The effects of recombinant human erythropoietin (rHuEPO) in diabetes-related healing defects were investigated by using an incisional skin-wound model produced on the back of female diabetic C57Bl/KsJ-m+/Leptdb/mice (db/db) and their normoglycemic littermates (db+/m). Animals were treated with rHuEPO (400 units/kg in 100 μl s.c.) or its vehicle alone (100 μl). Mice were killed on different days (3, 6, and 12 days after skin injury) for measurement of vascular endothelial growth factor (VEGF) mRNA expression and protein synthesis, for monitoring angiogenesis by CD31 expression, and for evaluating histological changes. Furthermore, we evaluated wound-breaking strength at day 12. At day 6, rHuEPO injection in diabetic mice resulted in an increase in VEGF mRNA expression (vehicle = 0.33 ± 0.1 relative amount of mRNA; rHuEPO = 0.9 ± 0.09 relative amount of mRNA; P < 0.05) and protein wound content (vehicle = 23 ± 5 pg/wound; rHuEPO = 92 ± 12 pg/wound; P < 0.05) and caused a marked increase in CD31 gene expression (vehicle = 0.18 ± 0.05 relative amount of mRNA; rHuEPO = 0.98 ± 0.21 relative amount of mRNA; P < 0.05) and protein synthesis. Furthermore, rHuEPO injection improved the impaired wound healing and, at day 12, increased the wound-breaking strength in diabetic mice (vehicle = 12 ± 2 g/mm; rHuEPO 21 ± 5 g/mm; P < 0.05). Erythropoietin may have a potential application in diabetes-related wound disorders. Diabetes 53: 2509–2517, 2004
Erythropoietin (EPO) is a hematopoietic factor regulating the proliferation and differentiation of erythroid precursor cells (16). The biological effects of EPO are mediated by its specific interaction with its cell surface receptor EPOR, a type 1 cytokine receptor that is present in erythroid progenitor cells as well as in several nonhematopoietic cell types.

Recombinant human EPO (rHuEPO) is therapeutically applied for stimulation of the erythroid lineage. Experimental evidence has demonstrated that this hormone can stimulate mitosis and induce differentiation and activation of numerous cell lines, such as those of endothelium, myocardium, and smooth muscle (17,18). The presence of EPO receptors on endothelial cells prompted several in vitro and in vivo investigations aimed to determine whether the hormone may directly affect some of the functions of these cells (19), and, more specifically, attention has been paid to the complex network of cytokines and growth factors involved in both the maturation of erythrocytes and the proliferation of endothelial cells (20). The hypothesis that hematopoietic and endothelial cells share a common hemangioblast progenitor is based on the finding (3) that both cell lineages express cell surface antigens like CD31 and CD34. In agreement with this hypothesis of the common ontogenesis of endothelial and hematopoietic cells, it has been shown that the hematopoietic factor EPO stimulates the proliferation and migration of cultured immortalized human umbilical vein endothelial cells (21), as well as human and bovine endothelial cells in vitro (22). EPO can stimulate the first initial phase of the angiogenic process (i.e., increase in cellular motility, breakdown of the cell matrix, and cellular proliferation) and the subsequent phase, which leads to the formation of the vascular cave structures. Moreover, the recently discovered interaction between EPO and VEGF (18) and the ability of EPO to stimulate endothelial cell mitosis and motility may be of importance in the complex phenomenon of wound healing (23).

The aim of this study was to assess the capacity of rHuEPO to improve wound repair in healing-impaired genetically diabetic mice.

### Erythropoietin in Diabetic Wound Healing

<table>
<thead>
<tr>
<th>Score</th>
<th>Epidermal and dermal regeneration</th>
<th>Granulation tissue thickness</th>
<th>Angiogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Little epidermal and dermal organization</td>
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<td>Altered angiogenesis (one to two vessels per site) characterized by a high degree of edema, hemorrhage, occasional congestion, and thrombosis</td>
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<tr>
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<td>Moderate granulation layer</td>
<td>Few newly formed capillary vessels (three to four per site), moderate degree of edema and hemorrhage, occasional congestion and intravascular fibrin deposition, absence of thrombosis</td>
</tr>
<tr>
<td>3</td>
<td>Complete remodeling of epidermis and dermis</td>
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<td>Newly formed capillary vessels (five to six per site), moderate degree of perivascular and interstitial edema and congestion, absence of thrombosis and hemorrhage</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>Very thick granulation layer</td>
<td>Newly formed and well-structured capillary vessels (more than seven per site) vertically disposed toward the epithelium and at the wound margins, slight degree of perivascular edema</td>
</tr>
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</table>

### RESEARCH DESIGN AND METHODS

All animal procedures were in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals.

Genetically diabetic female C57BL/Ks-m/+Lept+/m mice (db/db) and their normal littermates (db/+m) were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were 14 weeks old at the start of the experiments. Diabetic mice were obese, weighing 40–50 g, whereas nondiabetic littermates weighed 25–32 g. During the experiments, the animals were housed one per cage, maintained under controlled environmental conditions (12-h light/dark cycle, temperature ~23°C), and provided with standard laboratory food and water ad libitum.

The animals were divided into four groups of 21 animals each. The first and second groups, consisting of normoglycemic mice, were given, respectively, rHuEPO at a dose of 400 IU/kg s.c. in 100 μl or rHuEPO vehicle (100 μl of a NaCl 0.9% saline solution) for 12 days. The third and the fourth groups, consisting of diabetic mice, were treated, respectively, with rHuEPO at a dose of 400 IU/kg s.c. in 100 μl or with rHuEPO vehicle (100 μl of a NaCl 0.9% saline solution) for 12 days. After general anesthesia with ketamine hydrochloride (110 mg/kg), the hair on the back was shaved and the skin washed with povidone-iodine solution and wiped with sterile water. Two full-thickness longitudinal incisions (4 cm) were made on the dorsum of the mice, and the wound edges were closed with skin clips placed at 1-cm intervals. Seven animals for each group were killed after 3, 6, and 12 days, respectively, and the wounds were divided into three segments (0.8 cm wide and 1.2 cm long). The caudal and cranial strip was used for histology, whereas the central one was divided into two segments. The first one was used for molecular analysis and the second one for wound-breaking strength measurements (day 12 only).

### Evaluation of erythrocyte count and Hb

Peripheral blood for smears to evaluate the absolute number of circulating red cells and for Hb was obtained at 3, 6, and 12 days after wounding. Erythrocyte count was performed on stained smears according to the standard morphologic criteria for mouse.

### VEGF and CD31 expression

Total cellular RNA was extracted from incisional full-thickness wounds at different intervals after wounding. In brief, ~50 mg of tissue was homogenized with 800 μl RNAZOL STAT (Tel-Test, Friendswood, TX) in a microfuge tube, after which 80 μl chloroform was added. After vortexing and centrifugation, the aqueous phase was transferred to a new microfuge tube containing an equal volume of cold isopropanol and the RNA recovered by precipitation by chilling at ~8°C for 15 min. The pellet was washed with cold ethanol 70%, centrifuged, dried in a speed vacuum, and at the wound margins, slight degree of perivascular edema.

After dilution of the product with distilled water, 5 μl were used for each PCR, which contained the Taq polymerase (Perkin Elmer, Wellesley, MA), the buffer as supplied with the enzyme, each dNTP, and the specific primers designed to cross introns and to avoid confusion between mRNA expression and genomic contamination. We used the following primers: mouse VEGF

### Criteria to evaluate histological scores of wound healing

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sense 5’-GCA TCA GGC CAG CTA CTG C-3’ and antisense 5’-GCA GTA GCT GCG CTG ATA GCG C-3’; mouse CD31 sense 5’-AGG AAA GCC AAG GCC AAA-3’ and reverse 3’-TGT ACT GTC TTA AGT TCC-5’; and mouse β-actin sense 5’-GGTCAGAAGGATTCTAGTG-3’ and antisense: 5’-ATGCC CAATTGGAGATGACTG-3’.

The annealing temperatures were 49°C for VEGF, 53°C for β-actin, and 45°C for CD31. The optimal cycle number for mouse VEGF and CD31 was 25, and we used a PCR-negative and PCR-positive control without cDNA or with a known cDNA, respectively. After amplification, the products were electrophoresed through 2% agarose (ultrapure, molecular biology grade) and stained with 0.5 μg/ml ethidium bromide in Tris-borate ethylene diaminetetraacetic buffer.

Skin samples were homogenized in lysis buffer (1% Triton X-100; 20 mmol/l Tris/HCl, pH 8.0, 137 mmol/l NaCl, 10% glycerol, 5 mmol/l ethylenediamine tetraacetic acid, 1 mmol/l phenylmethyl-sulfonyl fluoride, 1% aprotinin, and 15 μg/ml leupeptin). Protein samples (40 μg) were denatured in reducing buffer (62 mmol/l Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.003% bromophenol blue) and separated by electrophoresis on an SDS (12%) polyacrylamide gel. The separated proteins were transferred onto a nitrocellulose membrane using the transfer buffer (39 mmol/l glycine, 48 mmol/l Tris, pH 8.3, 20% methanol) at 200 mA for 1 h. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) 0.01% Tween for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer’s protocol (Amersham Biosciences, Piscataway, NJ).

The CD31 protein signal was quantified by scanning densitometry using a bioimage analysis system (Bio-Profil). The results from each experimental group were expressed as relative integrated intensity compared with control nondiabetic skin measured with the same batch.

**Determination of VEGF in wounds.** The amount of VEGF in wounds was determined by enzyme-linked immunosorbent assay. Briefly, tissues were homogenized in 1.0 ml of 1× PBS containing Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN). Homogenates were centrifuged to remove debris and filtered through a 1.2-μm pore syringe filter. Analysis was performed with a commercially available human VEGF specific enzyme-linked immunosorbent assay kit. The amount of VEGF was expressed as picograms per milligram of protein.

**Histologic examination.** All tissue specimens were fixed in 10% neutral buffered formalin for at least 25 h at room temperature. After fixation, perpendicular sections to the anterior-posterior axis of the wound were dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Five-micron-thick sections were mounted on glass slides, deparaffinized, rehydrated with distilled water, and stained with hematoxylin and eosin according to routine procedures for light microscopy. As part of the histological evaluation, all slides were examined by a pathologist without knowledge of the previous treatment by means of an eyepiece grid under the microscope from ×20 to ×100 magnification. The following parameters were evaluated and scored: reepithelialization, dermal matrix deposition and regeneration, granulation tissue formation and remodeling, and angiogenesis. The edges of the wound in each of the sections as well as normal control wounds were used as comparison for scoring. Concerning angiogenesis, only mature vessels were counted and identified by the presence of erythrocytes in their lumen. In order to evaluate well-formed from poorly formed capillary vessels, the

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**FIG. 1.** Erythrocyte count (A) and blood Hb (B) in db/+×m and db+/db+ mice given vehicle or rHuEPO. Each point represents the mean ± SD of seven experiments. *P < 0.005*, rHuEPO versus vehicle.
following criteria were examined: presence or absence of edema, congestion, hemorrhage, thrombosis, and intravascular or intervascular fibrin formation. The histological score adopted in this study was performed and evaluated according to data in the literature (15,24) concerning wound healing in experimental models. The criteria used as histological scores of wound healing are summarized in Table 1.

Breaking strength. The maximum load (breaking strength) tolerated by wounds was measured blindly on coded samples using a calibrated tensometer (Instron, Canton, MA) as previously described (25). The ends of the skin strip were pulled at a constant speed (20 cm/min), and breaking strength was expressed as the mean maximum level of tensile strength (grams per millimeter) before separation of wounds.

Statistical analysis. All data were analyzed by Student’s paired t test. The results were expressed as means ± SD. The level for statistical significance was set at *P < 0.05.

RESULTS

Blood glucose and HbA1c levels. The diabetic patients were markedly hyperglycemic, with average glucose levels of 539 ± 32 mg/dl. Blood glucose levels in nondiabetic animals averaged 140 ± 8 mg/dl. We did not observe throughout the experiment (12 days) any significant weight loss or appearance of blood ketones in diabetic animals. Furthermore, mean blood HbA1c (Diastat analyzer; BioRad, Hercules, CA) was 8.4 ± 0.2% in db+/db+ mice and 3.1 ± 0.1% in db+/+m mice. These values did not change significantly throughout the experiments. The hyperglycemia produced classical signs of diabetes, including polydipsia, polyuria, and glycosuria.

Erythrocyte count and blood Hb. Erythrocyte count and Hb were measured either in normoglycemic db+/+m and db+/db+ mice throughout the study (Fig. 1). rHuEPO administration increased erythrocyte count and Hb in both normoglycemic animals (*P < 0.005 vs. vehicle) and diabetic animals (*P < 0.005 vs. vehicle) at day 12 (Fig. 1).

VEGF expression. In the wounds of nondiabetic mice, a marked induction of VEGF mRNA was found at days 3 and 6 after injury when compared with basal values (uninjured skin = 0.19 ± 0.04 relative amount of VEGF mRNA; *P < 0.01). Thereafter, VEGF expression declined at day 12 (Fig. 2A). Administration of rHuEPO augmented the early induction of VEGF mRNA at days 3 (*P < 0.05 vs. vehicle) and 6 (*P < 0.05 vs. vehicle) in normoglycemic animals (Fig. 2A).

In the wounds of diabetic mice, VEGF mRNA was markedly lower than in normoglycemic animals at day 3.
(P < 0.01) and at day 6 (P < 0.01) following skin wounding. The levels of the growth factor mRNA were still detectable at day 12 in diabetic mice. Administration of rHuEPO in diabetic mice increased VEGF mRNA levels at days 3 (P < 0.05 vs. vehicle) and 6 (P < 0.05 vs. vehicle), thus restoring the disturbed pattern of VEGF mRNA expression (Fig. 2A).

**VEGF production in wounds.** VEGF content in uninjured skin from normoglycemic and diabetic mice were very low or undetectable (results not shown). In the wounds of nondiabetic mice, VEGF protein levels increased, at days 3 and 6, when compared with basal values, declining thereafter to baseline by day 12 (Fig. 2B). Administration of rHuEPO in normoglycemic animals significantly increased wound content of the angiogenic growth factor at days 3 (P < 0.05 vs. vehicle) and 6 (P < 0.05 vs. vehicle) compared with vehicle.

The diabetic wounds showed a marked reduction in VEGF content when compared with nondiabetic wounds (Fig. 2B) both at day 3 (P < 0.01) and day 6 (P < 0.01), and the angiogenic factor was also slightly present in the wounds of untreated diabetic mice at day 12 (Fig. 2B). The administration of rHuEPO markedly enhanced the wound levels of VEGF in diabetic mice at days 3 (P < 0.05 vs. vehicle) and 6 (P < 0.05 vs. vehicle) and normalized the impaired secretion pattern (Fig. 2B).

**CD31 expression.** CD31 was investigated as a marker of neovessel formation. In fact, it represents a highly specific marker for vascular and endothelial cells. CD31 expression was very low in uninjured skin from normoglycemic and diabetic mice (results not shown). During normal repair, CD31 mRNA and protein expression was induced early after injury (day 3), thus indicating a strong angiogenic process at the wound site (Fig. 3A and B). The administration of rHuEPO enhanced CD31 mRNA expression at days 3 (P < 0.05 vs. vehicle) and 6 (P < 0.05 vs. vehicle) and protein synthesis at all time points (P < 0.05 vs. vehicle) in nondiabetic mice (Fig. 3A and B).

As shown in Fig. 3A, we found a markedly lower CD31 mRNA expression in db/db mice than in normoglycemic animals at both day 3 (P < 0.01) and day 6 (P < 0.01) (Fig. 3A), which was paralleled by the same pattern of reduction in CD31 protein synthesis at both time points (P < 0.01) (Fig. 3B). The administration of rHuEPO in diabetic

**FIG. 3.** A: CD31 mRNA expression in wounds from db+/+ and db/db mice given vehicle or rHuEPO. Each point represents the mean ± SD of seven experiments. §P < 0.01, db/db versus db+/-; *P < 0.05, db+/- treated with rHuEPO versus vehicle; #P < 0.05, db+/- treated with rHuEPO versus vehicle. B: Western blot analysis of CD31 protein in wounds from db+/- and db/db mice given vehicle or rHuEPO. Each point represents the mean ± SD of seven experiments. §P < 0.01, db/db versus db+/-; *P < 0.05, db+/- treated with rHuEPO versus vehicle; #P < 0.05, db+/- treated with rHuEPO versus vehicle.
mice markedly enhanced the wound levels of CD31 mRNA expression at days 3 ($P < 0.05$ vs. vehicle) and 6 ($P < 0.05$ vs. vehicle) and protein synthesis at days 3 ($P < 0.05$ vs. vehicle), 6 ($P < 0.05$ vs. vehicle), and 12 ($P < 0.05$ vs. vehicle), thus improving the disturbed pattern of CD31 expression and formation in diabetic mice (Fig. 3A and B).

**Histological results.** Figure 4 shows the histological scores of wounds throughout the experiment according to the criteria of Table 1. In normoglycemic animals ($db^{+}/+m$ mice) dermis remodeling and wound-closure process were complete (Fig. 4). In addition, the histological score of the wound healing indicated that the administration of rHuEPO qualitatively and quantitatively improved wound healing (Fig. 4). In particular, rHuEPO treatment showed a significant improvement in angiogenesis at day 6 (Figs. 4 and 5A and B).

Histological evaluation of diabetic wounds at day 6 disclosed incomplete reepithelialization, rested on a poorly formed and immature granulation tissue (Figs. 4 and 6A and B). A low vascular count was characterized by few newly formed capillaries (one to two per site) and vascular dilation at the peripheral edges of wounds (Figs. 4 and 6B). Perivascular fibrin deposition and increased adipose tissue were also observed (Fig. 6A).

Important differences characterized the diabetic wounds of mice treated with rHuEPO in comparison with wounds of mice administered the vehicle (Figs. 4 and 6C and D). The reepithelialization process was mild, and the granulation tissue appeared well formed (Fig. 6C). Angiogenesis was improved and moderate (four to six per site) (Figs. 4 and 6D).

Diabetic wounds of animals administered with vehicle at day 12 showed mild reepithelialization, with partially organized granulation tissue (Figs. 4 and 6E). Microvessel distribution gave a low to moderate count (i.e., three to five) per site (Figs. 4 and 6F). In contrast, moderate to complete reepithelialization and well-formed granulation tissue were observed in the diabetic wounds of mice treated with rHuEPO (Figs. 4 and 6G). In these animals, a moderate to high vascular count was evidenced (Figs. 4 and 6H).

**Breaking strength.** Wound-breaking strength for each group at day 12 is shown in Fig. 7. The wound-breaking strength of diabetic mice was significantly lower than that of normoglycemic animals ($P < 0.01$). The breaking strength of incisional diabetic wounds of mice treated with rHuEPO was higher than that of diabetic mice treated with vehicle ($P < 0.05$) (Fig. 7).

**DISCUSSION**

Angiogenesis, cell migration, inflammation, provisional matrix synthesis, collagen deposition, and reepithelialization play an integral role in skin repair. It is a multistep process characterized by an orderly sequence of events (26). Although much of the complexity of the angiogenic cascade has yet to be defined, it is known to result from a concerted interaction between the extracellular matrix, cells, and growth factors.

VEGF has a pivotal role in the initiation of angiogenesis based on its ability to induce the expression of proteases that digest components of the extracellular matrix that impede angiogenesis, to promote endothelial cell proliferation, and to prevent their apoptosis (27). Experimental
evident neoangiogenesis are visible. Bar /H11547 DIABETES, VOL. 53, SEPTEMBER 2004 2515 biological effects. These effects are mediated by a specific factor were severely reduced during the healing process. as well as the wound content of the mature angiogenic that in genetically diabetic mice the mRNA levels of VEGF experimental evidence (15) from our laboratory indicated release of the powerful angiogenic factor VEGF. Recent recent discovery (18) of an EPO receptor in human endothelial cells, and the synergy between VEGF and EPO, indicates that it may act as a direct, as well as an indirect, angiogenic factor. This is confirmed by the discovery of a common progenitor for hemapoietic and endothelial cells, the hemangioblast, as also suggested by the presence of common antigens on the surface of both cell types (28). In addition, VEGF and rHuEPO share several common regulatory properties: both are involved in the maturation of medullary precursors and use tyrosine kinases as intracellular messengers (29).

Recently, the role of the hematopoietic cytokine EPO was investigated (30) in an in vivo wound-healing model consisting of fibrin-filled chambers implanted subcutaneously in rats. EPO expression was markedly evident in macrophages, cells that play a pivotal role in the wound-healing process (30). Local exogenous recombinant EPO administration into the fibrin matrix significantly increased granulation tissue formation in a dose-dependent manner (30). Furthermore, soluble EPO receptor or anti-EPO monoclonal antibodies, both administered with the scope of neutralizing endogenous EPO, dose-dependently inhibited granulation tissue formation. This experiment unmasks an unrecognized physiological role of EPO during wound healing.

All these experimental evidences prompted us to study the effects of rHuEPO in diabetes-impaired wound healing. In our study, rHuEPO was successfully used to improve diabetes-impaired wound healing. As previously reported (15), an altered pattern of VEGF expression and formation was observed in the wound-healing process of diabetic db/db mice compared with the normal skin-repair process of normoglycemic db+/m mice. The application of rHuEPO significantly increased both VEGF mRNA and protein expression in the skin wound. The ameliorated VEGF pattern secretion was already evident at day 3 following rHuEPO administration, whereas “the hematopoietic effects” of the growth factor appeared only at day 12. This suggests that the effect on VEGF might not be necessarily mediated by the classical hematopoietic action of EPO, but in contrast might be due to a direct EPO stimulatory activity on the angiogenic factor and to its ability to stimulate endothelial cell mitosis. Furthermore, rHuEPO might also stimulate macrophage function and activity. In fact, the skin-repair process is characterized by two major events: angiogenesis mediated by endothelial cells and matrix formation regulated by fibroblasts, smooth muscle cells, and macrophages (the major cell type in granulation tissue), playing a central role in both the regulation of angiogenesis and matrix formation. Macrophages are in fact responsible for the production of cytokines and growth factors that are essential for the healing cascade.

evidence has demonstrated expression of VEGF and its receptors during wound healing (6).

Impaired wound healing activity in diabetes may result from several factors, including high blood glucose levels, blunted cell-mediated immunity, local ischemia, and reactive oxygen intermediates production. Indeed, the reduced oxygenation, such as that observed in local ischemia, prompts the production of extremely reactive metabolites (free oxygen radicals) that impair normal wounds by damaging keratinocytes, endothelial cells, and collagen metabolism. The overproduction of free radicals in diabetes may also cause an impairment in the production and release of the powerful angiogenic factor VEGF. Recent experimental evidence (15) from our laboratory indicated that in genetically diabetic mice the mRNA levels of VEGF as well as the wound content of the mature angiogenic factor were severely reduced during the healing process.

EPO is a glycoproteic hormone that possesses several biological effects. These effects are mediated by a specific binding with its cell surface receptor (EPOR), a type 1 cytokine receptor that is expressed in erythroid progenitor cells and in several nonhematopoietic cells. As a consequence, the EPO-EPOR signaling pathway has been implicated in several nonhematopoietic biological actions. EPO stimulates mitosis and induces the differentiation and activation of numerous cell lines, such as the endothelial, myocardial, smooth muscle, and mesangial cells. EPO acts as a growth factor, and the recent discovery (18) of an EPO receptor in human endothelial cells, and the synergy between VEGF and EPO, indicates that it may act as a direct, as well as an indirect, angiogenic factor. This is confirmed by the discovery of a common progenitor for hematopoietic and endothelial cells, the hemangioblast, as also suggested by the presence of common antigens on the surface of both cell types (28). In addition, VEGF and rHuEPO share several common regulatory properties: both are involved in the maturation of medullary precursors and use tyrosine kinases as intracellular messengers (29).

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FIG. 5. A: A db+/m mouse given vehicle (day 6) (original magnification ×40). Normal wound-healing process, good granulation tissue, and evident neoangiogenesis are visible. Bar = 100 μm. B: A db+/m given rHuEPO (day 6) (original magnification ×40). Angiogenesis is sustained, and the granulation tissue is better organized. Bar = 100 μm.
The molecular effects of rHuEPO correlated well with all of the histological parameters that were considered, including epidermal regeneration, thickness of granulation tissue, and formation of new well-structured capillary vessels. In fact, angiogenesis is central to granulation tissue formation because the in-growth of newly formed vessels is needed to ensure the supply of oxygen and nutrients to the regenerating tissue.

The effects on neoangiogenesis were therefore investigated by evaluating the expression of CD31, which represents a highly specific marker for vascular and lymphatic endothelial cells. Wound healing in genetically diabetic db+/db+ mice was characterized by a markedly blunted expression and formation of CD31 compared with the normal healing of normoglycemic db+/+ mice. This is in close agreement with previous data (31) showing a dramatic reduction in this endothelium-specific marker during wound healing in diabetic mice. Administration of rHuEPO significantly ameliorated the disturbed pattern of CD31 expression and formation, thus confirming that the growth factor improves the severely impaired angiogenic response in diabetic animals.

Interestingly, rHuEPO administration was also able to slightly improve the normal wound-healing process in

![FIG. 6](image-url)

**FIG. 6.** A: A db+/db+ mouse given vehicle (day 6) (original magnification x10). Incomplete reepithelialization, with poorly formed and immature granulation tissue, is observed. Bar = 100 μm. B: A db+/db+ mouse given vehicle (day 6) (original magnification x20). Few newly formed capillaries are shown, and vascular dilation is present at the edges of wound. Bar = 100 μm. C: A db+/db+ mouse given rHuEPO (day 6) (original magnification x10). Granulation tissue appears well formed, and mild reepithelialization is present. Bar = 100 μm. D: A db+/db+ mouse given rHuEPO (day 6) (original magnification x20). Moderate, improved angiogenesis is evident (four to five vessels per site). Bar = 100 μm. E: A db+/db+ mouse given vehicle (day 12) (original magnification x10). Mild reepithelialization with partially organized tissue is evident. Bar = 100 μm. F: A db+/db+ mouse given vehicle (day 12) (original magnification x20). Low to moderate angiogenesis (three to five vessels per site) is seen. Bar = 100 μm. G: A db+/db+ mouse given rHuEPO (day 12) (original magnification x10). Moderate to complete reepithelialization, with well-formed granulation tissue, is evident. Bar = 100 μm. H: A db+/db+ mouse given rHuEPO (day 12) (original magnification x20). Moderate to high vascular count. Bar = 100 μm.

![FIG. 7](image-url)

**FIG. 7.** Wound-breaking strength (day 12) in db+/+ m and db+/db+ mice given vehicle or rHuEPO. Each point represents the mean ±SD of seven experiments. §P < 0.01, db+/db+ versus db+/+ m; #P < 0.05, db+/db+ treated with rHuEPO versus vehicle.
normoglycemic \( db^{+/-} m \) mice. This evidence confirms the hypothesis that EPO has a physiological role in wound healing and suggests that, when used in sustained pharmacological doses, it may have powerful angiogenic effects. In conclusion, our results suggest that rHuEPO is able to improve wound healing by stimulating granulation tissue formation neovascularization and dermal regeneration. This might be of particular relevance in the clinical situation of disturbed and delayed wound repair that contributes to the development of diabetic ulcers.

REFERENCES