Inhibition of Lipid Peroxidation Restores Impaired Vascular Endothelial Growth Factor Expression and Stimulates Wound Healing and Angiogenesis in the Genetically Diabetic Mouse

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Impaired wound healing is a well-documented phenomenon in experimental and clinical diabetes. Experimental evidence suggests that a defect in vascular endothelial growth factor (VEGF) regulation might be associated with wound-healing disorders. We studied the involvement of lipid peroxidation in the pathogenesis of altered VEGF expression in diabetes-related healing deficit by using an incisional skin-wound model produced on the back of female diabetic C57BL/6J db/db mice and their normal (db/db+m) littermates. Animals were then randomized to the following treatment: raxofelast (15 mg kg−1 day−1 i.p.), an inhibitor of lipid peroxidation, or its vehicle (DMSO/NaCl 0.9%, 1:1 vol: vol). The animals were killed on different days (3, 6, and 12 days after skin injury), and the wounded skin tissues were used for histological evaluation, for analysis of conjugated dienes (CDs), as an index of lipid peroxidation and wound breaking strength. Furthermore, we studied the time course of VEGF mRNA expression throughout the skin-repair process (3, 6, and 12 days after skin injury), by means of reverse transcriptase-polymerase chain reaction, as well as the mature protein in the wounds. Diabetic mice showed impaired wound healing with delayed angiogenesis, low breaking strength, and increased wound CD content when compared with their normal littermates. In healthy control mice, a strong induction of VEGF mRNA was found between day 3 and day 6 after injury, while no significant VEGF mRNA expression was observed at day 12 after injury. In contrast, VEGF mRNA levels, after an initial increase (day 3), were significantly lower in diabetic mice than in normal littermates, and light induction of VEGF mRNA expression was also present at day 12 after injury. Similarly, the wound content of the angiogenic factor was markedly changed in diabetic mice. Administration of raxofelast did not modify the process of wound repair in normal mice, but significantly improved the impaired wound healing in diabetic mice through the stimulation of angiogenesis, re-epithelization, and synthesis and maturation of extracellular matrix. Moreover, raxofelast treatment significantly reduced wound CD levels and increased the breaking strength of the wound. Lastly, the inhibition of lipid peroxidation restored the defect in VEGF expression during the process of skin repair in diabetic mice and normalized the VEGF wound content. The current study provides evidence that lipid peroxidation inhibition restores wound healing to nearly normal levels in experimental diabetes-impaired wounds and normalizes the defect in VEGF regulation associated with diabetes-induced skin-repair disorders. Diabetes 50:667–674, 2001
normal aging, metabolic derangement such as diabetes, or therapeutic intervention.

Genetically diabetic mice (db/db mice) are useful as an animal model for wound-healing studies, since wound healing in these animals is markedly delayed when compared with nondiabetic littermates (5,6). Healing impairment is characterized by delayed cellular infiltration and granulation tissue formation, reduced angiogenesis, decreased collagen, and its organization (7–10). The mechanism of this alteration is thought to result from diabetes production of reactive free radicals that cause lipid peroxidation, which in turn impairs keratinocyte endothelial cells, fibroblasts, and collagen metabolism (11). Furthermore, the presence of a defect in VEGF regulation, characterized by an altered expression pattern of VEGF mRNA during skin repair in db/db mice, has been shown and thereby suggests that an impairment in VEGF regulation might be associated with wound-healing abnormalities seen in these animals (12). Therefore, the aim of our experiment was to investigate whether there is a link between altered VEGF regulation and increased lipid peroxidation in experimental diabetes-induced skin-repair abnormalities.

**RESEARCH DESIGN AND METHODS**

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

Genetically diabetic female C57BL/KsJ db/db mice and their controls (db/+ or +/+ mice) were obtained from the Jackson Laboratories (Bar Harbor, ME). The animals were 14 weeks old at the start of the experiments. They were obese, weighing 40–50 g, compared with their nondiabetic littermates, which weighed 25–32 g. The diabetic mice were markedly hyperglycemic with average glucose levels of 527 ± 25 mg/dl compared with 205 ± 9 mg/dl for the nondiabetic animals. The hyperglycemia produced classic signs of diabetes, including polydipsia, polyuria, and glycosuria.

During the experiments, the animals were housed one per cage, maintained under controlled environmental conditions (12-h light/dark cycle, temperature 22–23°C), and provided with standard laboratory food and water ad libitum. The animals were divided into four groups (21 animals each). The first and second groups, consisting respectively of diabetic and healthy control mice, were given raxoflactan, an inhibitor of lipid peroxidation (13), at a dose of 15 mg/kg i.p. for 12 days. The third group of diabetic mice and the fourth group of healthy control mice were treated with vehicle (DMSO/Nacl 0.9%, 1:1 vol/vol) for 12 days.

After general anesthesia with ketamine hydrochloride (110 mg/kg), hair on the back was shaved and skin was 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). The caudal and cranial strips were used for histology, while the central one was used for biochemical and molecular analysis and wound breaking strength measurements (only day 12).

**Histological evaluation.** The samples were fixed in 10% buffered formalin for light microscopic examination. After fixation, sections perpendicular to the anterior-posterior axis of the wound were dehydrated with graded alcohols and embedded in paraffin. Sections 5-μm thick of paraffin-embedded tissues were mounted on glass slides, rehydrated with distilled water, and stained with hematoxylin and eosin. As part of the histological evaluation, all slides were examined by a pathologist without knowledge of the previous treatment, using masked slides under the microscope at ×20 to ×100 magnification. The parameters measured were epidermal and dermal regeneration, granulation tissue thickness, and angiogenesis. The margins of the wound in each of the sections, as well as normal control wounds, were used as comparisons for scoring (Table 1). Concerning angiogenesis, only mature vessels that contained erythrocytes were counted. To evaluate well-formed vessels, the following parameters were considered: presence or absence of edema, congestion, hemorrhage, thrombosis, and intravascular or intervascular fibrin formation.

**Breaking strength.** The maximum load (breathing strength) tolerated by wounds was measured blindly on coded samples using a calibrated tensometer (Instron, Canton, MA) as previously described (14). 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.

**Conjugated dienes evaluation.** Estimation of the tissue content of conjugated dienes (CDs) was carried out to evaluate the extent of lipid peroxidation in wounds. Samples (0.2 mg tissue) were collected in polyethylene tubes and then washed with 1 ml butylated hydroxytoluene (BHT) (1 mg/ml in phosphate buffer).

The samples, after drying in absorbant paper, were frozen at 4°C until the analysis. The biochemical assay of CDs required previous lipid extraction from the tissue samples by chloroform/methanol (2:1). The lipid layer was dried under nitrogen atmosphere and then dissolved in cyclohexane. Wound contents of CDs were measured at 232 nm by using a spectrophotometric technique. The amount of wound CDs was expressed as AAB per milligram.

**VEGF expression.** Total cellular RNA was extracted from incisional full-thickness wounds at different intervals after wounding. In brief, ~50 mg tissue was homogenized with 500 μl RNAZOL STAT (Teltest, 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 was recovered by precipitation with chloroform/methanol (2:1). The RNA sample was then washed in 20 μl buffer. A 2-μg portion of total RNA was subjected to first-strand cDNA synthesis in a 20-μl reaction mixture containing the AMV reverse transcriptase (Superscript II; BRL), each dNTP, the specific primers, Tris-HCl, and MgCl2.

After dilution of the product with distilled water, 5 μl was used for each polymerase chain reaction (PCR), which contained the Taq polymerase (Perkin Elmer), 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. The following primers were used: mouse VEGF121 and mouse β-actin. The optimal cycle number for mouse VEGF121 was 25, and we used a PCR-negative and a PCR-positive control without cDNA or with a known cDNA, respectively. A portion of the PCR product was electrophoresed and transferred to a nylon membrane, which was prehybridized with oligonucleotide probes and radiolabeled with [32P]ATP by a T4 oligonucleotide kinase. After hybridization, filters underwent autoradiography in a darkroom with a fixed camera. The captured image, which was sent for image analysis (Bio-Profil software; Celbio, Milan, Italy), was subjected to a densitometric analysis.

**Determination of VEGF in wounds.** The amount of VEGF in wounds was determined by an enzyme-linked immunosorbent assay (ELISA). Briefly, tissues were homogenized in 1.0 ml of 1× phosphate-buffered saline containing complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Homogenates were centrifuged to remove debris and were filtered through a 1.2-µm pore syringe filter. Analysis was performed with a commercially available murine VEGF-specific ELISA kit (R&D Systems). The amount of VEGF was expressed as picograms per wound.

**Drug.** Raxofelast was supplied by Biomedica Foscama Research Center, Ferentino, Italy. The compound was administered intraperitoneally in DMSO/NaCl 0.9% (1:1 vol:vol). All substances were prepared fresh daily and administered in a volume of 1 ml/kg.

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

### RESULTS

**Histological results.** Figure 1 shows the histological scores of wounds throughout the experiment according to the criteria in Table 1.

Qualitative data regarding histological evaluation at day 12 are summarized in Table 2. In diabetic (db/db) mice treated with vehicle, edema and hemorrhage were prominent because of extensive endothelial cell damage with few incomplete newly formed capillary vessels (Fig. 2A). Most of endothelial cells showed swelling. The endothelium of capillaries was prominent, and extravasation of erythrocytes was observed. Histological sections showed little dermal and epidermal organization. Significant reduction of granulation tissue formation and incomplete matrix maturation and remodeling characterized by loose connective tissue in an irregular fashion were noted (Fig. 3A). These effects were probably related to vascular alterations and to subsequent prominent edema and hemorrhage. Scattered intravascular thrombi or interstitial fibrin were also observed. Moreover, generalized vascular congestion was present in all samples, whereas endothelial cells exhibited wide eosinophilic cytoplasm and irregularly shaped nuclei with prominent nucleoli.

On the other hand, in diabetic mice treated with raxofelast, re-epithelialization was moderate to complete with epidermal elongation spreading over two-thirds of the wound surface (Fig. 3B). Dermal regeneration was characterized by granulation tissue rich in fibroblasts, generally oriented parallel to the epidermal layer. A moderate amount of collagen fibrils and collagen bundles were organized in a more regular fashion than that seen in the db/db mice treated with vehicle (Fig. 2B). Newly formed capillary vessels were observed in moderate numbers in the dermis of the entire wound area. The number of profiles of small vessels was higher than that in diabetic nontreated mice. The differences between these two groups also consisted of a different structural preservation of the capillary wall and a different degree of edema, hemorrhage, and thrombosis. Scattered intravascular fibrin was sometimes seen. Thin capillary vessels were lined by lightly swollen endothelial cells with prominent nuclei and eosinophilic cytoplasm.

In the nontreated normal mice (db/+), epidermal regeneration and remodeling of the dermis was complete and almost similar to that in diabetic treated mice (Fig. 3C). New well-formed capillary vessels were disposed vertically toward the wound surface and were identical to

### TABLE 2

Main morphological characteristics of wounds

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treated db/db mice</th>
<th>Nontreated db/db mice</th>
<th>Nontreated and treated db/+ mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenesis</td>
<td>Well-oriented and well-formed capillary vessels in the entire wound area.</td>
<td>Few altered capillaries scattered in the entire wound area.</td>
<td>Vessels disposed vertically toward the epithelial surface in the edge site of the wound.</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Oval- and spindle-shaped fibroblasts parallel to the surface of the wound.</td>
<td>Stellate or spindle-shaped fibroblasts scattered throughout the granulation tissue.</td>
<td>Numerous spindle fibroblasts parallel to the surface of the wound.</td>
</tr>
<tr>
<td>Dermis</td>
<td>Slight edema and well-formed collagen matrix disposed in a regular fashion.</td>
<td>Few collagen fibrils or collagen bundles disposed in an irregular fashion; moderate edema.</td>
<td>Complete remodeling.</td>
</tr>
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</table>
those of normal dermis (Fig. 2C). Treatment with raxofelast did not significantly change the process of wound repair in normal (db/+ ) mice.

**Breaking strength.** The wound breaking strengths for each group at day 12 are depicted in Fig. 4. The breaking strength of incisional wounds from diabetic mice treated with raxofelast was higher than that of diabetic mice treated with vehicle. As a result of raxofelast administration, breaking strength of wounds from db/db mice treated with raxofelast was approximately the same as that in nondiabetic mice. No significant differences in breaking strength were observed between nondiabetic mice treated with raxofelast or vehicle.

**Conjugated dienes.** CDs were evaluated throughout the study. Very low CD levels, which were investigated as an index of lipid peroxidation, were found in db/+ mice during the wound-healing process; in addition, the administration of raxofelast in these animals did not cause any modification of this parameter (Fig. 5). In contrast, increased CD levels were observed in diabetic mice treated with vehicle, and raxofelast treatment succeeded in reducing the increased lipid peroxidation (Fig. 5).

**VEGF expression.** The top of Fig. 6 shows representative autoradiograms highlighting mRNA expression for VEGF in control and diabetic mice treated with vehicle or raxofelast. The bottom of the figure depicts quantitative data and indicates the relative amount of VEGF mRNA. Very low VEGF expression was found in unwounded skin of several groups of mice (data not shown). In the wounds of healthy control mice, a strong induction of VEGF mRNA was found between day 3 and day 6 after injury, while VEGF expression was not detectable at day 12; these data are in agreement with the evidence of a completed histological wound repair process in these mice. Administration of the lipid peroxidation inhibitor did not modify the pattern of VEGF expression in the wounds of healthy control mice.

In the wounds of diabetic mice, VEGF mRNA was markedly reduced at day 3 and day 6 after injury. Furthermore, mRNA levels for the growth factor were still detectable at day 12. In accordance with these findings, the histological results pointed out a markedly delayed wound-healing process in diabetic mice with altered angiogenesis and insufficient vessel formation. The inhibition of lipid peroxidation restored the pattern of VEGF mRNA expression in the wounds of diabetic mice (Fig. 6).

**VEGF production in wound.** To determine whether levels of the VEGF protein were altered in diabetic mice, wound homogenates were assayed for the presence of the angiogenic factor. The amounts of VEGF in uninjured skin from both normoglycemic and diabetic animals were low or undetectable (data not shown). In normal mice, levels of VEGF were increased at days 3 and 6, declining thereafter to baseline by day 12 (Fig. 7). The administration of raxofelast, an inhibitor of lipid peroxidation, did not change VEGF protein levels in the wounds of healthy control mice (Fig. 7). At day 3 and day 6, VEGF levels in wounds from diabetic mice were substantially diminished compared with nondiabetic mice, and the angiogenic factor was also slightly present in the wound of untreated diabetic rats at day 12 (Fig. 7). The administration of raxofelast significantly increased the levels of VEGF in the wounds from diabetic mice at days

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**FIG. 2.** Hematoxilin- and eosin-stained sections of wound specimens at day 12 from diabetic (db/db) mice treated with vehicle (A), a diabetic mouse treated with raxofelast (B), and a nondiabetic (db/+ ) non-treated mouse (C). (Original magnification x100.) A: Vascular changes are characterized by swollen endothelial cells, poorly formed capillary channels, and evident hemorrhage. B: A good re-epithelialization and well-formed granulation tissue is shown. Spindle-shaped and oval fibroblasts are oriented parallel to the epithelial surface. Neovascularization is characterized by well-structured capillary vessels and absence of hemorrhage. C: Well-formed collagen matrix with scattered newly formed capillary vessels lined by a single layer of endothelial cells with round-to-oval nuclei and absence of pathological alterations.
3 and 6; furthermore, the levels of the angiogenic factor declined to baseline by day 12 (Fig. 7) after raxofelast treatment.

**DISCUSSION**

Decreased healing capacity in diabetes is the result of multiple factors, including elevated blood glucose levels, suppressed cell-mediated immunity, local ischemia, and free radical generation. Inadequate oxygenation, such as that seen in local ischemia, causes production of extremely reactive metabolites (called free oxygen radicals) that impair normal wound healing by damaging keratinocyte endothelial cells, capillary permeability, and collagen metabolism (15).

Skin ischemia provides favorable conditions for formation of oxygen-derived free radicals by means of leukocytes, which are activated during ischemia. The release of oxygen radicals by adhered activated leukocytes causes additional damage because more leukocytes are attracted and the process is amplified (16). Under normal conditions, the generation of free radicals is counterbalanced by the presence of adequate endogenous antioxidant defenses (17), but when the generation of free radicals exceeds the capacity of the defenses, these highly active radicals may produce structural changes that may contribute to reversible or irreversible cell injury. Oxygen radicals cause tissue damage by lipid peroxidation of cellular and organelle membranes, disruption of the intracellular matrix, and alteration of important protein enzymatic processes (16, 18). These agents not only damage the lipids but also produce lipid hydroperoxides, secondary intermediates that can lead to a chain reaction of lipid peroxidation (19).

Experimental evidence has demonstrated expression of VEGF and its receptors during wound healing (20). High levels of VEGF mRNA were detected in keratinocytes at the wound edge and in keratinocytes that migrated to cover the wound surface (21). These findings suggest an important role of keratinocytes in wound angiogenesis. Because VEGF is highly specific for endothelial cells, it is likely to act in a paracrine manner on the sprouting capillaries of the wound edge and the granulation tissue. The exclusive detection of VEGF receptors in these cells supports this hypothesis (22). In our experiment, we compared the time course of VEGF expression during wound healing of healthy control mice and genetically diabetic db/db mice; the latter are characterized by a significant delay in the skin-repair process and have been widely used as a model for wound-healing disorders. In healthy control mice, a marked induction of VEGF expression was observed between day 3 and day 6. Expression returned to the basal level after the completion of the skin-repair process (day 12). In the wounds of db/db mice, the mRNA levels for VEGF were severely depressed during the first phase of the healing process, and low VEGF mRNA expression was also detectable at day 12. The data regarding VEGF protein in the wound indicated an overlapping alteration in diabetic mice. Furthermore, in agreement with this result, db/db mice showed an incomplete and altered skin-repair process at day 12.

The reason for the wound-healing defect in db/db mice is still not completely understood. The present data support the hypothesis that an altered pattern of VEGF mRNA expression might be, at least in part, one of the mechanisms underlying the diabetes-induced disorder in wound repair.

CD measurement is an indicative method for evaluating lipid peroxidation (23). The large amount of CDs found in treatment.

**FIG. 3.** Hematoxilin- and eosin-stained sections of wound specimens at day 12 from diabetic (db/db) mice treated with vehicle (A), a diabetic mouse treated with raxofelast (B), and a nondiabetic (db/+ ) non-treated mouse (C). (Original magnification ×25.) A: Evident edema and poorly formed granulation tissue, with altered epidermal and dermal organization and extravasation of erythrocytes. B: A good re-epithelialization and well-organized granulation tissue is shown. Connective tissue is highly cellular, mainly composed of fibroblasts organized in a regular fashion. C: Complete remodeling of epithelium and connective tissue.
the wound tissue of diabetic mice is consistent with the occurrence of free radical–mediated wound-healing damage. Lipid peroxidation is considered responsible for the impairment of endothelial cells, keratinocyte capillary permeability, and fibroblast and collagen metabolism. Therefore, we hypothesized that the increased lipid peroxidation might be one of the factors causing the defect in VEGF expression and finally producing the impairment in the wound-healing process. To test such a hypothesis, we treated diabetic mice with raxofelast, an inhibitor of lipid peroxidation.

The antioxidant activity of raxofelast and its deacetylated active metabolite IRFI 005 has been described in previous in vitro and in vivo studies (13). In addition, IRFI 005 has been shown to be a scavenger of superoxide anion, with a linear dose-response curve starting from 5 μmol/l. After systemic administration of raxofelast to rats, dogs, and humans, the plasma concentrations of the parent compound were very low, whereas high levels of IRFI 005 were found in plasma and tissue (24,25).

In our model, raxofelast was able to reverse the effects of diabetes on wound healing by reducing lipid peroxidation and edema and by stimulating re-epithelization, neovascularization, proliferation of fibroblasts, and synthesis and maturation of extracellular matrix. Thus, the degree of wound healing in \( db/db \) mice treated with raxofelast was approximately the same as that in control heterozygous (\( db/+ \) ) mice.

The beneficial effects of raxofelast on wound healing were also stressed by the increase in breaking-strength measurements. Finally, the inhibition of lipid peroxidation normalized the pattern of VEGF mRNA expression and secretion in diabetic mice, thus strongly supporting the idea that there might exist a close link between the deleterious phenomenon of lipid peroxidation and a defect in VEGF production. Indeed, the improvement in VEGF expression after raxofelast administration does not seem to be a consequence of a direct effect of the drug on the angiogenic factor. In fact, the vitamin E analog did not enhance VEGF expression in nondiabetic mice; further-
more, in vitro raxofelast (50 μmol/l) did not change the
ability of murine macrophages to secrete VEGF in re-
response to lipopolysaccharide (F.S. et al., unpublished
observations). The mechanism by which increased lipid
peroxidation impairs VEGF expression in diabetic mice
remains, at the moment, a matter of speculation. One
may speculate that the large production of unstable
reactive intermediates and hydroxyperoxides that oc-
curs during lipid peroxidation could cause structural
DNA changes that lead to an impairment in the trans-
duction mechanism.

Besides VEGF, other import growth factors, such as
platelet-derived growth factor and fibroblast growth fac-
tor, have been shown to be severely impaired during the
wound-healing process in diabetes (26,27). We must fur-
ther investigate whether enhanced lipid peroxidation also
plays a role in causing this dysfunction.

In conclusion, these results suggest that lipid peroxida-
tion and an altered pattern of VEGF mRNA expression
may contribute to deficient wound repair in genetically
diabetic mice.

FIG. 6. VEGF mRNA expression in wound specimens collected at different time points from db/+ and db/db mice treated either with vehicle (1 ml · kg−1 · day−1 i.p.) or raxofelast (15 mg · kg−1 · day−1 i.p.). The top panel shows representative autoradiograms highlighting VEGF mRNA expression. The bottom panel shows quantitative data and represent the mean ± SE of seven experiments. *P < 0.01 vs. db/+ mice; #P < 0.01 vs db/db mice treated with vehicle.

FIG. 7. VEGF levels in wound specimens collected at different time points from db/+ and db/db mice treated either with vehicle (1 ml · kg−1 · day−1 i.p.) or raxofelast (15 mg · kg−1 · day−1 i.p.). Each point represents the mean ± SE of seven experiments. *P < 0.01 vs. db/+ mice; #P < 0.01 vs db/db mice treated with vehicle.
This work was supported, in part, by a grant from the University of Messina (Fondi Ricerca d'Ateneo). There is no financial interest held by any of the investigators in the said company.

We thank Biomedica Foscama (Italy) for the generous supply of raxofelast.

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