Skin Graft Take Rates, Granulation, and Epithelialization

Dependence on Myeloid Cell Hypoxia-Inducible Factor 1α

Emre Vural, MD; Maaike Berbée, MD; Alison Acott, MD; Ross Blagg, MD; Chun-Yang Fan, MD, PhD; Martin Hauer-Jensen, MD, PhD

Objective: To compare the “take” rates of skin grafts between myeloid-selective hypoxia-inducible factor (HIF) 1α knockout (KO) and wild-type (WT) mice. Production of the α subunit of HIF–1α is increased in healing wounds, which stimulates expression of vascular endothelial growth factor (VEGF) to promote angiogenesis. Therefore, the take rate of skin grafts may be closely associated with the presence or absence of HIF-1α production in the recipient bed.

Design: The percentage of healthy graft areas obtained by planimetry and scores for epithelialization and granulation tissue formation obtained by histopathologic analysis were compared in 12 KO and 12 WT mice following skin grafting.

Results: The graft take rate was significantly impaired in the KO group (P = .009), whereas epithelialization (P = .46) or granulation (P = .41) tissue formation scores did not reveal any significant differences.

Conclusion: Hypoxia-inducible factor 1α in myeloid cells may be an important molecule for revascularization of avascular tissues such as skin grafts, probably owing to its stimulating effect on angiogenesis.


ONE OF THE MOST IMPORTANT factors involved in wound healing is granulation tissue formation and angiogenesis, which provide an infrastructure for epithelialization. Angiogenesis in healing wounds is closely associated with the production of vascular endothelial growth factor (VEGF), which promotes neovascularization by selectively targeting endothelial cells for proliferation.1 There is strong evidence in the literature that the angiogenic effect of VEGF in wound healing is primarily regulated by the α subunit of hypoxia-inducible factor 1 (HIF-1).2 Hypoxia-inducible factor 1 is a heterodimeric protein composed of α and β subunits, and the HIF-1α subunit is primarily regulated by tissue oxygen.3 In the presence of hypoxia, HIF-1α accumulates within the tissue instead of being degraded, which occurs constantly in normoxic state, and activates the expression of its target genes, including VEGF.4,5 It is well known that HIF-1α production is increased in healing wounds, which are hypoxic, at least at the initial stages of healing.6,7 This increased production of HIF-1α stimulates expression of VEGF and its receptors in healing wounds to promote angiogenesis.2

We have previously shown that exogenously administered VEGF can significantly increase “take” rates of transplanted avascular skin grafts either in healthy conditions or in irradiated recipient beds in a rat model.7,8 However, the role of endogenous VEGF production without external administration of VEGF in skin graft survival is unknown. We speculated that take rates of skin grafts would be closely associated with the presence or absence of HIF-1α production in the recipient bed as an initial step of angiogenesis. We performed this study to find out if the take rate of full-thickness skin grafts would be different between wild-type (WT) and myeloid-selective HIF-1α knockout (KO) mice.

METHODS

ANIMALS

This study was conducted in myeloid-selective HIF-1α KO mice and litter-mate WT mice after obtaining approval from the institutional animal care and use committee at the...
University of Arkansas for Medical Sciences. Animals were individually housed under standardized conditions with controlled temperature, humidity, and a 12-hour-day/12-hour-night light cycle. Animals had free access to water and standard mouse chow. Twelve adult male KO and 12 WT mice, weighing 25 to 30 g, were used. Myeloid-selective HIF-1α KO mice (HIF-1α-lysMcre) are double-mutant mice on a mixed Sv129/C57Bl/6/Cb.20 background. They carry as well a double-floxed HIF-1α gene as a Cre recombinase gene under control of the lysozyme M promoter (lysMcre).9 Wild-type mice lack the Cre recombinase gene. Breeders used to generate the mutant mice used in this study were on a mixed Sv129/C57Bl/6/CB.20 background and were kindly provided by R. S. Johnson, PhD, of the University of California at San Diego.9

SURGERY

On day 0, 12 male HIF-1α KO mice and 12 male WT mice were anesthetized using isoflurane inhalation anesthesia. The dorsum of the mice was shaved and prepared with alcohol. Subsequently, a circular area with a diameter of 20 mm was outlined on the dorsum at the midline using a surgical marking pen. The reason for choosing the dorsum at the midline for grafting was to minimize the mice’s access to the surgical site with their mouths as well as taking the advantage of natural convexity of this area to minimize the dead space under the graft. Under aseptic conditions, an incision was made along the marking using a No. 15 scalpel blade. After making this incision just below the panniculus carnosus muscle, the outlined skin was harvested as a full-thickness graft by separating it from the deep dorsal muscular fascia layer. To simulate the removal of excess fat from undersurface of the harvested full-thickness skin grafts in clinical conditions, the panniculus carnosus layer was removed from the undersurface of the skin graft. Following this maneuver, the graft was placed back into its donor site by securing the edges with interrupted nonabsorbable sutures. To eliminate potential dead space underneath the graft, 1 tacking suture was placed through the center of the graft into its recipient bed. The mice were then caged individually as an additional measure to minimize the trauma to the surgical site.

MACROSCOPIC ASSESSMENT OF THE SKIN GRAFTS

On day 14 following surgery, the skin grafted areas were macroscopically assessed by using planimetry, because this time frame has been shown to yield more objective assessment of the viability of the grafts, owing to epidermolysis that is almost invariably seen at earlier postoperative stages, which can be deceiving in the decision of graft viability.7,8 Areas with healthy graft tissue and areas that had healed by secondary intention after graft failure were identified. Regions with hair and/or follicles were considered to be healthy graft tissue, and areas with a smooth, whitish appearance without hair or follicles were considered to be areas that had healed by secondary intention owing to full-thickness loss (Figure 1). To calculate the size of the healthy regions and the regions healed by secondary intention, these areas were outlined on transparent paper that was placed on the skin-grafted dorsum. The transparency paper was digitally scanned, and the ratio of healthy area to the entire skin graft area was calculated by using computer software (Image Pro Plus, Silver Spring, Maryland) for each graft.

TISSUE PREPARATION

On day 14, after the macroscopic assessment of the skin grafts, the mice were humanely killed. The skin-grafted area was removed en bloc, including the recipient bed, and fixed in methanol-Carnoy solution (methanol, chloroform, and glacial acetic acid in a 6:3:1 ratio). Following this, representative parts composed of healthy graft areas and secondary intention healing were cut out of the main specimens, and 4-µm sections were obtained from each specimen for histopathologic evaluation.

HISTOPATHOLOGIC EVALUATION

Standard hematoxylin-eosin staining was performed on representative sections for histopathologic evaluation of epithelialization and granulation tissue formation. Each of these parameters was semiquantitatively evaluated for each representative slide under low-power (original magnification ×100) light mi-
Although grafts in the form of skin, dermis, cartilage, bone, fascia, and fat are indispensable tools of reconstructive surgery, their reliability may be questionable owing to their avascular nature, especially in the presence of less-than-optimal recipient bed conditions, such as diabetes mellitus, burns, or prior irradiation.10 Increasing the reliability of grafts, regardless of their nature, would provide an invaluable benefit to patients undergoing reconstructive surgery involving the use of graft materials.

Although extensive research is under way to improve wound healing by revealing the relationship between molecules such as HIF-1α and VEGF, most of these studies are being performed in models of excisional wounds with secondary intention healing. It has been shown that HIF-1α production is increased in wounds as a response to ischemia, which promotes expression of VEGF and VEGF receptors to increase angiogenesis.11 However, elimination of VEGF or its receptors from the wound does not cause delay in wound closure, despite impaired angiogenesis in such conditions.12,13 Therefore, impaired angiogenesis may not mean impaired wound healing, at least in secondary intention healing.13 Our study is somewhat in agreement with this because we were not able to demonstrate any difference in granulation tissue formation or epithelialization scores between KO and WT mice. This suggested that skin graft take rates were independent of granulation tissue formation and/or epithelialization potential of the wound. However, unlike secondary intention healing models, take rates of skin grafts showed noticeable impairment in HIF-1α KO mice, and this is most probably due to diminished angiogenesis causing poor revascularization of the grafts. Because HIF-1α deficiency in KO mice involved only myeloid cells and not all HIF-1α-producing cells, a decreased take rate of skin grafts in this group of animals should not be solely attributed to diminished VEGF production. It is known that myeloid-selective HIF-1α KO mice revealed limited attraction and differentiation of circulating angiogenic cells in the wound, which may be another reason for poor graft take rates in this group of animals.14 Furthermore, inhibition of proangiogenic factor HIF-2 in addition to HIF-1 has been shown to limit attraction and differentiation of circulating angiogenic stem cells even more, as a proof of involvement of more than 1 mechanism in wound angiogenesis.15 Regardless of the mechanism, it seems that relative HIF-1α deficiency in wounds repaired with skin grafts revealed poor take rates, probably owing to diminished angiogenesis within the grafted skin.

Despite the poor graft take rates in the HIF-1α–deficient mice in our study; granulation tissue formation and epithelialization scores were not significantly different in this group of animals when compared with WT animals. This may seem contradictory at first sight because one may expect poorer scores in these 2 major indicators of wound healing based on the finding of diminished graft take rates in HIF-1α deficiency. Although the KO mice used in this study were HIF-1α deficient selectively in myeloid cells, it is known that there are other sources of HIF-1α in wound healing in addition to myeloid inflammatory cells, such as basal keratinocytes at the wound edges.16 Therefore, HIF-1α deficiency in myeloid cell line alone may not cause a dramatic decrease in epithelialization because this process most probably continues from wound edges in both secondary intention healing and in skin grafting. Another explanation for the lack of difference in epithelialization scores between KO and WT mice would be the possibility of the absence of a direct relationship between HIF-1α

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**STATISTICAL ANALYSIS**

Comparison of the data obtained by planimetry for the percentage of healthy graft areas, as well as the data obtained by semiquantitative scoring for epithelialization and granulation tissue among WT and KO mice, was performed by using 2-tailed t test. The statistical significance was determined at P ≤ .05. All statistics were conducted using Microsoft Excel for Mac 2008 (Microsoft Inc, Seattle, Washington).

**RESULTS**

The mean (SEM) percentage take rates of skin grafts in WT and KO mice were 82.9 (6.5) and 56.3 (6.6), respectively (Figure 2). Epithelialization and granulation tissue formation scores did not show any statistically significant difference between WT and KO mice (see the Table for P values).

**COMMENT**

- **Figure 2.** Comparison of percentage “take” rates of skin grafts 14 days after surgery in wild-type (WT) and knockout (KO) mice.

- **Table.** Comparison of histopathologic parameters between wild-type (WT) and knockout (KO) mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Mean (SD)</th>
<th>WT SEM</th>
<th>KO Mean (SD)</th>
<th>KO SEM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelialization</td>
<td>1.8 (1.17)</td>
<td>0.35</td>
<td>2.17 (1.03)</td>
<td>0.30</td>
<td>.46</td>
</tr>
<tr>
<td>Granulation tissue formation</td>
<td>2.0 (1.00)</td>
<td>0.30</td>
<td>1.87 (0.89)</td>
<td>0.26</td>
<td>.41</td>
</tr>
</tbody>
</table>

Abbreviation: SEM, standard error of the mean.

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and the epithelialization process. The lack of difference in granulation tissue formation scores between KO and WT mice in our study is in agreement with previous observations.\textsuperscript{17} It has been previously shown that the wounds left for secondary intention healing did not show any difference between myeloid-selective HIF-1α KO and WT mice after 11 days of wounding, although the wounds filled out with granulation tissue faster in KO mice at the earlier phase, most probably owing to diminished nitric oxide production and eventual improvement in wound inflammation.\textsuperscript{17} Because our evaluation of grafted recipient beds took place 2 weeks after wounding, we may not have observed the earlier accelerating effect of HIF-1α deficiency on granulation tissue formation in this study. Obviously, HIF-1α seems to be a molecule that can affect wound healing through more than 1 mechanism, which may cause different outcomes in secondary intention healing (granulation tissue formation followed by epithelialization) and skin grafting (neovascularization of avascular skin).

Although we have not specifically looked at microvascular density in our study as a measure of angiogenesis in the grafted areas, statistically significant impairment in graft take rates in HIF-1α KO mice may suggest diminished angiogenesis, probably owing to diminished HIF-1α production in this group of animals. In addition to our previously shown improvement of skin graft take rates with exogenously administered VEGF in healthy and irradiated recipient beds,\textsuperscript{7,8} our current study provides evidence that presumed impairment of endogenous VEGF production owing to impaired HIF-1α expression in the environment may also interfere with skin graft take rates.

It seems that HIF-1α may serve as another molecule to be investigated as VEGF, in revascularization of avascular tissues such as grafts, or in increasing the survival of tissue flaps with diminished blood supply.\textsuperscript{18,19} These molecules may have potentials for therapeutic manipulation in the future, especially in situations with less-than-optimal neovascularization potential of tissue grafts or in ischemic conventional or microvascular flaps.

In conclusion, HIF-1α deficiency is related to poor skin graft takes, most probably owing to diminished angiogenesis.

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Correspondence: Emre Vural, MD, Department of Otolaryngology–Head and Neck Surgery, University of Arkansas for Medical Sciences, 4301 W Markham, Slot 543, Little Rock, AR 72205 (vuralemrea@uams.edu).

Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Vural, Berbee, and Hauer-Jensen. Acquisition of data: Vural, Berbee, Acott, and Blagg. Analysis and interpretation of data: Vural, Berbee, Fan, and Hauer-Jensen. Drafting of the manuscript: Vural, Berbee, Acott, Blagg, and Hauer-Jensen. Critical revision of the manuscript for important intellectual content: Vural, Fan, and Hauer-Jensen. Obtained funding: Hauer-Jensen. Administrative, technical, and material support: Vural, Berbee, Blagg, Fan, and Hauer-Jensen. Study supervision: Vural and Hauer-Jensen.

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REFERENCES