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Regulation of limb development by the sphingosine 1-phosphate receptor *S1p1/EDG-1* occurs via the hypoxia/VEGF axis

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Abstract

Angiogenesis, also known as new blood vessel formation, is regulated coordinately with other tissue differentiation events during limb development. Although vascular endothelial cell growth factor (VEGF) is important in the regulation of angiogenesis, chondrogenesis and osteogenesis during limb development, the role of other angiogenic factors is not well understood. Sphingosine 1-phosphate, a platelet-derived lipid mediator, regulates angiogenesis and vascular maturation via its action on the G-protein-coupled receptor $S1P_1$ (also known as EDG-1). In addition to vascular defects, abnormal limb development was also observed in $S1p_1^{-/-}$ mice. Here we show that strong induction of $S1P_1$ expression is observed in the blood vessels and the interdigital mesenchymal cells during limb development. Deletion of $S1P_1$ results in aberrant chondrocyte condensation and defective digit morphogenesis. Interestingly, the vasculature in the $S1p_1^{-/-}$ limbs was hyperplastic and morphologically altered. In addition, the hypoxia inducible factor (HIF)-1α and its response gene VEGF were induced in $S1p_1^{-/-}$ limbs. However, aberrant regulation of HIF-1α and VEGF were not observed in embryonic fibroblasts derived from $S1p_1^{-/-}$ mice, suggesting a non-cell autonomous effect of $S1P_1$ on VEGF expression. Indeed, similar limb defects were observed in endothelium-specific $S1P_1$ null mice in vivo. These data suggest that the function of $S1P_1$ in the developing vasculature is essential for proper limb development.

Keywords: Angiogenesis; Sphingosine 1-phosphate; Hypoxia; Vascular endothelial growth factor

Introduction

Cartilage formation is one of the early morphogenetic processes during embryonic development and involves a series of strictly coordinated events (Olsen et al., 2000). During limb development, cells from the lateral plate mesoderm migrate to the future bone areas and undergo precartilaginous condensation. Mesenchymal cells of the limb bud are differentiated into chondrocytes or fibroblasts whereas undifferentiated mesenchymal cells produce extracellular matrix (ECM). Chondrogenic cells become demarcated by ECM and acquire the characteristic limb skeletal pattern by undergoing a series of bifurcations and segmentation processes. Cartilage formation is then followed by endochondral ossification, during which chondrocytes in the avascular cartilage differentiate into hypertrophic cartilage, which is vascularized by the neighboring angiogenic precursor cells. Angiogenesis of the cartilage primordium occurs concomitantly with invasion of osteoblasts and bone formation (ossification). Whereas chondrocytes of the avascular cartilage produce inhibitors of angiogenesis, hypertrophic chondrocytes produce angiogenic stimulators that initiate vascularization, indicating the requirement for precise regulation of angiogenesis during chondrogenesis (Gerber et al., 1999).

During development, the vascular system forms by vasculogenesis (de novo vessel formation) and angiogenesis (vessel formation from preexisting vessels) (Carmeliet, 2003; Jain, 2003). In fact, the cardiovascular system is the

Abbreviations: S1P, sphingosine 1-phosphate; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia inducible factor-alpha; ECM, extracellular matrix; VSMC, vascular smooth muscle cells; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; PECAM, platelet endothelial cell adhesion molecule; MEF, mouse embryonic fibroblast; GLUT, glucose transporter.

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first functional organ system to develop in the vertebrate embryo and supply nutrients and oxygen to all tissues. Vascular development requires processes such as vessel formation, patterning, pruning, remodeling and maturation. Failure in any process during vascular development leads to embryonic lethality. Immature vessels formed by vasculogenesis and angiogenesis must be stabilized by mural cells, such as pericytes and vascular smooth muscle cells (VSMC) (Jain, 2003). During vessel stabilization, mural cells are recruited to invest the nascent endothelial tubes, thereby providing stability in the developing cardiovascular system with increasing demands and stresses.

Recent studies have made significant progress in defining the signaling pathways required for the recruitment and the differentiation of mural cells during vessel maturation. A number of signaling molecules, including platelet-derived growth factor (PDGF)-BB/PDGF-receptor-β, Smad5, endoglin and angiopoietin-1 have been identified as critical regulators of vessel maturation (Allende and Proia, 2002; Jain, 2003). Targeted disruption of the genes encoding these receptors and ligands in mice has been particularly informative in defining their roles. For example, PDGF-BB is essential for the recruitment of mesenchymal mural cell precursors expressing PDGF receptor-β to vessel walls (Leveen et al., 1994). Disruption of the *PDGF-B* or *PDGF* receptor-β genes in mice leads to microvasculature aneurysms, lethal hemorrhaging and edema in the perinatal stage due to a lack of pericytes (Hellstrom et al., 2001). Such signaling systems may also be important in vessel development/growth in the adult. For example, inhibition of vessel stabilization in mice by inhibiting PDGF-receptor-β together with the VEGF receptor resulted in additive inhibition of tumor angiogenesis and growth (Bergers et al., 2003). Thus, signaling systems for vessel stabilization are of importance not only in embryogenesis but also in adult physiology and pathology.

Our laboratory has defined the platelet-derived bioactive lipid sphingosine-1-phosphate (S1P) as a regulator of angiogenesis, vascular development and homeostasis (Hla, 2003; Hla et al., 2001). We originally cloned the $S1P_1$/EDG-1 (Endothelial Differentiation Gene-1) cDNA and showed that it encodes a high-affinity receptor for S1P (Hla and Maciag, 1990; Lee et al., 1998). In models of angiogenesis and vascular maturation in adult mice, S1P synergized with polypeptide growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) in the formation of mature vascular structures (Lee et al., 1999). Deletion of the *S1p1* gene in the mouse resulted in embryonic lethality due to defects in vascular stabilization (Liu et al., 2000), where defective mural cell investment to endothelial cells was observed. Interestingly, $S1p_1^{-/-}$ embryo also showed defective limb development; however, this was not emphasized in that study and detailed characterization of how S1P regulates limb development was not studied. In this study, we further characterized the underlying mechanism of how $S1P_1$ regulates limb development. Our data suggest $S1P_1$-regulated mechanisms in the developing limb bud in the coordination of angiogenesis and chondrogenesis.

Materials and methods

Animals

$S1p_1^{-/-LacZ}$ and *S1p1*-conditional mutant embryos were generated and genotyped as previously described (Allende et al., 2003; Liu et al., 2000). To obtain endothelial specific *S1p1* knockout (*S1p1 loxP/KoTie2-Cre*) and control littermates (*S1p1 loxP/KO*, *S1p1 loxP/WT Tie2-Cre* or *S1p1 loxP/WT*), *S1p1 loxP/loxP* mice were bred with *S1p1 WT/KO* mice expressing Cre recombinase under the control of the EC-specific promoter Tie2 (Allende et al., 2003).

β-galactosidase (LacZ) staining of embryos

Embryos were isolated in cold phosphate-buffered saline (PBS) and fixed in 0.2% glutaraldehyde and 1.5% paraformaldehyde in PBS, pH 7.4, at room temperature for 30 min, then washed in PBS. Staining was performed at 37°C in 0.02% X-gal, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in PBS overnight. The stained embryos were washed, postfixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm.

Whole-mount embryo immunostaining

Embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight, dehydrated through a methanol series and stored in 100% methanol at −20°C. The embryos were then bleached in 6% hydrogen peroxide/methanol for 1 h at room temperature and hydrated through a methanol series to 0.1% Tween 20/PBS (PBST). Hydrated embryos were incubated twice for 1 h in 4% BSA in PBST. The embryos were incubated with anti-CD31 antibodies, diluted 1:200 in 10% goat serum and 4% BSA in PBST at 4°C overnight. Embryos were washed with 4% BSA in PBST and incubated with peroxidase-conjugated goat anti-rat IgG in 10% goat serum and 4% BSA in PBST at 4°C overnight. DAB/hydrogen peroxide was used for visualization.

Alcian blue staining

Embryos were fixed in Bouin's solution (75% picric acid, 10% formaldehyde, 5% acetic acid) for 2 h. Embryos were then washed six to eight times in wash buffer (0.1% NH_4OH, 70% ethanol) until noncartilaginous tissues were white and equilibrated in 5% acetic acid twice for 1 h. Embryos were stained in 0.05% Alcian Blue 8XG in 5% acetic acid and rinsed twice for 2 h with 5% acetic acid. Stained embryos were cleared with methanol.

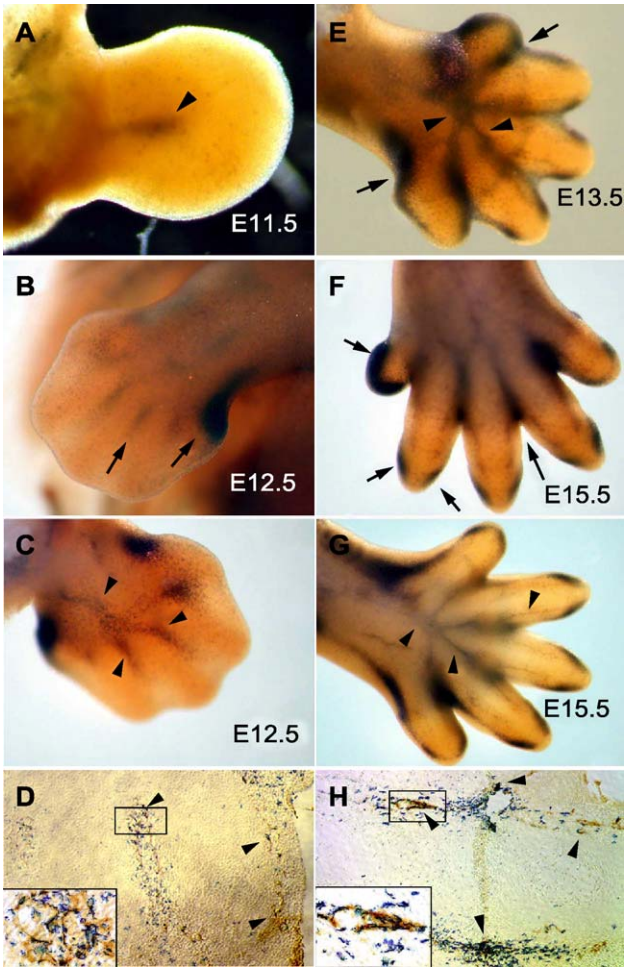

Fig. 1. $S1P_1$ expression in the developing limbs. E11.5 (A), E12.5 (B, C, D), E13.5 (E) and E15.5 (F, G, H) $S1p_1^{+/-LacZ}$ embryos were lacZ-stained. Longitudinal sections were counterstained with anti-CD31 antibody (D, H). (B, F) Dorsal view. (C, E, G) Ventral view. Arrowheads and arrows indicate blood vessels and apoptotic areas, respectively.

Northern blot analysis

Total RNA from limbs of different developmental stages of mouse embryos was isolated using the RNeasy kit™ (Qiagen Inc, Valencia, CA), as described by the manufacturer. Four micrograms of total RNA was subjected to Northern hybridization using oligonucleotide probes specific to the VEGF 3′ UTR at standard conditions. For hypoxic studies, mouse embryonic fibroblasts (MEFs) were incubated in hypoxic chamber gassed with 2% O_2–5% CO_2 and balanced with N_2 at 37°C for 16 h. For S1P stimulation, cells were serum-starved for 16 h and treated with 100 nM of S1P for 6 h. Ten micrograms of total RNA was used.

Western blot analysis

Limbs from E12.5 embryos were isolated and homogenized in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5% NP-40, protease inhibitors cocktail) by using the Dounce homogenizer. Homogenate was centrifuged at 1500 × *g* for 5 min, the pellet was washed with hypotonic buffer, resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitors cocktail) and incubated for 20 min on ice. Nuclear proteins were obtained by centrifugation at 15,000 × *g* for 20 min. Twenty micrograms of nuclear protein was subjected to gel electrophoresis and transferred to nitrocellulose membrane. HIF-1α was detected by monoclonal HIF-1α antibody (Novus Biological). Nuclear fraction was confirmed by immunoblot with the antibody for nuclear protein, lamin B1 (Zymed Laboratories Inc.).

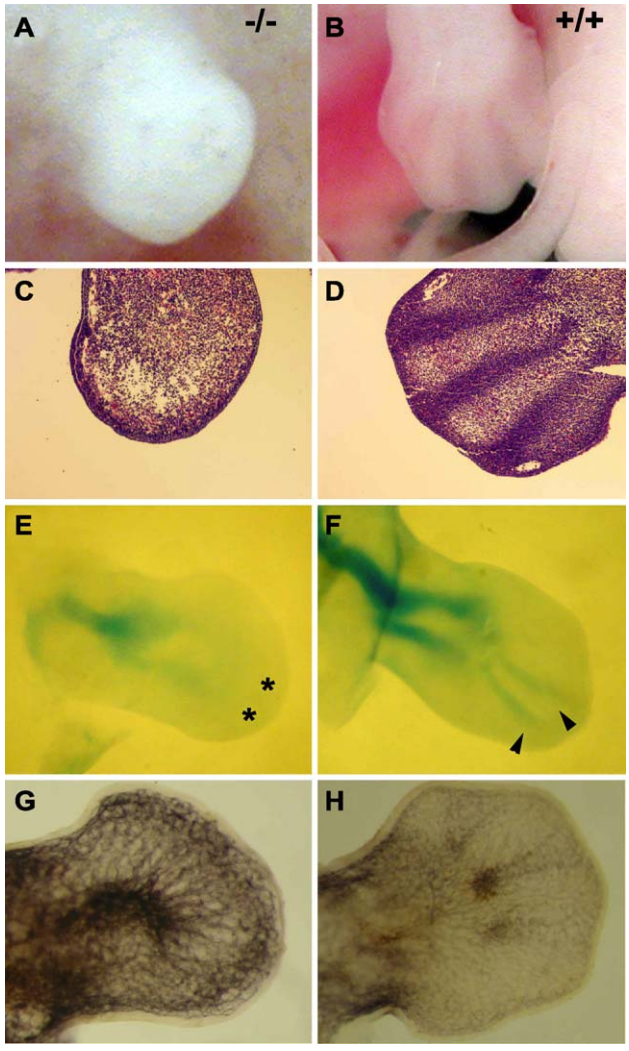

Fig. 2. Defective limb development in $S1p_1^{-/-}$ embryos. Limbs from $S1p_1^{-/-}$ (A, C, E, G) and wild-type (B, D, F, H) embryos at E12.5 were fixed, sectioned and stained with H&E (C, D). (A, B) Brightfield image. Cartilage formation was detected by Alcian blue staining (E, F). Arrowheads indicate cartilage primordia in the area where the digits will form. Asterisks indicate corresponding regions in $S1p_1^{-/-}$ limbs. Limb vasculature was visualized by whole-mount staining with anti-CD31 antibody (G, H). Note the high density of dilated blood vessels in the $S1p_1^{-/-}$ limb.

Results

Expression of S1P$_1$ during limb development

Previous studies have defined the expression of S1P$_1$ during midgestation mouse development by analysis of reporter gene expression (β-galactosidase) in *S1p$_1^{+/-}$* mice (Liu et al., 2000). In these studies, expression of S1P$_1$ was found in the developing vasculature, heart and forebrain. However, a more comprehensive analysis of expression in different stages of embryonic development has not been reported. We noticed that S1P$_1$ expression was more widespread at later stages of embryonic development (data not shown). In particular, S1P$_1$ expression was induced in the developing limb at E11.5 (Fig. 1A). At E12.5, expression was more prominent in the blood vessels and interdigital areas (Figs. 1B,C). Immunohistochemical staining of limb sections with the endothelial cell marker PECAM/CD31 on X-gal stained tissues showed that S1P$_1$ was expressed in vascular cells as well as limb mesenchymal cells (Figs. 1D,H). Most PECAM/CD31-positive endothelial cells were also positive for S1P$_1$. At later stages of development (E13.5–15.5), this pattern was more prominent in the tips of digits and interdigital areas (Figs. 1E–G). These data suggest that S1P$_1$ is strongly expressed in the areas of the developing limb undergoing apoptosis, tissue remodeling and vascular development.

Defective limb development in S1p$_1^{-/-}$ embryos

To gain further insights into the functional role of S1P$_1$ in limb development, developing limbs from wild-type and *S1p$_1^{-/-}$* embryos were analyzed. As shown in Fig. 2, *S1p$_1^{-/-}$* limbs showed major developmental defects. Even in early stages (E10.5–11.5) when the embryos were viable (with a heartbeat), the limbs appear short (data not shown). At E12.5 days of development, limb morphogenesis was aberrant as interdigital sculpting was absent (Figs. 2A,B). Histological analysis showed that chondrocyte condensation, which is required for digit formation, appeared to be impaired in *S1p$_1^{-/-}$* limbs (Fig. 2C vs. D). Whole-mount Alcian blue staining showed the normal development of stylopod and zeugopod but not autopod formation (Fig. 2E vs. F, asterisks). In addition to defective digit formation, knockout limbs were hypervascularized with dilated vessels (Fig. 2G vs. H). These data suggest that lack of S1P$_1$ results in limb morphogenetic defects and hypervascularity during development.

Non-cell-autonomous effect of S1P$_1$ on VEGF induction

It is known that *S1p$_1^{-/-}$* mice develop a vascular stabilization defect, resulting in vascular system failure and hemorrhage. This is apparent at E12.5 days of development, and by E15.5 100% of the embryos show lethal hemorrhage. To determine the molecular basis of the hypervascular defect in the limbs of *S1p$_1^{-/-}$* mice, we determined the expression level of the critical angiogenic factor, VEGF. As shown in Fig. 3A, VEGF levels were strongly induced in the limbs of *S1p$_1^{-/-}$* mice, suggesting that this may be an important event in the induction of hypervascular response. To test if VEGF induction in *S1p$_1^{-/-}$* mice is caused by cell-autonomous signaling of S1P$_1$, the expression levels of VEGF in wild-type and *S1p$_1^{-/-}$* MEF were compared by Northern blot analysis (Fig. 3B). VEGF expression in cells of both genotypes was comparable and was unaffected by S1P stimulation. However, hypoxia potently induced VEGF expression in both genotypes, suggesting that S1P$_1$ signaling per se in a cell-autonomous manner does not regulate VEGF expression. Rather, the enhanced VEGF expression occurs in vivo, most likely due to a systemic effect brought about by *S1p$_1$* gene deletion.

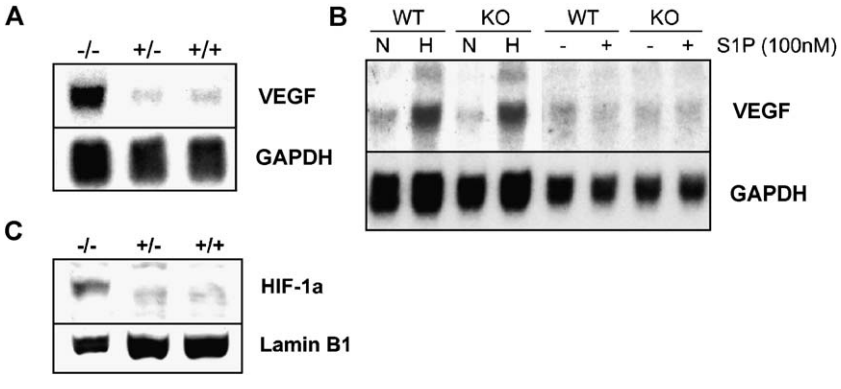

Fig. 3. Hypoxia and VEGF induction in *S1p$_1^{-/-}$* limbs. VEGF RNA level was determined by Northern hybridization using oligonucleotide probes specific to VEGF 3′ UTR. Four micrograms of total RNA from limbs of null (−/−), heterozygous (+/−) and wild type (+/+) embryos (A) and 10 μg from MEFs (B) were used. MEFs were incubated in hypoxic chamber for 16 h for hypoxia. For S1P stimulation, cells were serum-starved for 16 h and treated with 100 nM of S1P for 6 h. (C) The protein level of HIF1-α was determined in nuclear proteins isolated from null (−/−), heterozygous (+/−) and wild type (+/+) embryonic limbs. Nuclear fractionation was confirmed by nuclear marker protein, lamin B1. WT, *S1p$_1^{+/+}$* MEF; KO, *S1p$_1^{-/-}$* MEF; N, normoxia; H, hypoxia.

Hypoxia in $S1p_1^{-/-}$ embryonic limb

VEGF expression is directly induced by tissue hypoxia (Carmeliet, 2003). It is possible that in the growing peripheral tissue such as the developing limb, tissue hypoxia could be an important factor in the regulation of VEGF expression and angiogenesis. Indeed, the vascular stabilization defect in $S1p_1^{-/-}$ mice (Liu et al., 2000) could lead to exacerbated hypoxia. To test if hypoxia is a dominant inducer of VEGF in $S1p_1^{-/-}$ limbs, the level of hypoxia inducible factor (HIF)-1α polypeptide was compared. As shown in Fig. 3C, $S1p_1^{-/-}$ limbs showed much higher level of HIF-1α, suggesting that hypoxia upregulates VEGF expression, which leads to abnormal hypervascularization.

Defective limb development caused by the endothelium-specific loss of $S1P_1$

Although severe hypoxia is present in $S1P_1$ null limbs, resulting in enhanced VEGF expression, hypervascularization and limb defects, it is formally possible that the function of $S1P_1$ in nonvascular tissues of the developing limb, for example, the interdigital area, contributes to the observed phenotype. To test if vascular or nonvascular function of $S1P_1$ is responsible for the limb developmental defects, endothelial cell-specific $S1p_1^{-/-}$ embryos were examined. Interestingly, endothelial cell-specific $S1p_1^{-/-}$ mice also showed the same phenotypic defects in limb development as $S1P_1$ null embryos (Fig. 4). Compared with control littermate (Figs. 4A,C), knockout limbs showed defective chondrocyte condensation and hemorrhage (Figs. 4B,D). In addition, edema and dilated abnormal vasculature were also observed in some limbs (data not shown). These data indicate that loss of $S1P_1$ in endothelial cell abrogates normal limb development and support the notion that function of the receptor in the vasculature is primarily responsible for the observed defects in limb development.

Discussion

$S1P_1$ is known to be important in endothelial cell survival, migration, and morphogenesis (Hla et al., 2000, 2001). During embryonic development, intense expression of $S1P_1$ is detected in cardiovascular system, including the heart, dorsal aorta, intersomitic arteries and capillaries (Liu et al., 2000). In accordance with this expression pattern, $S1p_1^{-/-}$ embryos show defects in the developing vascular system, especially in the process of vascular stabilization. The phenotype of $S1p_1^{-/-}$ mice suggests a pivotal role of $S1P_1$ in embryonic vascular development. Immature vasculature is inefficient in the delivery of nutrients and oxygen to tissues, ultimately resulting in hypoxia. To meet the demand of oxygen, tissues in hypoxic environment produce angiogenic factors to stimulate blood vessel formation. A growing number of studies have emphasized the importance of HIF family of transcription factors on the regulation of expression of angiogenic factors (Pugh and Ratcliffe, 2003). Under hypoxic conditions, HIF-1α stabilization by the oxygen sensing machinery results in the increased level of HIF-1α, which induces the transcription of target genes, such as

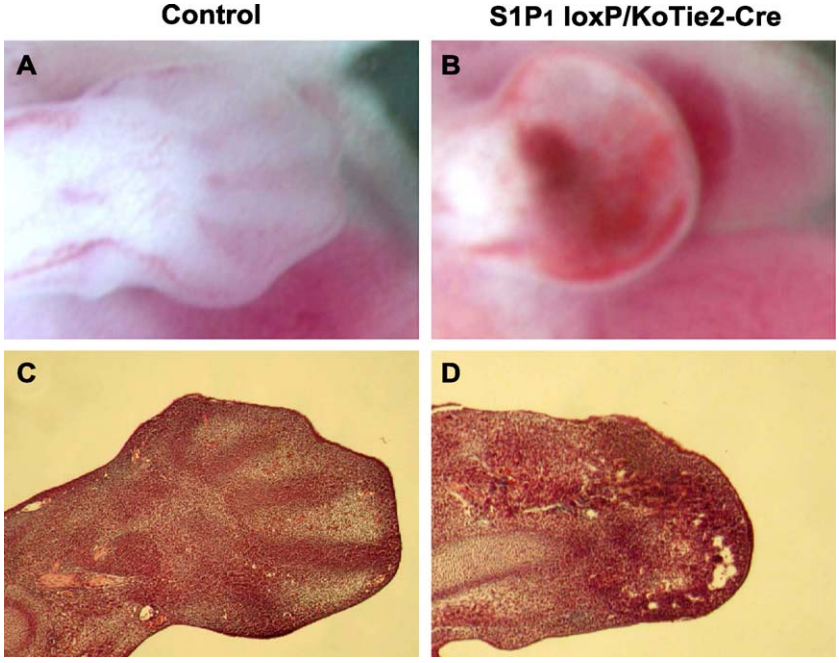

Fig. 4. Defective limb development in *$S1p_1$ loxP/KoTie2-Cre* mouse. *$S1p_1$ loxP/KoTie2-Cre* (B, D) and control littermate (A, C) embryos at E12.5 were isolated and photographed. Embryonic limbs were sectioned and stained with H&E.

VEGF, glucose transporter (GLUT-1), among others (Pugh and Ratcliffe, 2003). Significantly, high level of HIF-1α in $S1p_1^{-/-}$ limbs strongly suggests the presence of severe hypoxia in this tissue. Strong induction of VEGF mRNA and hypervascularization, therefore, may be caused by hypoxia in $S1P_1$ null limbs. However, hypoxia strongly induced VEGF expression in wild type and $S1p_1^{-/-}$ MEFs while S1P stimulation had no effect on VEGF expression level. These data suggest that impaired digit formation may occur secondarily to the vascular defects. This notion is strongly supported by the fact that endothelium-specific knockout of the $S1P_1$ receptor phenocopies the global knockout of $S1p_1^{-/-}$ (Allende et al., 2003).

It has been shown that angiogenic factors inhibited chondrocyte maturation, which is an important step in bone formation. Indeed, vascular regression is required for chondrogenesis in the developing limb of avian embryos (Yin and Pacifici, 2001). It is therefore likely that defective vascular development induced by lack of $S1P_1$ caused hypoxia, leading to enhanced VEGF expression and hypervascularization, which ultimately disturbs the limb morphogenesis and patterning. In addition, signaling between endothelial cells and surrounding tissues might also contribute to the phenotypic defects. Indeed, recent studies show that developing endothelial cells provide instructive cues to allow proper differentiation of the endoderm into pancreas and liver (Cleaver and Melton, 2003).

Interestingly, $S1P_1$ is expressed in the endothelial cells and interdigital mesenchyme, suggesting the possible role in angiogenesis and remodeling in the developing limb. As limb development ensues, expression of $S1P_1$ is also observed in the tips of digits where cells are removed by apoptosis. Programmed cell death has long been recognized as an important mechanism of normal embryonic development. In the developing limb, massive programmed cell death plays a critical role in controlling the amount of mesodermal tissue, sculpting overall limb shape and defining the digits. Cell death in the limb bud is regulated by several factors, including environmental conditions and interactions between epithelium and mesenchyme (Chen and Zhao, 1998). Removing the ectoderm overlying the interdigital area inhibited cell death, leading to the ectopic cartilage formation in the subjacent mesenchyme. While it is highly likely that $S1P_1$ signaling plays a role in tissue remodeling, further analysis of this function is not possible now since the profound vascular defects masked the outcome in the interdigital areas of $S1p_1^{-/-}$ limb.

Limb defects in the $S1p_1^{-/-}$ embryo are dramatic, which have not been reported in other angiogenic factor null mice. Only VEGF knockouts show a significant growth plate phenotype, whereas deletion of other angiogenic factors, including TGF-β1, -β3 and endostatin, do not result in phenotypic changes in the growth plate (Zelzer et al., 2002). Our finding that $S1P_1$ in endothelial cells is critical for proper limb development underscores the importance of this lipid mediator in development.

This study shows that sphingolipid signaling in the vasculature plays a profound role in the development of the limb tissue. It also points out that abnormal vascular development can induce dramatic changes in surrounding tissues. These mechanistic findings may have implications in the function of $S1P_1$ in adult physiology and pathology. During wound healing and tumor development, $S1P/S1P_1$ signaling may be important in angiogenesis and vascular maturation and thereby influence the growth of the juxtaposed tissues, such as the healing wound or the developing tumor. Such mechanisms should be considered in novel approaches to develop pharmacological tools to modulate the $S1P_1$ G-protein-coupled receptor.

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