Knockdown of the KINDLIN-2 Gene and Reduced Expression of Kindlin-2 Affects Vascular Permeability in Angiogenesis in a Mouse Model of Wound Healing

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Background: Angiogenesis is an important component of wound healing and tissue repair. Kindlin-2 is an integrin-associated protein, encoded by the KINDLIN-2 gene, which has been shown to affect cell adhesion and migration of cells, including endothelial cells. The aim of this study was to use a mouse model of wound healing to evaluate the effects of expression of KINDLIN-2 on angiogenesis in wound healing in vivo.

Material/Methods: Thirty-six male C57BL/6 mice were studied in an established model that used a wound created on the back. Mice were divided randomly into three groups: the normal group (n=12) received injections of normal (0.9%) saline; the KINDLIN-2(−) group (n=12) received injections of adeno-associated virus with small interfering (si) RNA targeting the KINDLIN-2 gene (AAV-KINDLIN-2-siRNA); and the control (group (n=12) received injections of adeno-associated virus containing a scrambled RNA sequence (AAV-control-RNA). Wound healing was analyzed by biochemical examination of the exudates and histology. Evans blue dye was injected into the caudal vein of each mouse, two weeks after wound healing to assess neovascular permeability.

Results: Wound healing was significantly delayed in the KINDLIN-2 gene knockdown mice (AAV-KINDLIN-2-siRNA) compared with that of the normal group and the control group, and neovascular permeability was increased. In the AAV-KINDLIN-2-siRNA group, blood vessels were shorter and thinner compared with the normal group and the control group.

Conclusions: In a mouse model of wound healing, KINDLIN-2 gene knockdown inhibited wound healing, and increased neovascular permeability in vivo.

MeSH Keywords: Dependovirus • Neovascularization, Pathologic • Wound Healing

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Material and Methods

Background

Angiogenesis is the development of new blood vessels and is a fundamental physiologic process that enables the supply of oxygen and nutrients to promote tissue repair and chronic inflammation. Angiogenic vessels initially consist of endothelial cells (ECs). New vessels in wounds sprout or form new vascular buds, either from pre-existing vessels or, more rarely, by the recruitment of circulating bone marrow-derived endothelial progenitor cells (stem cells) [2,3]. Although normal angiogenesis is part of the process of reparative wound healing and tissue repair, abnormal or excessive angiogenesis can result in diseases, such as diabetic retinopathy.

Kindlins are also known as fermitin family homolog-containing proteins and are a novel family of adaptor proteins that have key roles in multiple cell types and which promote the integrin cell signaling [4–7]. Three vertebrate kindlin family members include Kindlin-1, which is expressed in epithelial cells, Kindlin-2, which is expressed in most cells, and Kindlin-3, which is expressed by hematopoietic cells [4,8,9]. Kindlin-2 was the first of the Kindlin proteins to be discovered. Kindlin-2 is an integrin-associated protein, encoded by the KINDLIN-2 gene, which has been shown to affect cell adhesion and migration of cells, including endothelial cells, and is also expressed by cardiac muscle cells [10]. Kindlin-2 activates integrins by binding with the cytoskeletal protein, talin and recruits other proteins to adher to and control integrin activation [10]. Kindlin-2 regulates cardiac myogenesis and myogenic differentiation, the formation of intercalated discs, and embryonic development [11–15]. Knockdown of expression of the KINDLIN-2 gene can be lethal in laboratory mice that are KINDLIN-2(−/−) [16]. KINDLIN-2(+/−) mice have been shown to exhibit a reduced density and growth of intra-tumor angiogenic vessels [17]. These findings suggest that Kindlin-2, encoded by the KINDLIN-2 gene, might regulate angiogenesis in wounds.

Therefore, the aim of this study was to use a mouse model of wound healing to evaluate the effects of knockdown of KINDLIN-2 on angiogenesis in wound healing in vivo. A commercially available adeno-associated virus (AAV) vector for small interfering (si)RNA delivery into murine cells was used for the gene knockdown and wound healing was evaluated by biochemical examination, tissue histology, and new vessel permeability.

As a part of this study, Western blotting was performed as previously described [6]. Briefly, the wound tissues were weighed and lysed in lysis buffer. Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred

Ethical approval of the study protocol

All animal experiments were performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals, National Institutes of Health (NIH) and conformed to the guidelines of the Declaration of Helsinki. The study was approved by the Ethics Committee of Zhongshan Hospital, which is affiliated to Fudan University.

Experimental groups

Mice were bred in an SPF-grade experimental animal room. All surgical experiments were carried out using sodium pentobarbital anesthesia. Thirty-six male C57BL/6 mice, between 8–9 weeks old, were purchased from SLAC Laboratory Animals (Shanghai, China). KINDLIN-2 mouse knockdown models were prepared as previously described [18–20].

Mice were divided randomly into three groups: the normal group (n=12) received injections of normal (0.9%) saline; the KINDLIN-2(−) group (n=12) received injections of adeno-associated virus with small interfering (si)RNA targeting the KINDLIN-2 gene (AAV-KINDLIN-2-siRNA); and the control (group (n=12) received injections of adeno-associated virus containing a scrambled RNA sequence (AAV-control-RNA). Then, 5×10^10 v.g. of AAV-KINDLIN-2-siRNA (Jikai Biotechnology, Shanghai, China) or AAV-control-siRNA (Jikai Biotechnology, Shanghai, China) in 30 µL of normal saline was injected intradermally into the dorsal skin of mice; 30 µL of normal saline alone was used for the normal group. The wound model was evaluated 14 days after injection, following anesthesia using sodium pentobarbital.

Mouse wound model

The mouse wound model was created as previously described, and for all 36 mice included in the study [21,22]. Following the induction of anesthesia, the dorsal skin was shaved and disinfected using 75% ethanol. Skin wounds were created using a 6-mm skin punch and scissors. Five days after wounding, healing tissues were removed from some mice for further experiments, including immunofluorescence and Western blot, and from the remainder of the mice to evaluate wound healing and blood-vessel permeability. After four weeks, the mice were euthanized by cervical dislocation under anesthesia.

Western blot

Western blotting was performed as previously described [6]. Briefly, the wound tissues were weighed and lysed in lysis buffer. Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred
to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Then, the PVDF membranes were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA). The blots were visualized using enhanced chemiluminescence (ECL) reagent. The antibodies used were a rabbit monoclonal antibody to Kindlin-2 (Sigma-Aldrich, Saint Louis, MO, USA) and a rabbit monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to analyze blot density.

Immunofluorescence

Immunofluorescence analysis of tissue was performed as previously described [23,24]. Briefly, mouse skin tissues were sectioned using a cryostat microtome in a freezing chamber. First, the sections were permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Sections were incubated in blocking solution for 60 min at room temperature. Immunostaining was done using primary antibodies that included a rat anti-CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit anti-Kindlin-2 antibody (Sigma-Aldrich, Saint Louis, MO, USA) with overnight incubation at 4°C, followed by incubation with the fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 h at room temperature. Six samples were selected from each group of mice, and four image views were captured from each sample, by immunofluorescence microscopy.

Macroscopic analysis of the wound area

The wound area was photographed using a digital EOS40D camera (Canon, Tokyo, Japan) at one, three, six, and eight days post-wounding. The wound area was calculated using ImageJ software.

Evaluation of blood vessel permeability

Vascular permeability was measured as previously described [17]. Two weeks after the mouse skin wounds had healed, an intravenous injection of Evans Blue dye (EBD) 100 µL (Sigma-Aldrich, Saint Louis, MO, USA) and mustard oil was applied to the dorsal skin. Thirty minutes after injection with EBD, skin samples of similar size were removed, weighed, and photographed. Then, the EBD was extracted with 1 mL of formamide overnight at 60°C using constant agitation. The amount of EBD extracted was measured using a spectrophotometer at 610 nm. The skin was dried for 72 h at 60°C, weighed, and the absorbance was measured.

Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using a Kolmogorov-Smirnov normality test. Between-group comparison of means was performed by one-way analysis of variance (ANOVA). P<0.05 was considered as statistically significant.

Results

The effects of KINDLIN-2 gene knockdown, expression of the Kindle-2 protein, and cell migration in the mouse skin wounds

Western blotting (Figure 1) and immunofluorescence (Figure 2) were used to assess the levels of Kindlin-2 protein expression...
Figure 2. The effects of KINDLIN-2 gene knockdown following intradermal injection of adeno-associated virus with small interfering (si)RNA targeting the KINDLIN-2 gene (AAV-KINDLIN-2-siRNA) on the migration of Kindlin-2 (+) cells in mouse skin wounds. (A–D) Intradermal injection of AAV-KINDLIN-2-siRNA reduced the number of KINDLIN-2(+) cells around the wounds and decreased the number of KINDLIN-2(+) cells (H, I) migrating from the wound edge into the wound. (E–G) KINDLIN-2(+) cells in the normal group with intradermal injection of saline; the KINDLIN-2(+) cells are shown around the wound surface. A large number of KINDLIN-2(+) cells are shown to migrate from the wound edge into the wound (J, K). Bars: 200 microns. Magnification: ×20. (L) The number of KINDLIN-2(+) cells migrating from the wound edge to the wound surface. AAV-KINDLIN-2-siRNA – adeno-associated virus with small interfering (si)RNA targeting the KINDLIN-2 gene. Data are expressed as the mean ±SEM.
in the mouse skin samples in the three study groups. In the normal wound and control groups, there was no significant difference in expression levels of Kindlin-2 protein (P>0.05) (n=3) (Figure 1A). However, in the KINDLIN-2(–) group of mice that received injections of adeno-associated virus with small interfering (si)RNA targeting the KINDLIN-2 gene (AAV-KINDLIN-2-siRNA), there was significantly reduced expression of the Kindlin-2 protein when compared with the normal group (P<0.05) (n=3). Kindlin-2 protein was highly expressed in the normal group, and many Kindlin-2-positive cells (396±49 per field) migrated from the wound edge to the wound surface (Figure 2). However, in the KINDLIN-2(–) or AAV-KINDLIN-2-siRNA group, the number of Kindlin-2-positive cells was significantly reduced to 138±20 per field (P<0.05).

The effects of KINDLIN-2 gene knockdown and wound healing in the mouse skin wounds

To determine the effects of Kindle-2 on wound healing, the mouse skin wounds in the three study groups were observed wounds for ten days. Wound healing was significantly delayed in the AAV-KINDLIN-2-siRNA group (or KINDLIN-2(–) group) (P<0.05 (n=6) (Figure 3). The percentage wound area on day-9 in the AAV-KINDLIN-2-siRNA group (24±2%) was significantly greater when compared with the control group (7±4%) and with the normal group (5±2%) (P<0.05) (n=6). There was no significant difference in wound healing between the control group and the normal group (P>0.05) (n=6). These findings indicated that in the mouse wound healing model, the expression of the KINDLIN-2 gene encoding the Kindle-2 protein was required for normal wound healing and that knockdown of the KINDLIN-2 gene resulted in delayed and less effective wound healing.

The effects of KINDLIN-2 gene knockdown and angiogenesis and vascular permeability in the mouse skin wounds

Angiogenesis plays a key role in the healing of skin wounds [25]. Kindlin-2, as a regulator of integrins, is essential for maintenance of the integrity and function of tissues in adults [26,27]. The role of the KINDLIN-2 gene and expression of its protein product, Kindlin-2 were evaluated. Five days after skin wounding, immunostaining of skin sections for endothelial cells was performed using an endothelial cell-specific antibody, CD31, which showed a significant increase of vessel numbers (123±5 per field) in the AAV-KINDLIN-2-siRNA group compared with the normal group (30±8 per field) (P<0.05) (n=3) (Figure 4). Also, in the AAV-KINDLIN-2-siRNA group, blood vessels were shorter and thinner, and dermal tissues were thicker. Two weeks (14 days) after the skin wounds had healed, the vascular permeability in healed wounds was assessed using Evans blue dye (EBD) staining (Figure 5). The permeability of blood vessels in the AAV-KINDLIN-2-siRNA group was significantly increased (to 159±25%) when compared with the normal group (P<0.05)
These findings indicated that in the mouse wound healing model, the expression of the KINDLIN-2 gene encoding the Kindle-2 protein was required for normal vascular permeability in angiogenesis and that knockdown of the KINDLIN-2 gene affected wound healing by disrupting neovascular permeability and not by increasing the number of angiogenic blood vessels.

Discussion

Kindlin-2 is an integrin-associated protein, encoded by the KINDLIN-2 gene, which has been shown to affect cell adhesion and migration of cells, including endothelial cells. The aim of this study was to use a mouse model of wound healing to...
evaluate the effects of expression of KINDLIN-2 on angiogenesis in wound healing in vivo. The findings of this study showed that KINDLIN-2 gene knockdown inhibited wound healing, and increased endothelial cell permeability in vivo, and resulted in abnormal new vessel morphology.

Kindlin-2 is essential for embryonic development as Kindlin-2-deficient embryonic stem cells have shown reduced adhesion to various substrates of the extracellular matrix, such as laminin-111, laminin-332, and fibronectin, which can lead to embryo death [14]. Knockdown of the KINDLIN-2 gene that encodes Kindlin-2 has been reported to lead to a significant reduction in the invasive properties of cancer cells through nuclear factor kappa B (NF-κB)-dependent upregulation of expression of matrix metalloproteinase (MMP)-9 and MMP-2 [28].

Although a previously published study has shown that Kindlin-2 plays an important role in wound healing by regulating the function of fibroblasts [29], the role of Kindlin-2 in skin wound healing and angiogenesis has not been previously studied in detail. Kindlins and talins bind to the β-cytoplasmic tails of integrins for optimal integrin activation [25]. Kindlins have also been implicated in ‘outside-in’ signaling across integrins, and their capacity to regulate the function of integrin adhesion receptors has recently been studied [5,30,31].

Integrins, such as αvβ1, αvβ3, and αvβ5 are highly expressed in cells of the vasculature, especially by proliferating endothelial cells [32]. Disruption of binding of Kindlin-2 to the β-cytoplasmic tails of integrins can lead to defects in endothelial cell migration in vitro and to the developmental and tumor angiogenesis in vivo [33]. The hypothesis that drove this study was that expression of the KINDLIN-2 gene, and its protein Kindlin-2 regulated angiogenesis, vascular permeability, and wound healing by interacting with integrins, allowing interaction with β-/γ-catenin and actin filaments, linking vascular endothelial cadherin-based cell junctions to the actin cytoskeleton [34].

Angiogenesis is associated with cancer, diabetes mellitus-based complications, and inflammatory diseases. Research has shown that two alpha-v integrin pathways contribute to angiogenesis: one that depends on alpha vb3 and a second one that is potentiated by alpha vb5 [35].

Fibroblast growth factor receptor (FGFR) signaling is thought to be essential for vascular development. Fibroblast growth factor (FGF) signaling in endothelial cells is required for the response to injury but not for vascular homeostasis. Studies have shown that mice lacking FGFR1/2 in endothelial cells show a significant reduction in neovascular growth and tissue repair [35] and that FGF signaling is a key positive regulator in maintaining the integrity of endothelial cell barriers [36,37]. Previously published studies have also shown that antagonists to integrin αvβ3 suppress angiogenesis induced by FGFR2 [38].

Conclusions

Previously published studies support the findings of the present study, that the expression of the KINDLIN-2 gene and the Kindlin-2 protein induces normal angiogenesis, and aids the integrity of the endothelial cells and barriers related to integrin and fibroblast growth factor receptor (FGFR) signaling. According to the findings of the present study, combined with those of previously published studies, we propose that there is a Kindlin-2-integrin-FGF axis that regulates angiogenesis in wound healing. However, the mechanism of this pathway is not known and requires further study.

Conflict of interest

None.

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