Fibromodulin Enhances Angiogenesis during Cutaneous Wound Healing

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Background: Fibromodulin (FMOD) plays a critical role in the wound-healing process. Our previous studies revealed that FMOD deficiency led to marked alterations in adult wound healing characterized by delayed dermal cell migration, postponed wound closure, and increased scar formation, all accompanied by impeded angiogenesis. Therefore, the aim of this study was to reveal the effect of FMOD on angiogenesis during the wound-healing process.

Methods: In vivo angiogenic effects of FMOD were assessed by a chick embryo chorioallantoic membrane assay, a Matrigel (BD Bioscience, Franklin Lakes, N.J.) plug implant assay, and rodent primary closure wound models. In vitro angiogenic effects of FMOD were recorded by cell invasion and dimensional and topological parameters of human umbilical vein endothelial cells.

Results: We provided evidence that FMOD significantly enhanced vascularization: first, FMOD boosted blood vessel formation on the chorioallantoic membrane; second, FMOD markedly stimulated capillary infiltration into Matrigel plugs subcutaneously implanted in adult mice; and finally, FMOD robustly promoted angiogenesis in multiple adult rodent cutaneous wound models. Furthermore, FMOD administration restored the vascularity of fmod−/− mouse wounds. In support of this, FMOD endorsed an angiogenesis-favored microenvironment in adult rodent wounds not only by upregulating angiogenic genes but also by downregulating angiostatic genes. In addition, FMOD significantly enhanced human umbilical vein endothelial cell invasion and tube-like structure formation in vitro.

Conclusions: Altogether, we demonstrated that in addition to reducing scar formation, FMOD also promotes angiogenesis. As blood vessels organize and regulate wound healing, its potent angiogenic properties will further expand the clinical application of FMOD for cutaneous healing of poorly vascularized wounds. (Plast Reconstr Surg Glob Open 2014;2:e275; doi: 10.1097/GOX.0000000000000243; Published online 23 December 2014.)

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A fundamental problem of retarded wound healing is lack of a functional extracellular matrix (ECM) to stimulate, direct, and coordinate healing. For instance, deficiency of a single ECM molecule, fibromodulin (FMOD), in an adult mouse cutaneous wound model resulted in delayed dermal fibroblast migration, delayed granulation tissue formation, delayed wound closure, and subsequently increased scarring. FMOD is a broadly distributed small leucine-rich proteoglycan (SLRP), which regulates ECM assembly, organization, and degradation via binding with collagens. FMOD plays an essential role in cell fate determination and fetal scarless wound healing. In addition, our previous studies have demonstrated that FMOD controls significant aspects of adult cutaneous wound healing. Compared with their wild-type (WT) counterparts, FMOD-null mice have reduced fibronectin deposition, unorganized collagen architecture, altered transforming growth factor (Tgfβ) signaling, and reduced dermal fibroblast infiltration followed by impeded angiogenesis. On the other hand, FMOD administration in both adenoviral and protein forms reduced scar formation in adult cutaneous wounds. Specifically, we have demonstrated that FMOD significantly promoted fibroblast migration into the wound area, aiding timely wound closure and reduced scar formation.

Because newly generated blood vessels provide nutrients to support active cells, promote granulation tissue formation, and facilitate clearance of debris, wound healing cannot occur without angiogenesis, a process of neovascular formation by endothelial cells (ECs). Our previous studies revealed that retarded mouse wound healing is associated with markedly reduced blood vessel regeneration, suggesting a direct relationship between FMOD and angiogenesis. In this study, the effects of FMOD on angiogenesis under both uninjured and wounded scenarios were investigated.

MATERIALS AND METHODS

Ethics Statement
All animal surgeries were performed under institutional approved protocols provided by Chancellor’s Animal Research Committee at University of California, Los Angeles (protocol number: 2000-058).

In Ovo Chick Embryo Chorioallantoic Membrane Assay
The in ovo chorioallantoic membrane (CAM) assay was performed as previously described. Fertilized chicken eggs (Charles River Labs, North Franklin, Conn.) were incubated at 37°C and 60% relative humidity in an egg incubator. On day 3, 5-ml albumin was withdrawn from the pointed end of the egg. Rectangle windows were cut into the shell as a portal of access for the CAM. On day 10, 2.0 mg/ml FMOD in 30 μl 1:3-diluted growth-factor-reduced Matrigel (BD Biosciences, Franklin Lakes, N.J.) was loaded on an autoclaved 5 × 5-mm polyester mesh layer (grid size: 530 μm; Component Supply Company, Fort Meade, Fla.) and incubated for 45 minutes at 37°C for gel formation before transplantation onto the CAM. A non-FMOD phosphate buffered saline (PBS) control was transplanted onto the same CAM with a 1-cm distance. On day 13, CAMs were excised and photographed. The capillary area density directly under the mesh was measured by ImageJ (National Institutes of Health, Bethesda, Md.).

Matrigel Plug Assay
Four hundred μl of growth-factor-reduced Matrigel containing 0 or 4.0 mg/ml FMOD was subcutaneously injected into the abdomen of adult 129/sv male mice, which were harvested with the overlying skin 14 days post injection.

Wound Generation
Four (per adult male 129/sv mouse) or 6 (per adult male Sprague-Dawley rat) full thickness, 10 mm
Histology and Immunohistochemistry Staining

After fixation in 4% paraformaldehyde, samples were dehydrated, paraffin-embedded, and sectioned at 5-μm increments for hematoxylin and eosin (H&E), picrosirius red (PSR), and histology and immunohistochemistry staining. PSR-coupled polarized light microscopy (PSR) was used to identify the wound area. Blood vessels were identified and quantitated by von Willebrand factor (Abcam Inc., Cambridge, Mass.).

Gene Expression Assay

RNA was isolated using RNeasy Mini Kit with DNase treatment (Qiagen, Valencia, Calif.) and 1.0 μg mouse RNA was used for reverse transcription with iScript Reverse Transcription Supermix for real time-quantitative polymerase chain reaction (Bio-Rad Laboratories, Hercules, Calif.). Quantitative real time polymerase chain reaction (qRT-PCR) was performed with TaqMan Gene Expression Assays (Life Technologies, Grand Island, N.Y.) and SsoFast Probes Supermix with ROX (Bio-Rad Laboratories) on a 7300 Real-Time PCR system (Life Technologies). Meanwhile, 2.5 μg RNA isolated from adult rat wounds was injected into RT² First Strand Kit (Qiagen) for reverse transcription. qRT-PCR was performed in a 96-well format of rat wound-healing RT² PCR Array (Qiagen) according to the manufacturer’s protocol. Three different cDNA templates were tested. Concomitant glyceraldehyde 3-phosphate dehydrogenase (gapdh) was used as a housekeeping standard. Data analysis was achieved by the manufacturer’s online services (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

Cell Culture

Passages 3–6 human umbilical vein endothelial cells (HUVECs) were cultured in Medium 200PRF supplied with Low Serum Growth Supplement according to manufacturer instruction (Life Technologies).

Tube-like Structure Formation Analysis

Technologies Endothelial Tube Formation Assay protocol provided by Life Technologies (http://www.lifetechnologies.com/us/en/home/references/protocols/cell-and-tissue-analysis/cell-proliferation-assay-protocols/angiogenesis-protocols/endothelial-cell-tube-formation-assay.html) was used to assay tube-like structure (TLS) in vitro. Briefly, a 24-well plate was coated with 100 μl/well reduced growth factor basement membrane matrix for 1 hour at 37°C before being seeded with 2.5 × 10⁴ HUVECs in Medium 200PRF supplied with different doses of FMOD. Five images per well and 4 wells per treatment were documented after 4 hours by using an Olympus fluorescent microscope (Center Valley, Pa.). Images were assessed by recording dimensional and topological analyses with ImageJ (http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analys-er-for-ImageJ.html&amp;lang=en#outil_sommaire_0).

RESULTS

FMOD Promotes Vascularization in Uninjured Scenarios

FMOD-administrated CAMs showed a 1.5 times greater proportion of blood vessels with large diameters than the PBS control (Fig. 1), confirming that FMOD promotes vasculogenesis during development. As angiogenesis in adults may differ in important ways from the process during development, a predocumented Matrigel plug assay was used to confirm the proangiogenic action of FMOD.
in vivo. FMOD markedly elevated angiogenesis in Matrigel plugs subcutaneously implanted in adult mice, whose capillary densities were 4-fold that of non-FMOD plugs. (See figure, Supplemental Digital Content 1, which displays Matrigel plugs subcutaneously injected into the abdomen of adult 129/sv male mouse, http://links.lww.com/PRSGO/A77.) Thus, FMOD is a proangiogenic factor in uninjured scenarios.

**FMOD Is Important for Angiogenesis during Wound Healing**

In agreement with our previous studies at day 7 post injury, vascular generation in adult fmod−/− mouse skin wounds at day 14 post injury was diminished by approximately 50% as compared with the age-matched WT wounds (Fig. 2). [See Supplemental Digital Content 2, which displays H&E staining and a PSR-coupled polarized light microscopy demonstration of adult mouse cutaneous wounds (outlined by dashed lines) at day 14 post injury, http://links.lww.com/PRSGO/A78.] On the contrary, exogenous FMOD administration restored vascularity of fmod−/− wounds to the same level as that of FMOD-treated WT wounds, further signifying that FMOD deficiency was responsible for the reduced angiogenesis in fmod−/− mouse wounds (Fig. 2). Additionally, capillary density of FMOD-treated adult WT mouse skin wounds was approximately 2.6 times

![Fig. 1. Effects of FMD on vascularization assessed by in ovo CAM assay. Macropscopic photographs (A) and computerized quantitation (B) showed significantly increased more capillary generation on 30 μl 2.0 mg/ml FMOD-treated CAMs than on PBS-control groups. Significant differences compared by paired t test (P < 0.05) are marked with asterisks (n = 5). Bar = 500 μm.](image)

![Fig. 2. von Willebrand factor staining of adult mouse cutaneous wounds. Sections of PBS-treated WT (A), FMOD-treated WT (B), PBS-treated fmod−/− (C), FMOD-treated fmod−/− (D) wounded mouse skin at day 14 post injury, whose wound capillary density was quantitated (E). Wound areas are outlined by dashed lines and blood vessels are indicated by red arrowheads. (See Supplemental Digital Content 2, which displays H&E staining and a PSR-coupled polarized light microscopy demonstration of adult mouse cutaneous wounds (outlined by dashed lines) at day 14 post injury, http://links.lww.com/PRSGO/A78.] FMOD: 0.4 mg/ml × 50 μl/wounds. Significant differences compared by Mann-Whitney analysis (P < 0.05) are marked with asterisks: red asterisk indicates significance resulting from fmod knockout, and blue asterisks indicate significance resulting from FMOD administration. Bar = 200 μm.)
Fig. 3. von Willebrand factor (vWF) staining of rat mouse cutaneous wounds at day 14 post injury. PSR-coupled polarized light microscopy demonstrated the wound area (A; B; outlined by dashed lines), whereas the blood vessels were identified by histology and immunohistochemistry staining against vWF (C; D; red arrowheads) and were quantitated (E). H&E staining of the identical wounds were shown in Supplemental Digital Content 3 (which displays H&E staining of adult rat cutaneous wounds at day 14 post injury, http://links.lww.com/PRSGO/A79). FMOD: 0.4 mg/ml × 50 μl/wounds. Significant differences compared by Mann-Whitney analysis (P < 0.05) are marked with asterisks. Bar = 200 μm.

Fig. 4. Gene expression in adult WT and fmod−/− mouse cutaneous wounds. Expression levels of vegf (A) and angpt1 (B) were measured by real-time PCR and were normalized to uninjured adult WT skin tissue (dashed lines). FMOD: 0.4 mg/ml × 50 μl/wounds. Data are presented as mean ± SD (n = 3 different cDNA templates, each template underwent reverse transcription from an RNA pool of 3 wounds harvested from 3 different animals, a total of 9 wounds from 9 animals per treatment were used). Significant differences compared by 2-sample t test (P < 0.05) are marked with asterisks: red asterisks indicate the significance from fmod knockout, and blue asterisks indicate the significance that resulted from exogenous FMOD administration.
no considerable difference in angpt1 expression in unwounded skin tissues was observed between adult WT and fmod−/− mice, transcription levels of angpt1 were significantly lower in fmod−/− wounds after wound closure compared with that of age-matched WT mouse wounds (Fig. 4B). Interestingly, FMOD-treated WT and fmod−/− adult mouse wounds had similar vegf and angpt1 levels at day 14 post injury (Fig. 4), which was correlated to their similar wound capillary densities (Fig. 2). Considering the fact that fmod−/− wounds have decreased vascularity which can be rescued by exogenous FMOD administration, these data are highly associated with Vegf’s critical angiogenic function during granulation tissue formation and Angpt1’s important mediation of vessel remodeling and maturation.

Numerous angiogenic and angiostatic factors have been identified in the past. To further enrich our knowledge of how FMOD affects angiogenesis-related genes during wound healing, a RT² PCR Array for rat wound healing (Qiagen) was employed for high-throughput gene expression analysis in an adult rat cutaneous wound model. As seen in the adult mouse data shown above, FMOD administration elevated both angpt1 and vegf expression (Fig. 5). Moreover, FMOD not only upregulated the expression of angpt1 and vegf but also upregulated expression of other angiogenic genes, such as tgfα (which stimulates chemotactic response, proliferation, and Vegf expression of ECs), fibroblast growth factor (fgf)2 (which induces EC proliferation, migration, and Vegf secretion), platelet-derived growth factor (pdgf)α [which escorts connective tissue cells (such as fibroblasts and mast cells) into the wound area to produce angiogenic factors and enhances angiogenic effects of Vegf and Fgf2], and colony stimulation factor (csf)3 (which recruits monocytes to trigger the synthesis of angiogenic cytokines) after wound closure (Fig. 5). On the other hand, FMOD reduced the levels of angiostatic genes including interferon (ifn)γ (which inhibits EC growth and Vegf expression) and blocks capillary growth induced by Fgf and Pdgf), tgfβ1 (which hinders activation of differentiated ECs for sprouting and thus maintains endothelial quiescence), and plasminogen (plg) which inhibits EC proliferation and their response to Fgf and Vegf) after wound closure (Fig. 5). Therefore, FMOD endorsed an angiogenesis-favoring gene expression network in adult rodent wound models.

**FMOD Prompts EC TLS Formation In Vitro**

To explore the direct effects of FMOD on EC sprouting, the initial step of angiogenesis, primary HUVECs were seeded in Geltrex matrix (Life Technologies), which contains laminin, collagen IV, elastin, and heparin sulfate proteoglycans to model a wound-healing angiogenic situation. HUVECs spontaneously acquired elongated morphology and formed a capillary network in the gel, clearly visible by 3 hours post seeding (Fig. 6A). A broad range of FMOD (10–250 µg/ml) markedly enhanced HUVEC TLS formation and subsequently established polygon structures referred to as complex meshes (Fig. 6A). Quantitative analyses demonstrated that FMOD significantly increased both dimensional (total length of cellular TLS network per area) and topological parameters (number of junctions, branches, and meshes per area) (Fig. 6B) of HUVEC TLSs. In agreement with previous studies that revealed the positive rela-

![Fig. 5. RT² PCR Array for angiogenic and angiostatic gene expression during adult rat cutaneous wound healing. Gene expressions at day 7 (A) and 14 (B) post injury are shown. Angiogenic genes include angpt1, vegf, tgfα, fgf2, pdgfα, and csf3, whereas angiostatic genes include ifnγ, tgfβ1, and plg. FMOD: 0.4 mg/ml × 50 µl/wounds. Data are presented as mean ± SD (n = 3 different cDNA templates, each template underwent reverse transcription from an RNA pool of 3 wounds harvested from 3 different animals, a total of 9 wounds from 9 animals per treatment were used) and normalized to uninjured rat skin tissue (dashed lines). Significant differences compared by 2-sample t test (P < 0.05) are marked with asterisks.](image-url)
Fig. 6. TLS formation by HUVECs on Geltrex matrix in vitro. A, Light microscopy of HUVECs spontaneously formed TLSs (outlined). B, Dimensional and topological parameters of the HUVEC TLS network were quantified. Significant differences compared by Mann-Whitney analysis ($P < 0.05$) are marked with asterisks ($n = 16$). Bar = 200 μm.

**DISCUSSION**

Angiogenesis, a process of neovascular formation from preexisting blood vasculature by sprouting, splitting, and remodeling of the primitive vascular network, results from multiple signals acting on ECs regulated by diverse groups of growth factors and ECM molecules.\(^\text{31,43,53}\) Until now, most studies on angiogenesis focused on soluble factors, such as Vegf and Fgf2.\(^\text{29–32,41}\) However, increasing reports reveal that cell-ECM interaction is also critical for EC growth, differentiation, apoptosis, and response to soluble growth factors.\(^\text{10,55,56}\) For instance, blockage of EC-ECM interactions inhibits neovascularization relationship between EC migration and polygon structure formation,\(^\text{54}\) we found that FMOD significantly stimulated HUVEC invasion through the Geltrex matrix in vitro (Fig. 7). Therefore, FMOD exhibits its angiogenic function, at least partially, via promotion of EC migration/invasion.

Fig. 7. In vitro invasion assay of HUVECs. Data are presented as mean ± SD ($n = 6$) and normalized to non-FMOD PBS-treated control group. Significant differences compared by 2-sample t test ($P < 0.05$) are marked with asterisks. One-way analysis of variance revealed that there is no significant difference between 10, 50, and 250 μg/ml FMOD groups.
in vivo and TLS formation in vitro. These findings indicate that successful angiogenesis requires a dynamic temporally and spatially regulated interaction between ECs, angiogenic factors, and surrounding ECM molecules such as SLRPs.

SLRPs are a family of proteins, including decorin, lumican, and FMOD, that are present within the ECM of all tissues. As recent studies have shown that SLRPs interact with a diversity of cell surface receptors, cytokines, growth factors, and other ECM components resulting in modulation of cell-ECM cross-talk and multiple biological processes, the common functionalities of SLRPs are far beyond their structural functions in the ECM. Specifically, intense studies present a controversial function of decorin in angiogenesis: decorin is angiogenic during development and normal wound healing but is antiangiogenic during tumor angiogenesis due to its ability to interfere with thrombospondin-1, suppress endogenous tumor VEGF production, and evoke stabilization of pericellular fibrillar matrix. Additionally, Niewiarowska et al revealed that lumican inhibits angiogenesis by reducing proteolytic activity of ECs. However, unlike decorin and lumican, our current study revealed that FMOD is an angiogenic ECM molecule. Although FMOD and lumican present close homology and share the same binding region on type I collagen, their diverse influences on angiogenesis and epithelial migration further support the hypothesis that FMOD and lumican do not seem to be functionally redundant, especially during cutaneous wound healing.

In this study, we demonstrated that not only did FMOD markedly enhance vasculogenesis during development, as documented by the in vivo CAM assay, but it also significantly stimulated angiogenesis as evidenced by the Matrigel plug assay and capillary density measurements in adult rodent cutaneous wound models. Additionally, impaired wound angiogenesis in fmod−/− mice could be restored by exogenous FMOD administration. At the cellular level, we confirmed that FMOD boosted HUVEC migration/invasion and TLS formation in vitro. Our previous studies also found that without considerable influence on EC proliferation, FMOD promoted EC cell adhesion, spreading, and actin stress fiber formation for vascularization in vitro. Thus, FMOD is an angiogenic ECM molecule that directly modulates EC behaviors. In addition to ECs, mural cells (such as fibroblasts and pericytes) and inflammatory cells (such as monocytes and mast cells) also contribute to wound angiogenesis. By stimulating expression of various angiogenic factors including angpt1, vegf, tgfα, tgfβ, pdgfr, and csf3, FMOD also activated these angiogenesis-related cells in vivo during the wound-healing process. In contrast, Ifnγ and Plg are antiangiogenic, proinflammation molecules involved in wound healing. Additionally, Plg, in particular, also plays an important role in re-epithelialization, as keratinocyte migration over the wound is delayed in Plg-deficient mice. In this study, FMOD administration reduced ifnγ and plg levels and increased angiogenesis in adult rodent wounds, which is highly correlated with our previous observation that cutaneous wounds of fmod−/− mice exhibited extended inflammation, elevated epithelial migration, and insufficient angiogenesis. Moreover, Tgfβ1, a multipotent growth factor that regulates wound healing, promotes EC differentiation in a Vegf-independent manner at early stages of development but inhibits sprouting angiogenesis in differentiated ECs. Thus, lower tgfβ1 transcription after wound closure could also contribute to enhanced angiogenesis in FMOD-treated wounds. Consistent with previous studies, FMOD administration induced a proangiogenic microenvironment for wound healing in vivo by stimulating angiogenic factors and reducing angiostatic molecules.

**CONCLUSIONS**

In summary, as one of the pioneer groups investigating the influence of SLRPs on wound healing, we elucidated the angiogenic properties of FMOD in wounded scenarios, which function at least partially by promoting EC activation and infiltration in the wound area. Although translation from the preclinical to the clinical setting can be difficult due to an increased number of external factors such as bacterial inhibition, taken together, current studies suggest that FMOD maintains the potential to be an attractive therapeutic candidate for wound management, especially for patients suffering from impaired wound healing due to aberrant cellular infiltration and insufficient angiogenesis, such as in the cases of diabetic wounds.
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