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PDGF mediates TGFβ-induced migration during development of the spinous process

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ABSTRACT

Mechanisms mediating closure of the dorsal vertebrae are not clear. Previously, we showed that deletion of TGFβ type II receptor (*Tgfbr2*) in sclerotome in mice results in failure in the formation of the spinous process, mimicking spina bifida occulta, a common malformation in humans. In this study, we aimed to determine whether missing dorsal structures in *Tgfbr2* mutant mice were due to defects in mesenchymal migration and to clarify mechanism of TGFβ-mediated migration. First, we showed that gross alterations in dorsal vertebrae were apparent by E16.5 days in *Tgfbr2* mutants. In addition, histological staining showed that the mesenchyme adjacent to the developing cartilage was thin compared to controls likely due to reduced proliferation and migration of these cells. Next, we used a chemotaxis migration assay to show that TGFβ promotes migration in mixed cultures of embryonic sclerotome and associated mesenchyme. TGFβ stimulated expression of PDGF ligands and receptors in the cultures and intact PDGF signaling was required for TGFβ-mediated migration. Since PDGF ligands are expressed in the sclerotome-derived cartilage where *Tgfbr2* is deleted and the receptors are predominantly expressed in the adjacent mesenchyme, we propose that TGFβ acts on the sclerotome to regulate expression of PDGF ligands, which then act on the associated mesenchyme in a paracrine fashion to mediate proliferation, migration and subsequent differentiation of the adjacent sclerotome.

Introduction

In vertebrates, the axial skeleton is composed of cartilage and bone produced by paraxial mesoderm. The vertebral column is the central part of the axial skeleton, and it includes the vertebral body, the neural arch, and the intervertebral discs between adjacent vertebral bodies. During development, the paraxial mesoderm gives rise to the segmental units of the somite. The somites then subdivide into the dorsal dermomyotome and the ventral sclerotome under the induction of signals from the notochord and the floor plate of the neural tube (Pourquie et al., 1993). Sclerotome, a population of undifferentiated mesenchymal cells, differentiate to form three subcompartments: ventral, lateral, and dorsal, with each giving rise to distinct parts of the vertebrae and ribs (Christ et al., 2000). Following a process of patterned anterior–posterior fusion of consecutive sclerotomes, the vertebrae forms as a cartilaginous template that is later converted into bone by endochondral ossification.

Spina bifida (SB), or failure of the neural tube and vertebrae to close at the midline, is one of the most common (1–2 cases per 1000 births) congenital malformations in humans leading to infant mortality or severe disability. This condition is divided into four categories with spina bifida occulta (SBO) as the mildest form that only involves the vertebrae. As with many other problems, SB appears to result from a combination of genetic and environmental risk factors, but the major cause of SB is believed to be failure of neural tube closure during the first 4 weeks of embryogenesis. However, accumulating evidence suggests that SB can result from defects in the somatic mesoderm surrounding the neural tube (Furumoto et al., 1999; Payne et al., 1997; Pickett et al., 2008; Stottmann et al., 2006).

The transforming growth factor beta (TGFβ) superfamily contains signaling molecules that regulate many aspects of cell physiology including skeletal development. TGFβ signals through a dual receptor system of type I and type II transmembrane serine/threonine kinases (Janssens et al., 2005). Upon TGFβ binding to type II receptor (TGFbR2), which is a constitutively active kinase, type I receptor (TGFbR1) can be recruited and becomes phosphorylated and activated. The canonical Tgfbr2 signaling pathway is mediated by a group of transcription factors called Smads. Smads directly regulate the transcription of target genes (Janssens et al., 2005). The role of members of the TGFβ superfamily in specific aspects of skeletal development and pathology is most clearly illustrated in mice and humans with mutations or targeted deletions in their respective genes (Kingsley, 1994; Serra and Chang, 2003). Targeted deletion of the mouse *Tgfb2* gene results in several skeletal abnormalities including failure in the dorsal closure of the neural arches suggesting an important role for the TGFβ2 ligand in vertebrae development (Sanford et al., 1997). Little is known about the mechanisms of neural arch closure or of TGFβ action in this process.

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Platelet-derived growth factor (PDGF) is a potent mitogenic and chemotactic factor for cells of mesenchymal origin, including chondrocytes and mesenchymal stem cells. The PDGF signaling family consists of four different polypeptide chains, designated A, B, C and D, as well as two receptors α (PDGFRα) and β (PDGFR β). The four PDGF chains assemble into five different dimeric isoforms: PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD. PDGF-BB, AB and CC can bind and activate both PDGFRα and β, whereas PDGF-AA binds only PDGFRα (Bornfeldt et al., 1995). Upon binding to the receptor, PDGF ligands transduce signals to regulate many biological functions of cells, including proliferation, migration, differentiation, and apoptosis (Heldin and Westermark, 1999). The PDGF ligand–receptor system has also been implicated to play roles in wound healing (Barrientos et al., 2008), tendon and cartilage repair (Molloy et al., 2003; Schmidt et al., 2006), atherosclerosis (Ross, 1993), and tumorigenesis (Sun et al., 2005). A recent study showed that the PDGFRα pathway plays a central role in regulating formation of the spinous process (Pickett et al., 2008). Disruption of PDGFRα signaling in the mesenchyme surrounding sclerotome-derived cartilage led to SBO whereas disruption in the sclerotome itself had no effect on vertebral development (Pickett et al., 2008). Since PDGF ligands were shown to be expressed in sclerotome derived tissues and the receptors were expressed in the surrounding mesenchyme, it was suggested that paracrine signaling between the sclerotome and surrounding mesenchyme is required for closure of the neural arches and formation of the spinous process (Pickett et al., 2008). Deletion of *Pdgfc* in mice also resulted in SBO and it was suggested that PDGF-C acts through PDGFRα to mediate development of the vertebrae (Ding et al., 2004). TGFβ induces the expression of PDGF ligands in many cell types, such as mesangial cells (Haberstroh et al., 1993) and endothelial cells (Taylor and Khachigian, 2000), suggesting that TGFβ might mediate physiological and pathological processes by regulating PDGF signaling. In fact, various studies have implicated PDGF acting in concert with TGFβ in development of organ fibrosis, including pulmonary and hepatic fibrosis (Trojanowska, 2008).

Our previous results showed that deletion of *Tgfbr2* in Col2a expressing tissue in mice resulted in alterations in the formation of the vertebrae and intervertebral disc (IVD) (Baffi et al., 2004). These mice demonstrated small vertebrae and the spinous process failed to form, mimicking SBO in humans. These results suggested that TGFβ plays an important role in regulating embryonic development of the axial skeleton; however, the mechanism of this action has not been clarified. In this study, we aimed to determine whether the missing dorsal structures in *Tgfbr2* mutant mice were due to defects in proliferation and migration, and to further clarify the underlying mechanism of migration mediated by TGFβ. We first show that TGFβ signaling is required for dorsal closure of vertebrae and formation of the spinous process, and that gross disruption to development is evident by E16.5 days. We then used a chemotaxis migration assay to show that TGFβ signaling promotes the migration of sclerotome-associated mesenchyme, and that the downstream effects of PDGF are required. We propose that TGFβ acts through PDGF in a paracrine manner to regulate migration of the mesenchyme adjacent to the sclerotome and subsequent formation of the spinous process.

Material and methods

Mice

All experiments were carried out with the approval of the UAB institutional animal care committee. The generation of *Tgfbr2*$^{fl/fl}$ mice was previously described (Chytil et al., 2002). *Col2a-Cre* mice were obtained from Jackson laboratories (Ovchinnikov et al., 2000). The *Rosa26*/+ reporter strain was obtained from Jackson labs (Soriano., 1999). *Col2a-Cre* mice were crossed to *Tgfbr2*$^{fl/fl}$ mice to obtain *Col2a-Cre*$^{+}$;*Tgfbr2*$^{fl/wt}$ mice, which were subsequently crossed to *Rosa26*/*Rosa26* mice to get the mice with a genotyping of *Col2aCre*$^{+}$;*ROSA26*/+;*Tgfbr2*$^{fl/wt}$. These mice were then used to backcross with *Tgfbr2*$^{fl/fl}$ to get *Col2aCre*$^{+}$;*ROSA26*/+;*Tgfbr2*$^{fl/wt}$ as controls and *Col2aCre*$^{+}$;*ROSA26*/+;*Tgfbr2*$^{fl/fl}$ as the experimental group.

Whole mount X-gal staining

Embryos were collected with the day of the vaginal plug designated as E0.5. Whole-mount X-gal staining was performed on the staged embryos as described (Chai et al., 2000). Between five and ten embryos of each genotype were generated. Briefly, embryos were taken and skin was removed before the fixation in 4% paraformaldehyde for 2 h at room temperature. The fixed embryos were soaked in the permeabilization solution for 1 h, followed by the staining solution overnight at room temperature. Stained embryos were post-fixed in 4% paraformaldehyde overnight at 4 °C and then cleared with glycerol for photography. Alterations in the dorsal most edge of the vertebra were measured as the length of the gap between the edges of the neural arches at the level of the second lumbar vertebrae in E15.5, E16.5 and E17.5 day embryos. Measurements were taken from 5 mice of each genotype. Two embryos from each group were used for sectioning. Frozen sections (10 μm) were prepared by embedding the stained embryos in OCT (Tissue-Tek).

Histology, immunostaining, and cell proliferation

At least two E15.5, 16.5, and 17.5 day embryos from each group were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned at 6 μm. For histological analysis, sections were stained with either hematoxylin and eosin or alcian blue. For immunofluorescence and immunohistochemical staining, sections were de-waxed and hydrated, followed by antigen retrieval by boiling the sections in citrate acid buffer (pH 6.0) for 20 min. After blocking in 5% BSA for 1 h, the sections were incubated overnight at 4 °C with a 1:100 dilution of primary antibodies. Antibodies used for this experiment include Ki67 (NeoMarkers) and anti-mouse PDGF-BB (Abcam). Biotinylated goat-anti-rabbit IgG or biotinylated rabbit-anti-goat IgG (1:200, Vector lab) was added as the secondary antibody. Fluorescent signal was detected with avidin-conjugated Cy3, with DAPI as counterstain. Color was developed with DAB (Vector laboratories), with hematoxylin as counterstain.

Proliferation of sclerotome and associated mesenchymal cells was detected by immunostaining for Ki67 on deparaffinised sections as described above. Ki67-positive cells and DAPI-stained total cells were counted from five randomly selected boxed areas in the region of neural arch. Percentage of proliferation was obtained by dividing the number of Ki67-positive cells by the total number of cells. Data were presented by multiplying 100. Significance was determined by T-test.

Micromass culture of sclerotome and associated mesenchyme

Sclerotome and associated mesenchyme ventral to the neural tube were isolated from E11.5 day mouse embryos. The micromass culture was set up using a method described previously (Sohn et al., 2010). Briefly, mesenchymal cells were dissociated into a single cell suspension with incubation in 1 mg/ml collagenase D at 37 °C for 30 min and reconstituted at a density of 1×10^{7} cells/ml. Twenty microliters of cell suspension was dropped into each well of a 24 well plate. After a pre-incubation time of 1 h at 37 °C to allow cells to attach, the cultures were then flooded with F-12:DMEM (1:1) containing 10% FBS, 50 μg/ml ascorbic acid, 10 mM β-glycerolphosphate, 2 mM glutamine, antibiotics with or without different concentrations of TGFβ1. For the cells infected with adenovirus, virus was added to the cell drops with 2 μl per 20 μl drop (100 MOI, Multiplicity of Infection).