracic aortic aneurysms and aortic dissections (Pannu et al., 2007; Zhu et al., 2006). Thus, it is clear that the pathobiological processes that lead to the development of aneurysms include abnormalities in either vascular ECM proteins, vSMC or in a combination of both.

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The integrin family of heterodimeric transmembrane receptors connects the extracellular matrix to the actin cytoskeleton and is thought to participate in vSMC differentiation, ECM organization and mechano-sensing *in vivo* (Hynes, 2002; Li et al., 2003; Sun et al., 2005; Xiao et al., 2007). While many of the integrins have been shown to contribute to vSMC function *in vitro*, β1 integrin is considered to play a particularly significant role as it partners with multiple α subunits and thus constitutes a highly represented receptor (Martinez-Lemus et al., 2003). Furthermore, *in vitro* studies demonstrated that β1 integrin is important for the differentiation of stem cells into smooth muscle and in the organization of collagen and fibronectin fibers (Li et al., 2003; Xiao et al., 2007). Though *in vitro* and *ex vivo* studies are informative, these studies cannot fully recapitulate the *in vivo* context.

Global inactivation of β1 integrin results in peri-implantation embryonic lethality around embryonic day 5.5 (E5.5), several days prior to smooth muscle cell differentiation in the embryo (Fassler and Meyer, 1995; Stephens et al., 1995). Consequently, cell-specific deletion experiments through Cre-lox systems have been critical to elucidate the specific contributions of this gene within a cellular compartment. To determine the role of β1 integrin in differentiated smooth muscle cells, we independently crossed two different β1 integrin flox alleles to the sm22αCre recombinase mouse (Holtwick et al., 2002). The use of the sm22α ensured that deletion occurred in a population of cells that were committed to the smooth muscle fate and had initiated their differentiation program. Mutant mice were embryonic lethal at late gestation and exhibited interrupted aortic arch, aneurysms and failure to assemble ECM proteins. Interestingly, the phenotype was mostly confined to the branches of the aortic arch and compromising the brachiocephalic and carotid arteries. The defects did not extend to the dorsal aorta. Deletion of β1 integrin in neural crest using the wnt1Cre confirmed that absence of the protein did not affect migration of smooth muscle precursors to the aortic arch. These findings establish a critical period in development (E15.5 to E18.5) that requires β1 integrin for the assembly of the vascular wall. The data also highlights an essential contribution of β1 integrin in the protection against aneurysms that is confined to the ascending aorta and emerging/proximal carotid arteries.

Materials and methods

Mice

$\beta1^{e3}$ (Raghavan et al., 2000), sm22αCre (Holtwick et al., 2002) and wnt1Cre (Danielian et al., 1998) mice were purchased from Jackson Labs. We also used the previous published $\beta1^{fl}$ mouse (Potocnik et al., 2000). Cre recombinase expressing mouse lines were independently bred to the $\beta1^{fl}$ and $\beta1^{e3}$ transgenic lines. Additionally, $\beta1^{e3}$; sm22αCre + and $\beta1^{e3}$; wnt1Cre mice were crossed to the ROSA26R (Soriano, 1999) line, also obtained from Jackson Labs. Genotyping for these lines was performed as previously described (Turlo et al., 2010; Zovein et al. 2010). The vaginal plug in conjunction with developmental staging was used to determine the embryonic age of the embryos evaluated. Experiments were evaluated and approved by the Animal Research Committee at the University of California, Los Angeles.

Histological analysis

β-galactosidase staining was performed as described previously (Turlo et al., 2010). Immunostaining for β1 integrin on the $\beta1^{fl}$ dorsal aorta was done on vibratomed sections without an unmasking step. Tissue was dissected and placed in 2% paraformaldehyde overnight. Sections were obtained after the tissue was embedded in agarose and vibratomed into 300–500 μm sections. Specimens were then rinsed in 1X PBS and placed in blocking solution (1x PBS pH7.8, 5% donkey serum, 0.3% triton) for 1 h. Next, sections were incubated in blocking buffer with primary antibody overnight following standard immunostaining techniques as previously described (Zovein et al., 2010). All other immunostaining was performed on histological sections also following protocols described (Zovein et al. 2010, for detailed protocols visit: http://www.mcdb.ucla.edu/Research/Arispe/index.php). Unmasking of epitopes was done in 10 mM sodium citrate at pH 6.0 and heated to 100 °C for 15 min. Sections were blocked in 5% donkey serum in 1X PBS with 0.3% triton. Antibodies used in immunostaining were β1 integrin (1:100, Millipore, MAB 1997), FITC-α-smooth muscle actin (1:200, Sigma, F3777), myh11 (1:200, ABD Serotec), calponin (1:200, Abcam), tropoelastin (1:200, abcam), fibronectin (1:200, abcam), laminin (1:200, Sigma), collagen IV (1:200, abd serotec) and sm22α (1:200, Abcam). Samples were imaged using a 10×, 40× or 100× objective in a Zeiss LSM 510 META confocal microscope.

Western blot

For Western blots on E15.5 and P(0) animals, the whole thoracic aorta was extracted in RIPA buffer (Lee et al., 2006). Tissue for Western blot of E10.5 embryos was collected from the pharyngeal arch region of the embryo. Tissue was first snap frozen and subsequently extracted in RIPA buffer. Total protein concentrations were obtained using DC protein assay (Bio-Rad) on the Bio-Rad Molecular imager Chemi Doc XRS + using Image Lab Software v3. Equal total protein levels (generally 5–10 μg) were loaded per lane. Antibodies used for Westerns included: β1 integrin (1:1000, Millipore, AB1952), α-tubulin (1:1000, Sigma, T5168), GAPDH (1:5000, Millipore, MAB374) and α-smooth muscle actin (1:5000, Sigma, A2547).

Results

Deletion of β1 integrin in smooth muscle results in perinatal/early postnatal death

While the expression of β1 integrin in mature vSMCs is well established (Mechtersheimer et al., 1994), the initiation of β1 integrin expression in this cell population has not been characterized. Thus, we crossed mice carrying the β1 integrin loxP flanked allele ($\beta1^{fl}$) that includes a lacZ reporter to mice with the sm22αCre (sm) transgene (Fig. 1A) (Holtwick et al., 2002; Potocnik et al., 2000). Excision of the DNA between the loxP sites concurrently results in genetic deletion and reporter activation providing a faithful read-out of β1 integrin promoter activity. We chose to use the sm22αCre as this gene is expressed at the onset of smooth muscle differentiation (Yang et al., 2010). From this cross we found β-galactosidase (β-gal) positive cells are present as early as E9.5 in the dorsal aorta and heart (Fig. 1B). By E15.5 all smooth muscle cells of the aortic arch and carotids are positive for β-gal (Fig. 1C). This expression persists into maturity and it includes all smooth muscle cells in the vessel wall suggesting a constitutive need for this gene in vSMC even after development has ceased (Fig. 1D). In addition to vSMCs, staining for β-gal in smR26R and $sm\beta1^{fl/wt}$ embryos confirmed sm22αCre also targets early cardiomyocytes, where the β1 integrin promoter is active as early as E8.5 (Figs. 1B, C and Fig. S1). Overall, we found that expression of β1 integrin essentially mirrors the sm22α promoter, as per ROSA26R β-gal evaluations (data not shown).

In addition to functioning as a reporter of β1 integrin promoter activity, the $\beta1^{fl}$ allele also promotes deletion of β1 integrin. Generation of $sm\beta1^{fl/fl}$ mice revealed that these mice died embryonically or shortly after birth. No live animals with the mutation were found after postnatal day 5 [P(5)] (Table 1). At the neonate stage, $sm\beta1^{fl/fl}$ animals were easily recognized among their wild type littermates, as per their pale appearance (Fig. S3A). Previous work by our lab revealed differences in phenotypes depending on the β1 integrin