Postnatal Recapitulation of Embryonic Hedgehog Pathway in Response to Skeletal Muscle Ischemia

Roberto Pola, MD, PhD*; Leona E. Ling, PhD*; Tamar R. Aprahamian, BS; Elena Barban, MD; Marta Bosch-Marce, PhD; Cynthia Curry, BS; Michael Corbley, PhD; Marianne Kearney, BS; Jeffrey M. Isner, MD†; Douglas W. Losordo, MD

Background—Hedgehog (Hh) proteins are morphogens regulating epithelial–mesenchymal signaling during several crucial processes of embryonic development, including muscle patterning. Sonic (Shh), Indian (Ihh), and Desert (Dhh) hedgehog constitute the repertoire of Hh genes in humans. The activities of all 3 are transduced via the Patched (Ptc1) receptor. Recent observations indicate that exogenous administration of Shh induces angiogenesis. Here, we studied whether the endogenous Hh pathway, in addition to its functions during embryogenesis, plays a physiological role in muscle regeneration after ischemia in adults.

Methods and Results—We found that skeletal muscle ischemia induces strong local upregulation of Shh mRNA and protein. In addition, the Ptc1 receptor is activated in interstitial mesenchymal cells within the ischemic area, indicating that these cells respond to Shh and that the Shh pathway is functional. We also found that Shh-responding cells produce vascular endothelial growth factor under ischemic conditions and that systemic treatment with a Shh-blocking antibody inhibits the local angiogenic response and the upregulation of vascular endothelial growth factor.

Conclusions—Our study shows that the Hh signaling may be recapitulated postnatally in adult and fully differentiated muscular tissues and has a regulatory role on angiogenesis during muscle regeneration after ischemia. These findings demonstrate a novel biological activity for the Hh pathway with both fundamental and potential therapeutic implications. (Circulation. 2003;108:479-485.)

Key Words: genes, hedgehog | ischemia | muscle, skeletal | angiogenesis | tissue regeneration

In the past decade, there has been increasing appreciation of the fact that pathways studied predominantly during embryogenesis and known to be relatively silent during normal adult life may be recruited postnatally in response to tissue injury.1 Hedgehog (Hh) proteins are morphogens that act in a wide variety of tissues during embryonic development2–5 and regulate epithelial–mesenchymal interactions that are crucial to morphogenesis of the nervous system, somite, limb, lung, gut, hair follicle, and bone.3,7,10–12 There are 3 highly conserved Hh genes in mammals: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh).13 The interaction of Hh proteins with their specific receptor patched-1 (Ptc1) inactivates the repression of the transmembrane protein smoothened (Smo), leading to activation of the transcription factor Gli,14–16 the principal mediator of the Hh signaling pathway. Gli induces expression of downstream target genes of the Hh signaling pathway. Thus, Ptc1 and Gli are both components and transcriptional targets of the Hh signaling pathway.

Previous observations have suggested that Hh might also be involved in the vascularization of certain embryonic tissues. First, transgenic overexpression of Shh in the dorsal neural tube is associated with hypervascularization of neuroectoderm,19 whereas a knockout of the zebrafish Shh homologue results in disorganization of the endothelial precursor cells and inability to form the dorsal aorta or axial vein. In addition, Shh-null mice lack proper vascularization of the developing lung.4 More recently, it has been reported that vasculogenesis in the mouse embryo is regulated by Ihh.20 Finally, very recently, we found that cells in the adult cardiovascular tissues express Ptc1 and can respond to exogenous administration of Shh by upregulating Ptc1.21 We also demonstrated that Shh induces neovascularization in 2 different murine models of angiogenesis and upregulates 2 families of angiogenic growth factors, including vascular endothelial growth factor (VEGF) and angiopoietins.21

The aim of this study was to investigate whether the endogenous Hh pathway is physiologically involved in the...
revascularization of ischemic tissue in adults. We used a murine model of muscle regeneration by inducing ischemia of the hindlimb. Then, we observed the expression pattern of different components of the Hh pathway, including Shh, Dhh, Ihh, and Ptc1, and studied the relationship between Hh activation, VEGF expression, and angiogenesis. We found that Shh is activated in the regeneration after ischemia and that interstitial cells within the ischemic area strongly express Ptc1, indicating the postnatal activity of the Hh signaling pathway. We found that Ptc1 expression was associated with VEGF production and angiogenesis. Inhibition of Shh inhibits its endogenous angiogenesis and VEGF production in the ischemic hindlimb. Our data suggest a novel and unexpected physiological role for Shh.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Me), male or female nls-Ptc1-lacZ mice, or their wild-type littermates (Ontogeny, Inc) were used for the ischemic hindlimb experiments. All the experiments were conducted in accordance with the St Elizabeth’s or Biogen Institutional Animal Care and Use Committee.

Murine Ischemic Hindlimb Model

Ischemia was induced in 8- to 12-week-old C57BL/6J mice, nls-Ptc1-lacZ mice, and their wild-type littermates as described previously.

In Situ Hybridization for Hh Members

Skeletal muscles were harvested 4 and 7 days after surgery and immediately immersion-fixed overnight in 4% paraformaldehyde, paraffin-embedded, and sectioned longitudinally at 7 to 8 μm. Shh, Ihh, and Dhh in situ hybridization was performed with digoxigenin-labeled sense and antisense cRNA probes.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

Mice were killed 4 and 7 days after ischemia, and reverse transcription–polymerase chain reaction (RT-PCR) was performed as described previously. The primer sequences were as follows: Shh forward, GAGCAGACCGCTGATAGCT; Shh reverse, AGAGATGCCAACGGCATTAC; Dhh forward, CGCAAGC-CGGCTGATAGC; Dhh reverse, CGGAGCGCTAGAGCGTTCA; Ihh forward, CAAAGCGGCTAGGAGCTTTC; Ihh reverse, AGC-GACCGCGAGGAT. The probe sequences were as follows: Shh 6FAM, AGAGGTGCAAGACAGAGAG-MGBNFQ; Dhh 6FAM, AGCGTGGCAAAAGAG-MGBNFQ; Ihh 6FAM, AGGTCATCGAGACTCA-MGBNFQ.

ELISA

Mice were killed 4 and 7 days after ischemia, and hindlimb muscle specimens were harvested and homogenized in lysis buffer. Shh protein levels were determined by ELISA as described previously.

Western Blotting for Ptc1

Protein extracts from the skeletal muscles of mice killed 7 days after ischemia were used for Western blotting analysis of Ptc1 expression as described previously. Densitometric analysis was performed (NIH Imaging program) to allow for quantitative comparison of protein expression.

Immunofluorescence and Immunohistochemistry

Ischemic and contralateral muscle specimens were harvested 7 days after induction of ischemia, and frozen sections were processed as detailed previously. Primary antisera were goat polyclonal IgG anti-Shh C-terminus, goat polyclonal IgG anti-Ihh C-terminus, and goat polyclonal IgG anti-Dhh C-terminus (Santa Cruz Biotechnology). Horse anti-goat IgG horseradish peroxidase–conjugated antibody (1:500 dilution) (Vector Laboratories) was used as secondary antiserum. Staining was visualized by using FITC-conjugated streptavidin (Pharmingen). For vimentin immunostaining, muscles were fixed in 1% paraformaldehyde for 2 hours. The staining was done on frozen sections with anti-vimentin goat serum (Sigma) compared with normal goat serum (Sigma) using horseradish peroxidase–conjugated donkey anti-goat secondary antibody (Jackson Immunoresearch) or rhodamine-conjugated donkey anti-goat antibody (Santa Cruz Biotechnology). Staining for VEGF was performed with a rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology) with a biotinylated goat anti-rabbit Ig as secondary antibody.

LacZ Immunofluorescence and Histochemistry in nls-Ptc1-lacZ Mice

For β-gal immunofluorescence staining, hindlimb muscles from nls-Ptc1-lacZ mice were harvested and processed as described previously.

Inhibition of the Hh Pathway and Analysis of Local VEGF Expression and Analysis of the Angiogenic Response to Ischemia

Unilateral hindlimb ischemia was induced as described above in 8- to 12-week-old C57BL/6J mice. Animals were treated with daily intraperitoneal injections of 10 mg/kg 5E1 blocking antibody or the same amount of 1E6 control antibody. 5E1 blocks the binding of Shh to Ptc1; it was obtained from Curis Inc and Dr Thomas Jessell (Columbia University) and prepared as purified IgG1 in PBS. Seven days after induction of ischemia, mice were killed. Hindlimb muscle specimens were harvested, processed, and analyzed by Western blotting for VEGF expression. For analysis of the response to ischemia, animals were divided into 2 groups: the first group received a total dose of 1.25 mg 5E1, delivered systemically via a osmotic pump over a period of 21 days, and the second group received an equal amount of 1E6. Ten animals in each group were studied. At days 7, 14, 21, and 28 after induction of ischemia, blood flow was measured with a laser Doppler perfusion imaging system as described previously.

Analysis of capillary density was performed as described previously.

Statistical Analysis

All results are expressed as mean ± SD, with the exception of the real-time RT-PCR results, which are presented as mean ± SEM. Group differences were analyzed by ANOVA or Student’s t test. Differences were considered statistically significant at a value of P < 0.05.

Results

Shh Signaling Pathway Is Activated in Ischemic Regenerating Skeletal Muscles

The expression of Shh mRNA was increased at 4 and 7 days after injury in ischemic compared with nonischemic skeletal muscle, as detected by in situ hybridization (Figure 1a). Shh mRNA in ischemic muscle was strongly induced, particularly in the interstitial regions. Expression of Ihh was also slightly elevated in ischemic muscle, whereas little Dhh expression was detected at 4 or 7 days after ischemic injury (Figure 1b).

Upregulation of Shh was confirmed and quantified by real-time RT-PCR: Shh expression increased 10±3.3-fold in day 4 ischemic muscles (P<0.01) and 16±3.8-fold at day 7 (P<0.0007) (Figure 2a and data not shown). No significant increment of Dhh and Ihh expression was documented.
transduction pathway and Ptc1 expression is known to occur.

Shh is a downstream transcriptional target of the Shh signal during muscle regeneration after ischemia in adults. Because ischemic tissue produces Shh and expresses the Ptc1 gene, indicating that the Shh pathway is physiologically active (Figure 3, e–g). These data demonstrate that cells in the ischemic area, within and around skeletal muscle fibers, are immunopositive for Shh within 7 days after induction of ischemia (Figure 3, a and b). No positive immunostaining in ischemic muscle was observed at this time point for either Ihh or Dhh (data not shown).

Immunofluorescence analysis demonstrated that several cells are immunopositive for Shh within 7 days after induction of ischemia (Figure 3, a and b). Strong positive signal for Shh mRNA is present in interstitium of ischemic muscles at both days 4 and 7 after surgery, whereas no signal is detected in contralateral nonischemic specimens (a); Ihh mRNA seems slightly elevated in ischemic muscle (b). Very little Dhh expression is detected at 4 or 7 days after ischemic injury (b) (H&E indicates hematoxylin and eosin; Shh AS, Shh Antisense probe; Shh S, Shh Sense probe; Ihh AS, Ihh Antisense probe; Dhh AS, Dhh Antisense probe).

Figure 1. Shh mRNA is upregulated during skeletal muscle regeneration after ischemia. In situ hybridization for Shh, Ihh, and Dhh 4 and 7 days after ischemia. Strong positive signal for Shh mRNA is present in interstitium of ischemic muscles at both days 4 and 7 after surgery, whereas no signal is detected in contralateral nonischemic specimens (a); Ihh mRNA seems slightly elevated in ischemic muscle (b). Very little Dhh expression is detected at 4 or 7 days after ischemic injury (b) (H&E indicates hematoxylin and eosin; Shh AS, Shh Antisense probe; Shh S, Shh Sense probe; Ihh AS, Ihh Antisense probe; Dhh AS, Dhh Antisense probe).

The concentration of Shh protein in ischemic and contralateral muscles was studied by ELISA and was shown to be increased at day 4 ($P<0.03$) and day 7 ($P<0.02$) (Figure 2b). Ptc1 upregulation was verified by Western blotting (Figure 2c) and normalized for tubulin expression, was also statistically significant ($P<0.01$) (Figure 2d).

Immunofluorescence analysis demonstrated that several cells are immunopositive for Shh within 7 days after induction of ischemia (Figure 3, a and b). No positive immunostaining in ischemic muscle was observed at this time point for either Ihh or Dhh (data not shown). Immunofluorescence staining for $\beta$-gal, performed in nls-Ptc1-lacZ mice, demonstrated the expression of PtC1 in several cells in the ischemic tissue (Figure 3, c and d). Interestingly, both Shh- and PtC1-positive cells appeared to be interstitial cells widely distributed in the ischemic area, within and around skeletal muscle fibers. By performing double immunofluorescence staining for Shh and $\beta$-gal in nls-Ptc1-lacZ mice, we demonstrated that Shh and PtC1 are coexpressed in the same cells (Figure 3, e–g). These data demonstrate that cells in the ischemic tissue produce Shh and express the PtC1 gene, indicating that the Shh pathway is physiologically active during muscle regeneration after ischemia in adults. Because PtC1 is a downstream transcriptional target of the Shh signal transduction pathway and PtC1 expression is known to occur in response to Shh signaling, expression of Ptc1 constitutes evidence of active Shh signaling in ischemic skeletal muscle. In addition, these findings suggest that in this model, an autocrine interaction occurs between the Shh ligand and its receptor Ptc1.

Shh and Ptc1 Activation Occur in Interstitial Mesenchymal Cells and Are Associated With VEGF Production

To determine the identity of the Shh-producing and -responding cells during ischemia, we performed further immunohistochemical analyses. These interstitial cells were not positive for the endothelial cell marker CD31 or the smooth muscle cell marker $\alpha$-SM-actin (data not shown). In contrast, we found that Shh- and Ptc1-positive cells stained positive for vimentin, consistent with mesenchyme-derived fibroblasts (Figure 4, a–d).

We also analyzed the relationship between the activated, endogenous Shh pathway and VEGF in ischemic skeletal muscle. We found that Ptc1-positive interstitial cells located within the ischemic area were strikingly immunopositive for VEGF (Figure 4e). The colocalization in the same cells of Ptc1 and VEGF suggests that the Shh signaling pathway may stimulate, either directly or indirectly, VEGF expression within the neovascular foci. No Ptc1 (X-gal) or VEGF staining was observed in either the contralateral or control muscles (data not shown).

The time course of upregulated Ptc1 expression after the onset of ischemia, inferred from whole-mount X-gal staining of nls-Ptc1-lacZ hindlimbs, was characterized by Ptc1 upregulation beginning 4 days after ischemia, peaking at day 7, and decreasing significantly by day 14 after ischemia (Figure 4f).

Inhibition of Shh Signaling Pathway Impairs Local VEGF Upregulation

To determine whether the Shh signaling pathway is necessary for VEGF upregulation during ischemia, we used a Shh-neutralizing antibody (5E1). After unilateral hindlimb ischemia had been induced in mice, animals were treated for 7 days with systemic injections of 5E1 or control antibody (1E6). Local VEGF expression was studied by Western blotting in both ischemic and contralateral muscles. As expected, mice treated with the 1E6 control antibody exhibited a physiological upregulation of VEGF in the ischemic hindlimbs (Figure 5a). In contrast, animals treated with the 5E1 antibody did not upregulate VEGF in the ischemic hindlimb (Figure 5a). Comparison of VEGF expression in ischemic muscle, normalized for tubulin expression, indicated a statistically significant difference between mice treated with 5E1 versus 1E6 ($P<0.01$) (Figure 5b).

Inhibition of the Shh Signaling Pathway Decreases the Angiogenic Response to Ischemia

Hh-blocking antibody (5E1) or a control antibody (1E6) was administered by continuous subcutaneous infusion through an osmotic pump for 3 weeks after induction of hindlimb ischemia. Twenty-eight days after induction of ischemia, blood flow was significantly lower in animals treated with the Shh-blocking antibody ($P<0.01$) (Figure 5c). Capillary den-
sity was assessed by CD31 immunostaining and was significantly reduced as well ($P<0.0001$) (Figure 5, d and e). These results indicate that the activation of the Shh pathway is a prerequisite for the postnatal angiogenic response to skeletal muscle ischemia.

**Discussion**

The Hh pathway has been studied and characterized extensively during embryogenesis. The vast majority of these prenatal studies have focused on the role of Hh family members in the regulation of epithelial–mesenchymal interactions crucial to limb, lung, gut, hair follicle, and bone formation,3–6 including a possible role during vascularization of certain embryonic tissues.4,19,20,27–29 In contrast, a role for the Hh family in the regulation of postnatal tissue regeneration and revascularization has received limited attention.30–32 We recently demonstrated that exogenous administration of Shh induces neovascularization in both corneal and ischemic hindlimb models of angiogenesis.21 Shh stimulates fibroblasts in vitro to produce a combination of potent angiogenic factors, including the 3 major isoforms of VEGF, Ang-1, and Ang-2.21 Shh seems to act as an indirect angiogenic agent and may trigger neovascularization through Shh/Ptc1 signaling specifically in mesenchymal cells.21

Following these observations, we investigated the hypothesis that the Hh pathway may be postnatally recapitulated in response to skeletal muscle ischemia and discovered that in adult mice, a strong upregulation of Shh and Ptc1 occurs during regeneration of ischemic skeletal muscle. These findings are consistent with previous reports in the literature describing the association of both ischemia and tissue regeneration with the reactivation of genes involved in fetal transcription programs.33–35

After ischemia, Ptc1 expression occurs in interstitial mesenchymal fibroblasts. The ability of fibroblasts to respond to Shh stimulation has already been demonstrated: eg, fibroblasts respond to Shh stimulation in vitro,21 physiologically express Ptc1 in adult perineural sheaths and dermis,6,9 and upregulate Ptc1 and VEGF during Shh-induced corneal neo-
vascularization. Taken together, these data strongly suggest that fibroblasts are central mediators of Shh activity during muscle regeneration.

In our ischemic model, interstitial mesenchymal fibroblasts also expressed Shh. The coexpression of Shh and Ptc1 in the same cells indicates the presence of an autocrine mechanism regulating Shh signaling in ischemic muscle. Such an autocrine mechanism has already been described in adult pancreas, in which Dhh and Ihh are coexpressed with Ptc1 in pancreatic β-cells and regulate insulin production.

In muscle regeneration after ischemia, a crucial role is played by angiogenesis. In this study, we show that Ptc1-positive interstitial fibroblasts within the ischemic area produce VEGF. We also show that the inhibition of Shh signaling is sufficient to decrease local VEGF upregulation. Similarly, ischemia-induced angiogenesis is decreased by inhibition of the Shh pathway. These results indicate that although Ptc1-positive fibroblasts do not represent the majority of VEGF-producing cells during ischemia, the activation of the Shh signaling pathway is crucial for the overall production of VEGF and the related angiogenic response. Indeed, interstitial fibroblasts are important supporting cells, and their function, modulated by Shh, might be fundamental for stimulating the angiogenic activity of neighboring cells.

The mechanism by which ischemia and/or hypoxia upregulates Shh expression remains uncertain. The promoter regions of Hh family members are not known to include a hypoxia-inducible factor sequence, and no data are available about the possible interactions between Shh- and hypoxia-inducible factor pathways in regulating VEGF synthesis and stabilization. Interestingly, it has been reported that in mice with a deletion of the hypoxia-response element in the VEGF promoter, fibroblasts are still able to upregulate VEGF under hypoxic conditions. This finding is apparently specific for fibroblasts and, in association with the ability of these cells to produce VEGF on Shh stimulation, indicates that fibroblasts...
may have hypoxia-independent mechanisms to upregulate VEGF, potentially involving direct regulation by the Hh pathway transcriptional factor Gli. However, no Gli response elements are present in the VEGF promoter region. Hh can, however, also induce a Gli-independent pathway, which activates the orphan nuclear receptor COUPTF-II.37 Interestingly, COUPTF-II–null embryos are defective in maturation of the primary vascular plexus.38 Thus, it is possible that the induction of angiogenic growth factors by Hh occurs via COUPTF-II activation in mesenchymal cells (Figure 6).

The development of functional neovascularization in regenerating tissues requires precise spatial–temporal regulation of cell proliferation, migration, interaction, and differentiation. The role of Shh as a morphogen may be relevant to its potential activity to orchestrate appropriate postnatal angiogenesis after tissue injury. The activation of components of the Hh pathway during ischemia and the reduced angiogenesis observed after inhibition of Hh suggest a crucial role for these morphogens in the pathophysiology of muscle regeneration. In addition, these results open the possibility that members of the Hh family might play a role in the development of angiogenesis–related diseases, such as diabetic retinopathy or tumor angiogenesis. Finally, influencing angiogenesis by modulating the Hh pathway might have important implications for both proangiogenic and antiangiogenic therapeutic strategies.

Acknowledgments

This project was supported in part by National Institutes of Health grants HL-53354, HL-57516, HL-60911, HL-63414, HL-63695, AG-16323, and HL-66957 and the Shaughnessy Center for Clinical Genetics. Dr Pola is a recipient of a grant for Young Investigators from the A. Gemelli University Hospital (Rome, Italy) and the Italian Ministry of University and Scientific and Technological Research. We also acknowledge Norm Affaire for performing the Taqman experiments, the Beth Israel–Deaconess In Situ Hybridization Core Facility, and Dr Urs Berger for his excellent technical assistance.

References


Postnatal Recapitulation of Embryonic Hedgehog Pathway in Response to Skeletal Muscle Ischemia

Roberto Pola, Leona E. Ling, Tamar R. Aprahamian, Elena Barban, Marta Bosch-Marce, Cynthia Curry, Michael Corbley, Marianne Kearney, Jeffrey M. Isner and Douglas W. Losordo

_Circulation_. 2003;108:479-485; originally published online July 14, 2003;
doi: 10.1161/01.CIR.0000080338.60981.FA

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circ.ahajournals.org/content/108/4/479