TGFβ is required for the formation of capillary-like structures in three-dimensional cocultures of 10T1/2 and endothelial cells

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Abstract

New vessels form de novo (vasculogenesis) or from pre-existing vessels (angiogenesis) in a process that involves the interaction of endothelial cells (EC) and pericytes/smooth muscle cells (SMC). One basic component of this interaction is the endothelial-induced recruitment, proliferation and subsequent differentiation of pericytes and SMC. We have previously demonstrated that TGFβ induces the differentiation of C3H/10T1/2 (10T1/2) mesenchymal cells toward a SMC/pericyte lineage. The current study tests the hypothesis that TGFβ not only induces SMC differentiation but stabilizes capillary-like structures in a three-dimensional (3D) model of in vitro angiogenesis. 10T1/2 and EC in Matrigel™ were used to establish cocultures that form cord structures that are reminiscent of new capillaries in vivo. Cord formation is initiated within 2–3 h after plating and continues through 18 h after plating. In longer cocultures the cord structures disassemble and form aggregates. 10T1/2 expression of proteins associated with the SMC/pericyte lineage, such as smooth muscle α-actin (SMA) and NG2 proteoglycan, are upregulated in these 3D cocultures. Application of neutralizing reagents specific for TGFβ blocks cord formation and inhibits expression of SMA and NG2 in the 10T1/2 cells. We conclude that TGFβ mediates 10T1/2 differentiation to SMC/pericytes in the 3D cocultures and that association with differentiated mural cells is required for formation of capillary-like structures in Matrigel™.

Introduction

Blood vessel formation occurs during development, as part of wound repair, and during the reproductive cycle. It is also characteristic of a number of pathologies including retinopathy of prematurity, diabetic retinopathy and tumor vascularization [1–3]. In vasculogenesis EC arise from mesenchymal precursors, which assemble to form nascent vessels [4], whereas in angiogenesis proliferation of EC from pre-existing vessels initiates new vessel formation. On the abluminal vessel surface are pericytes and smooth muscle cells (SMC). There are two current concepts regarding the mechanism by which pericytes and SMC are recruited to new vessels: cell recruitment from nearby mesenchymal tissue and recruitment of SMC from pre-existing vessels. The recruitment and differentiation of pericytes and SMC appear to be EC-dependent and may occur by similar mechanisms in vasculogenesis and angiogenesis [5–7].

An increasing number of factors have been identified that appear to be involved in vessel assembly by regulating the cell–cell interactions that promote vessel growth. Among these is the potent angiogenic factor, vascular endothelial growth factor (VEGF), which induces EC proliferation, migration and lumen formation [8–10]. Targeted disruptions of VEGF [11, 12] and use of neutralizing reagents [13] indicate that VEGF is required for both vasculogenesis and angiogenesis. There is also evidence to suggest that platelet-derived growth factor BB (PDGF-B) mediates the local proliferation and recruitment of mesenchymal cells toward the forming vessel [6, 7]. The PDGF-B [5] and the PDGFR-β receptor [14] deficient mice are both embryonic lethal and have abnormalities in some populations of pericytes and vascular SMC. Further evidence for factors involved in vessel formation comes from the gene knockout studies of the angiopoietins (ang) and their cognate receptor, Tie 2 [reviewed in 15]. The absence of ang 1 [16] or Tie 2 [17] and the
overexpression of ang 2 [18] result in similar phenotypes, characterized by a disorganized vasculature and a lack of mature vessels.

The current study is focused on elucidating the involvement of TGFβ in vessel assembly. Evidence from knockout studies suggests that TGFβ is involved in vessel formation. Fifty percent of the mice lacking the TGFβ1 gene product died at about embryonic day 10 (E10) with abnormal yolk sac hematopoiesis and vasculogenesis [19]. The yolk sac vessels were large and leaky with apparently abnormal adhesion between EC [19]. Mice lacking the TGFβ Type II serine/threonine kinase receptor had a similar yolk sac phenotype, with additional abnormalities in the development of other organ systems [20]. More recent evidence of a role for TGFβ in vessel development comes from examination of the endoglin gene product. Endoglin binds to TGFβ family members and their receptors and is thought to contribute to TGFβ signaling [21]. Mice deficient in endoglin show vascular abnormalities and die by E11.5 [22]. Early stages of vasculogenesis seem to be normal in these mice, but the vessels appear immature and are not invested with SMC. The inherited genetic disorder hemorrhagic telangiectasia results from a mutation in the endoglin gene [23] and these patients have arterial and venous malformations that are characterized by large, leaky vessels with variable SMC investment. Thus, TGFβ signaling is important for some aspects of vessel formation, although its precise roles and the target cell types are as yet unclear.

TGFβ is a pleiotropic cytokine that has been shown to affect the differentiation of a number of cell types, including SMC [24, 25]; and TGFβ expression is correlated with SMC differentiation in a rat artery injury model in vivo [26]. We have shown previously that there is localized activation of TGFβ upon contact of SMC with EC [27]. In addition, we have demonstrated that there is a TGFβ-dependent induction of SMC differentiation when undifferentiated mesenchymal cells contact EC [6]. TGFβ and an activating protein, urokinase plasminogen activator, have been localized to interdigitating sites between EC and pericytes in granulation tissue undergoing angiogenesis, indicating spatial and temporal colocalization in vivo [28]. These results suggest that local activation of TGFβ in the microenvironment of a developing vessel may have an important regulatory role in the differentiation of SMC and the subsequent maturation and stabilization of vessels.

In the current study, we report a three-dimensional (3D) model of vessel assembly that is dependent upon heterotypic cell–cell interactions in Matrigel™. We tested the hypothesis that TGFβ promotes the differentiation of vascular SMC and that this differentiation is important for vessel stabilization. We have targeted the role of TGFβ in this system with the use of neutralizing agents to TGFβ and find that TGFβ is required for differentiation of SMC and the formation of capillary-like cord structures in 3D cocultures of 10T1/2 cells and EC.

Materials and methods

Cell culture

C3H/10T1/2 mouse mesenchymal cells (10T1/2, ATCC, Rockville, MD) and bovine aorta-derived EC were grown in Dulbecco’s modified Eagle’s medium (DMEM, JRH Biosciences, Lenexa, Kansas), supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Logan, Utah), 233.6 µg/ml glutamine, 80 units/ml penicillin, 80 µg/ml streptomycin (Irvine Scientific, Santa Ana, California) and 25 mM glucose (Sigma Chemical Company, St Louis, Missouri). These culture conditions reduced the basal level of smooth muscle α-actin (SMA) expression in the 10T1/2 cells. The homogeneity of the EC were confirmed by Dil-acetylated LDL uptake (Biomedical Technologies, Stoughton, Massachusetts) according to the manufacturer’s protocol. The cells were used under passage 20 and were maintained at confluence for 3–5 days prior to use. The cells were routinely trypsinized for passage and collection using 0.05% trypsin/0.53 mM EDTA (GibcoBRL, Grand Island, New York). Cell counts were determined using a Coulter Counter (Coulter Corporation, Miami, Florida).

Vital dye labeling

10T1/2 and EC were labeled with PKH67 (Sigma) and PKH26 (Sigma), respectively, according to the manufacturer’s recommended protocol. In brief, cells were trypsinized to a single cell suspension and counted. The cells were resuspended at a density of 2×10⁶ cells/100 µl of diluent and transferred to a polypropylene tube. An equal volume of 40 µM PKH dye was added and mixed with gentle swirling. The cells were exposed to the dye for 3 min at room temperature. The dye uptake was terminated with addition of 200 µl of FCS and 7 ml of PBS to the cell suspension. The cells were then centrifuged over a 3 ml bed of FCS and further washed with 10 ml of complete DMEM. To allow for full recovery from the labeling procedure, the cells were plated for 24 h after labeling prior to use in coculture experiments. The excitation and emission spectra for PKH 26 are 551 and 567 nm, and for PKH 67 are 490 and 502 nm, respectively.

3D cultures

The 3D cultures were established by polymerizing 200 µl of Matrigel™ basement membrane extract (Fisher Scientific, Pittsburgh, Pennsylvania) on 12 mm glass coverslips in 24 well tissue culture plates. Cells were plated on the Matrigel™ in assay medium consisting of normal growth medium with 2% FCS. Cells in 3D cultures were visualized using a Dualband filter set for FITC/TRITC (510402 Excitation Filter, Micro Video Instruments, Avon, Massachusetts) on a Nikon Inverted Microscope. Photos were taken using a Nikon camera and Ektachrome 100 ASA film. Slide images were scanned and