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RESEARCH ARTICLE

Discordant effects of a soluble VEGF receptor on wound healing and angiogenesis

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Soluble receptors to vascular endothelial growth factor (VEGF) can inhibit its angiogenic effect. Since angiogenesis is involved in wound repair, we hypothesized that adenovirus-mediated gene transfer of a soluble form of VEGF receptor 2 (Flk-1) would attenuate wound healing in mice. C57Bl/6J and genetically diabetic (db/db) mice (each n=20) received intravenous (i.v.) injections of recombinant adenoviruses (10^9 PFU) encoding the ligand-binding ectodomain of VEGF receptor 2 (Flk-1) or cDNA encoding the murine IgG2α Fc fragment (each n=10). At 4 days after gene transfer, two full-thickness skin wounds (0.8 cm) were created on the dorsum of each animal. Wound closure was measured over 9–14 days after which wounds were resected for histological analysis. Prior to killing, fluorescent microspheres were systemically injected for quantitation of wound vascularity. Single i.v. injections of adenoviruses encoding soluble Flk-1 significantly decreased wound angiogenesis in both wild-type and diabetic mice. Fluorescence microscopy revealed a 2.0-fold (wild type) and 2.9-fold (diabetic) reduction in wound vascularity in Flk-1-treated animals ($p<0.05$). Impairment of angiogenesis was confirmed by CD31 immunohistochemistry. Interestingly, despite significant reductions in wound vascularity, wound closure was not grossly delayed. Our data indicates that while VEGF function is essential for optimal wound angiogenesis, it is not required for wound closure.
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Introduction

Angiogenesis is a crucial biological process involved in both physiological and pathophysiological conditions that is regulated by a delicate balance of both proangiogenic and antiangiogenic factors.[1] Recent elaboration of mechanisms regulating angiogenesis has led to extensive efforts to identify and exploit potential agents stimulating angiogenesis under circumstances when the recruitment of new blood vessels is desired, for instance in coronary heart disease or peripheral arterial disease.[2–7] Conversely, the need for inhibitors of angiogenesis directed against angiogenesis in pathophysiological conditions such as tumor growth or proliferative diabetic retinopathy has arisen.[8]

Among angiogenic growth factors, ligands for receptor tyrosine kinases (RTKs) such as vascular endothelial growth factor (VEGF) have been identified to play an essential role in vascular development.[9] This molecule was initially identified for its ability to induce vascular leak and permeability. Later, it was shown that VEGF promotes differentiation, proliferation, migration and tube formation of endothelial cells *in vitro*, all of which are key elements in early stages of angiogenesis. The six members of the VEGF family that have been characterized and purified to date (VEGF-A through VEGF-E and PlGF) interact with three high-affinity RTKs designated as VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1 and VEGFR-3/Flt-4.[10]

Recently, VEGF antibodies or gene transfer with soluble forms of VEGF receptors (sFlt-1 and sFlk-1) have been introduced as novel antiangiogenic strategies to disrupt tumor growth. Gene transfer with sFlt-1 or sFlk-1 markedly reduced tumor growth by inhibiting tumor angiogenesis in various animal tumor models.[11–15] Furthermore, in corneal micropocket assays in rodents, gene delivery significantly attenuated neovascularization induced by sustained release VEGF pellets.[11,16]

Wound healing is a complex, dynamic process in which angiogenesis has been hypothesized to play an integral part since the formation of new blood vessels is a necessary step that allows a variety of mediators and regulators to reach the center of the healing process.[17] VEGF appears to play a crucial role during the proliferative phase of wound healing. VEGF levels measured in wound fluids from surgical wounds increase postoperatively, and harvested wound fluid induces a neovascular response in the rat corneal micropocket assay that is significantly attenuated by VEGF neutralization.[18] Other investigators have shown that VEGF release by epidermal keratinocytes appears to play an important role during the proliferative phase of cutaneous wound healing.[19,20]

The use of antiangiogenic therapy as a new treatment modality for cancer requires the evaluation of possible

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adverse effects on physiologic angiogenic processes such as wound healing. In the present study, we investigated the role of VEGF on wound closure and wound angiogenesis in a murine excisional wound model by analyzing the antiangiogenic properties of sFlk-1 after adenoviral gene transfer. Since patients undergoing antiangiogenic therapy might have pre-existing vascular complications due to risk factors, such as hypertension, diabetes or hypercholesterolemia, the effect of gene transfer was analyzed in both wild-type and healing-impaired genetically diabetic mice.

Material and methods

Animals

Wound healing was analyzed in 12-week-old female C57BL/6J (wild type) mice (n=20, ~20–25 g), and in age- and sex-matched genetically diabetic mice (BKS.Cg-m+/+Lepr^{db}, n=20, ~35–45 g; Jackson Laboratories, Bar Harbor, ME, USA). Genetically diabetic mice served as a known mouse model of impaired wound healing. Diabetic animals homozygous for the point mutation in the leptin receptor gene are obese, hyperglycemic and insulin resistant.[21] All mice had free access to tap water and rodent chow and were housed individually in a temperature-controlled animal facility with a 12-h light/dark cycle. The study protocol was approved by the Administrative Panel on Laboratory Animal Care of Stanford University (APLAC).

Adenoviral construction

Murine Flk-1 cDNA encoding the signal peptide and the ectodomain was fused to a murine IgG21α Fc fragment and the fusion gene was then released with *Xba*I and *Bam*HI and inserted in the polylinker of the adenovirus shuttle vector HIHG Add2 as previously described in detail.[11] In the resulting construct, Flk1-Fc expression is controlled by the human cytomegalovirus promoter and the rabbit β-globin intron and polyadenylation signal. For the control Fc fragment, a cDNA encoding the murine IgG2α Fc cDNA was released with *Xho*I and *Xba*I and ligated into HIHG Add2. Viruses were produced by infection of 293 cells, banded over CsCl gradients, and dialyzed into 4% sucrose.[11]

Transfection of animals and determination of transgene expression

Gene transfer of the adenoviral construct for Fc or Flk-1 was performed by tail vein injection (10^9 PFU). Blood samples were obtained from all animals by retroorbital bleeding under anesthesia 3 days after viral administration and a day prior to killing. The murine Flk1-Fc concentrations were determined by sandwich ELISA using anti-murine Flk-1 (Pharmingen, San Diego, CA, USA) and anti-murine IgG2αFc-horseradish peroxidase (Jackson Immuno Research, West Grove, PA, USA) as primary and secondary antibodies.[11] Notably, investigators involved in creating the wounds, and evaluating wound closure and angiogenesis, were blinded to the treatment groups.

Murine excisional wound model

At 4 days after gene transfer, animals were subjected to wounding as previously described in detail.[22] Briefly, mice were anesthetized using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg), and the dorsal surface was shaved and disinfected with povidone–iodine solution and alcohol swabs. Two full-thickness excisional wounds, each 0.8 cm in diameter, were created on the back of each animal using a disposable skin punch biopsy tool (Acuderm Inc., Fort Lauderdale, FL, USA). The distance between both wounds was approximately 1 cm. Tincture benzoin solution (Paddock Laboratories, Minneapolis, MN, USA) was applied to the wound perimeter and the wounds were covered with a transparent, bio-occlusive dressing (Opsite, Smith and Nephew Medical Limited, Hull, UK). At the end of the surgical procedure, all the animals were given 1.0 ml of 0.9% saline solution intraperitoneally.

Analysis of wound closure

Wound size was documented with a digital camera (Nikon Coolpix 995, Nikon, Japan) on day 0, 5, 9 (wildtype mice) or day 0, 5, 9 and 14 (diabetic mice). Images were analyzed using NIH image 1.60 software by tracing the wound margin with a fine resolution computer mouse and calculating pixel area. Pixel counts were then related to a circular filter paper of the same diameter as the original wound that served as a reference on every image. The measurements were performed in duplicate and mean values of consecutive tracings were computed and expressed as percentage of closure from the original wound. Finally, mean wound closure out of the two wounds in each animal was calculated.

Resection of wounds

Animals were euthanized on day 9 (wild-type mice) or day 14 (diabetic mice) for further analysis of the wounds. Different time points of killing were chosen based on our previous experience showing a rapid wound closure in C57BL/6J mice (almost 100% after 14 days).[22] In contrast, in diabetic mice wound healing is complete after 5–6 weeks. In order to ensure sufficient expression levels of the adenoviral construct, diabetic mice were killed 14 days after wounding.

To exclude animals with bacterial wound contamination from statistical analysis, wound fluid from both wounds was incubated on MacConkey II and mannitol salt agar plates (Becton Dickinson, Cockeysville, MD, USA).

Prior to resection of the wounds, space-filling carboxylate-modified fluorescent microspheres were injected into the left ventricle (0.2 μm, Molecular Probes, Eugene, OR, USA) to visualize neovascularization in the wounded area.

Histomorphometry and immunohistochemistry

The upper wound including a ~1–2 mm margin of surrounding normal skin was excised and longitudinally cut in half through the least-healed portion. Half of the wound was fixed in 10% formalin and embedded in paraffin, and 5 μm sections from the paraffin blocks were stained with hematoxylin and eosin (Sigma, St Louis, MO, USA). The other half of the wound was placed in prelabeled base molds with frozen tissue matrix (OCT, Sakura Finetek Inc., Torrance, CA, USA) and immediately snap-frozen in isopentane solution cooled in liquid nitrogen for immunohistochemistry.

Histological analysis was performed by a surgical pathologist as previously described.[22] Briefly, each slide was given a histological score ranging from 1 to 12 that took into account the amount of inflammatory cells, fibroblast accumulation, the presence and quantity of granulation tissue, collagen deposition, neovascularization, and the extent of epithelial migration (1–3, none to minimal cell accumulation, no granulation tissue or epithelial travel; 4–6, thin, immature granulation that is dominated by inflammatory cells but has few fibroblasts, capillaries or collagen deposition; minimal epithelial migration; 7–9, moderately thick granulation tissue, can range from being dominated by inflammatory cells to more fibroblasts and collagen deposition, extensive neovascularization, epithelium can range from minimal to moderate migration; 10–12, thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition, epithelium partially to completely covering the wound).[22,23]

Capillary density (CD31 staining cells/HPF) was assessed by counting cells staining for the endothelial antigen CD31 in high-power fields and deriving an average value. Immunohistochemical staining of endothelial cells was performed using a monoclonal antibody against CD31 (MEC13.3, BD Pharmingen, San Diego, CA, USA). Antibody binding was visualized via a three-step staining procedure using a biotinylated polyclonal anti-rat Ig-G secondary antibody and the streptavidin horseradish peroxidase together with the DAB detection system. Since wounds from diabetic mice were extremely friable, we were unable to obtain utilizable sections for CD31 immunohistochemistry.

Local VEGF expression within wounds was analyzed by immunohistochemistry using a polyclonal antibody against mouse VEGF (R&D Systems Inc., Minneapolis, MN, USA).

All slides for immunohistochemistry were counterstained with hematoxylin. Controls for immunostainings included incubations with irrelevant class- and species-matched immunoglobulins, and incubations in which the primary antibody was omitted.

Fluorescence microscopic evaluation of wound vascularity

The second wound was excised, snap-frozen in OCT and stored at -80°C until further use. Cryostat cross-sections (10 μm) were made from the mid-portion of each wound and fixed in ice-cold acetone. Wound vascularity, as reflected by the presence of fluorescent microspheres, was analyzed using fluorescence microscopy as previously described ($20\times$ objective, Laborlux S, Leitz, Wetzlar, Germany).[22] Digital images with maximum fluorescence signal were captured from four different cross-sections. The percentage of fluorescent pixels was quantified using image analysis software (Image-Tool 2.02 software, University of Texas Health Sciences Center at San Antonio, San Antonio, TX, USA). The mean percentage of fluorescent pixels out of the four cross-sections served as an index of angiogenic response.

Statistical analysis

All data are given as mean $\pm$ s.e.m. Statistical significance was tested using the two-tailed Student *t*-test for unpaired comparisons between groups. Pearson correlation coefficients were calculated when indicated. Statistical significance was accepted at $P<0.05$. In cases of multiple comparisons, a *post hoc* correction with the Bonferroni procedure was performed.

A total of six animals were excluded from statistical analysis. Two animals died shortly after the surgical procedure, one animal had to be killed due to macroscopic signs of wound infection and three mice were excluded due to bacterial growth of wound fluid on agar plates.

Results

Effect of transgene expression on wound vascularity and local VEGF secretion

The effect of Ad Flk1-Fc or the control virus Ad Fc on wound vascularity and wound closure was analyzed in 17 C57Bl/6J (n=10 Fc; n=7 Flk-1) and 17 diabetic mice (n=8 Fc; n=9 Flk-1). Transgene expression was verified in all animals. The mean plasma levels of Flk–Fc 3 days after injection of adenoviruses were 7.86 ± 1.0 mg/ml and thus within the previously reported range.[11]

Strikingly, single i.v. injections of adenoviruses encoding sFlk-1 markedly reduced wound vascularity in both wild-type and diabetic mice. Fluorescence microscopy of cross-sections obtained from wounds resected after 9 (wild type) or 14 days (diabetic) revealed a $50.3\pm5.3\%$ reduction in wound vascularity in wild-type mice (P=0.013) and a $65.0\pm8.5\%$ reduction in diabetic mice (P=0.002; Figure 1). The reduction in wound vascularity between wild-type and diabetic mice was not statistically different (50.3 ± 5.3 *versus* $65.0\pm8.5\%$, P=0.194). Decreased wound vascularity was confirmed by immunohistochemistry showing markedly reduced staining for CD31 in Flk-1-treated animals (Figure 2b). Notably, we observed a marked reduction – in some animals a complete absence – of blood vessels in the dermis as opposed to the hypodermis (Figure 2b). To corroborate this finding, we performed a semiquantitative analysis by counting CD31-positive cells per high-power field within the dermis and hypodermis of wounds and in adjacent unwounded tissue in C57Bl/6J mice (each n=5). In unwounded skin (dermis and hypodermis), CD31 cell density was similar between Fc- and Flk-1-treated mice (Figure 2c). In contrast, within wound dermis and hypodermis microvessel density was markedly reduced

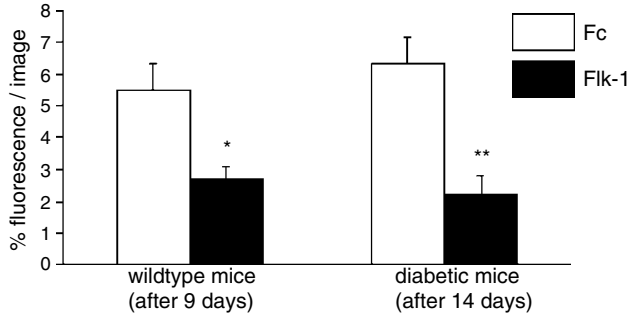

Figure 1 *Impact of adenoviral gene transfer with sFlk-1 on wound vascularity. Fluorescent microsphere analysis (% fluorescent pixels/image) in wild-type and diabetic mice. Wound vascularity was significantly reduced in Flk-1-treated mice (*$P<0.05$, **$P<0.01$).*

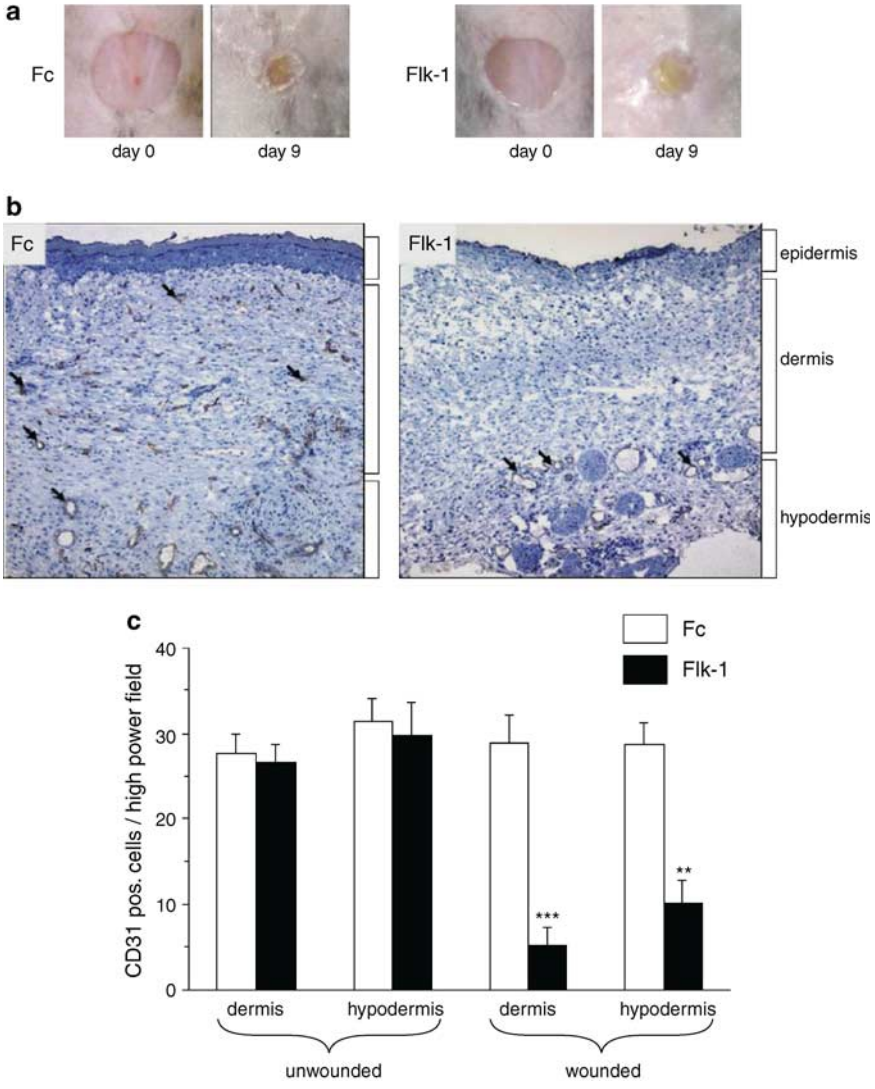

Figure 2 *(a) Macroscopic aspects of resected wounds from wild-type mice treated with Fc or Flk-1 at day of wounding and day of resection. (b) CD31 immunohistochemistry of frozen tissue sections of wounds from wild-type mice resected 9 days after wounding. Wound vascularity (as indicated by the number of dermal vessels highlighted by positive staining for CD31; see black arrows) is markedly decreased in Flk-1-treated mice; notably, the wound dermis of the Flk-1-treated animal is avascular as opposed to the hypodermis where CD31 immunoreactivity can be detected (10 × objective). (c) Semiquantitative analysis of microvessel density. Capillary density was assessed by determining an average value for CD31-expressing cells per high-power field in unwounded and wounded dermis and hypodermis of C57Bl/6J mice treated with Fc or Flk-1 (**P<0.01, ***P<0.001).*

in Flk-1-treated animals (dermis: 29.0±3.9 *versus* 5.1±2.6 positive cells/field, $P=0.001$; hypodermis: 28.6±3.1 *versus* 10.0±3.2 positive cells/field, $P=0.003$; Figure 2c).

In order to determine whether antiangiogenic gene transfer with sFlk-1 affects local VEGF expression within wounds, VEGF immunohistochemistry was performed. Staining for VEGF was most prominent in keratinocytes in both Fc- and Flk-1-treated mice (Figure 3). Interestingly, VEGF immunoreactivity tended to be stronger in the wound dermis and hypodermis of Flk-1- *versus* Fc-treated mice (Figure 3), consistent with a compensatory response to VEGF blockade.

Effect of transgene expression on wound closure

Wild-type mice. As previously observed by our group,[22] wound healing was rapid in wild-type mice. Average wound closure after 9 days in all wild-type animals ($n=34$ wounds, including both Flk-1 and Fc) was 83.1±2.5%. In Flk-1-treated mice, the degree of wound closure was 11.1±2.7% less after 5 days ($P=0.034$, Figure 4a) and 14.8±2.6% less after 9 days ($P=0.001$, Figure 4a) when compared to Fc-treated mice. Thus, although significant, these differences in wound closure rate between Fc- and Flk1-Fc-treated mice were small in relation to the ~50% reduction in wound vascularity observed by fluorescent microsphere analysis after 9 days (Figure 1), and the even more pronounced reduction in microvessel density observed by CD31 immunohistochemistry in the wound dermis (~82% reduction) and wound hypodermis (~65% reduction, Figure 2c).

Diabetic mice. As previously described[22] wound healing in diabetic mice was markedly impaired compared to wild-type mice ($P<0.0001$ for days 5 and 9). The average wound closure after 14 days in all diabetic animals was

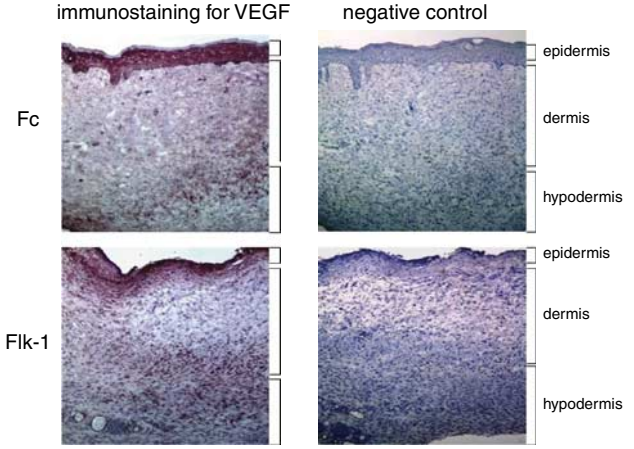

Figure 3 *Immunohistochemistry for VEGF. Staining for VEGF was most prominent in keratinocytes. VEGF immunoreactivity tended to be more pronounced in the wound dermis and hypodermis in Flk-1 as compared to Fc-treated mice. Negative control sections (omission of primary antibody) from the same area are shown on the right panel (10 × objective).*

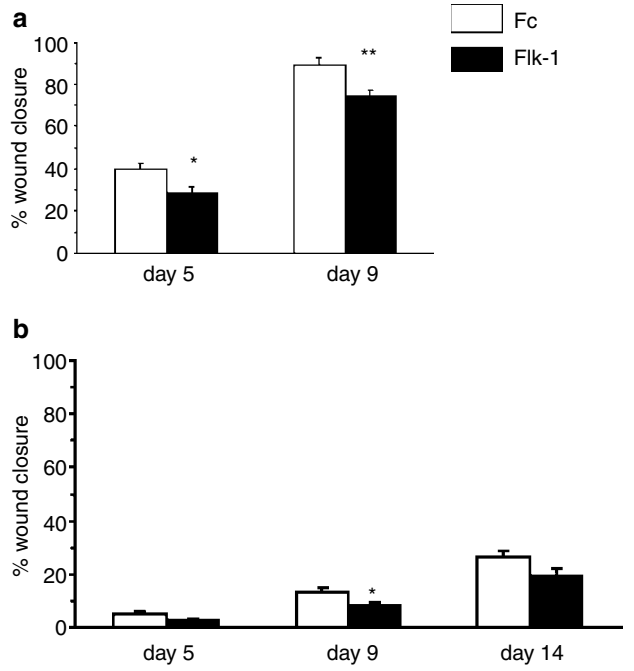

Figure 4 *Effect of antiangiogenic gene transfer with sFlk-1 on wound closure. (a) Wound closure in wild-type mice after 5 and 9 days. Wound closure was significantly delayed in Flk-1-treated mice at both time points. (b) Wound closure in diabetic mice after 5, 9 and 14 days. Wound closure was only significantly different between Fc- and Flk-1-treated mice after 9 days, but not after 5 or 14 days (*$P<0.05$, **$P<0.01$).*

22.8±2.0%. Again, the degree of wound closure tended to be lower in Flk-1- compared to Fc-treated mice at all time points; however, differences were only statistically significant after 9 days (4.6±1.1%, P=0.042, Figure 4b). After 14 days, wound closure was not statistically different between Fc- and Flk-1-treated diabetic mice (7.0±2.4%, P=NS, Figure 4b). The modest reductions in wound closure were in contrast to the marked reduction in wound vascularity (~65%) observed in Flk-1-treated diabetic animals.

Correlation of wound vascularity with wound closure.

Consistent with the visual discrepancy between CD31 immunoreactivity (Figure 2b, c) and wound closure, there was little correlation between vascularity (as assessed by fluorescence microsphere analysis) and wound closure. In wild-type animals, there was only a modest correlation between wound vascularity and wound closure after 9 days (r=0.51, $P<0.05$, Figure 5a). Similarly, in diabetic mice, the correlation between wound vascularity at day 14 and the degree of wound closure at day 9 (only time point at which wound closure was found to be significantly different in these mice) was weak (r=0.50, $P<0.05$, data not shown) and no longer observed when wound vascularity at day 14 was plotted against wound closure at day 14 in these mice (r=0.28, P=NS, Figure 5b).

Histological scores (ranging from 1–12) were not significantly different between the two treatment groups although scores tended to be lower in Flk-1-treated mice (wild-type mice: 9.0±0.6 *versus* 7.1±0.9, P=NS; diabetic mice: 5.5±1.3 *versus*. 4.0±1.0, P=NS).

Discussion

In the present study, we demonstrated that adenoviral-mediated gene transfer of the soluble VEGF receptor Flk-1 markedly inhibits wound angiogenesis in C57Bl/6J and genetically diabetic mice. The results clearly demonstrate the importance of VEGF in wound angiogenesis as indicated by both sFlk-1-induced reductions in CD31 staining and fluorescent microsphere determination of vascularity. Unexpectedly, there was a significant discordance between the marked effect of sFlk-1 on vascularity and its modest effects on wound closure. Although angiogenesis is thought to be a critical determinant of wound healing angiogenesis,[17,24–26] a severe impairment in wound angiogenesis was not accompanied by an equally severe impairment in wound healing.

Indeed, in the diabetic mice, there was a profound impairment in wound angiogenesis that was not matched by an equal impairment in wound closure. Likewise, wound healing in wild-type mice remained relatively rapid even when vascularity was markedly reduced by administration of the soluble VEGF receptor, with substantial portions of wound dermis from Flk–Fc-treated mice appearing avascular.

Previous studies have outlined a striking antiangiogenic effect of adenoviral-mediated gene transfer with either Flk-1 or Flt-1 in mouse tumor models.[11–14] In these studies, gene delivery with either Flk-1 or Flt-1 elicited a ~80% suppression in tumor growth that was associated with decreased tumor microvessel density.[11] Similar antiangiogenic properties were demonstrated in the corneal micropocket assay in which Flk-1 and Flt-1 strongly inhibited corneal neovascularization.[11,16] Based on these findings, we hypothesized that gene transfer with soluble VEGF receptors might also impair wound healing. Of note, animals in this study were injected with the same systemic dose (10^9 PFU) of the respective adenoviruses that were found to be highly effective in suppressing tumor growth and corneal neovascularization in the above-mentioned studies.[11,16]

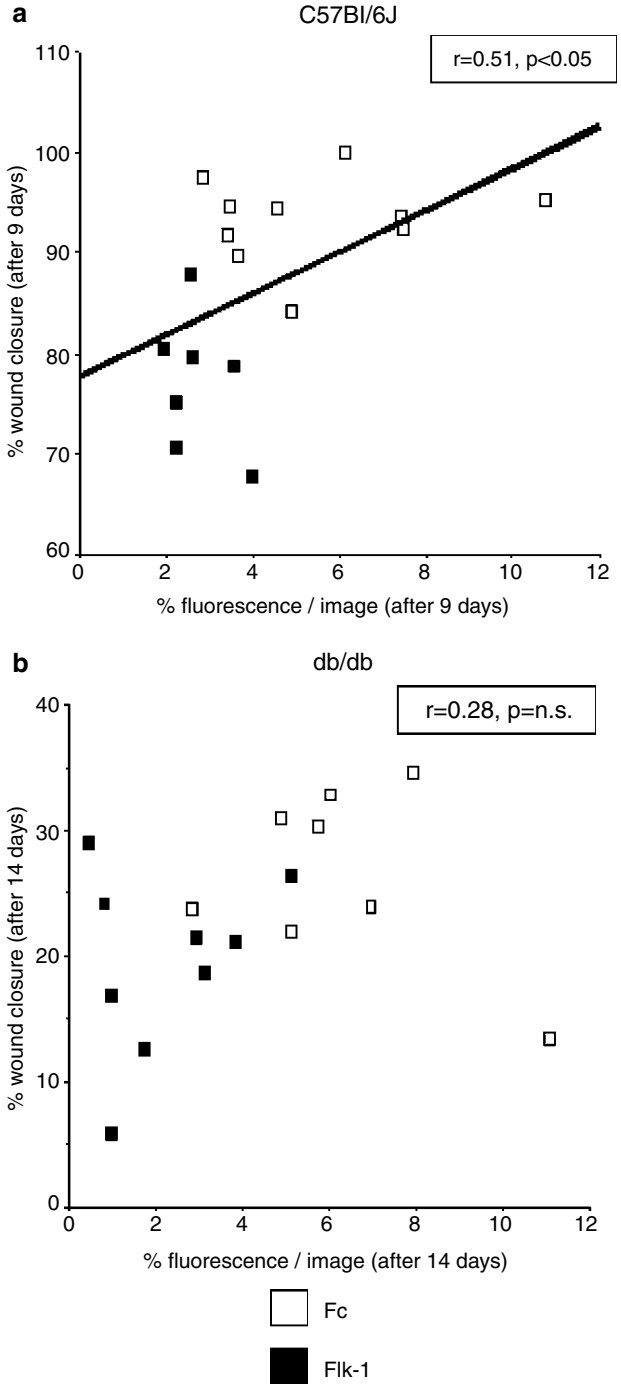

Figure 5 *Bivariate correlation analysis between wound vascularity (as determined by fluorescent microsphere analysis) and wound healing (as determined by area of the wound) in C57Bl/6J and db/db mice. In C57Bl6J mice, there was only a weak correlation between wound angiogenesis and the degree of wound closure after 9 days. In db/db mice, no correlation was seen between wound angiogenesis at day 14 and the degree of wound closure at day 14.*

The lack of a profound effect of antiangiogenic therapy with Flk-1 on wound closure is consistent with reports from a recent publication using the same wound healing model in C57Bl/6J mice.[27] In this study, topical treatment (over 4 days) with retroviruses encoding a signaling-defective, truncated VEGF type 2 receptor significantly reduced angiogenesis and granulation tissue formation without delaying wound closure.[27] However, retroviral infection was performed on the wound directly without quantitation of infection of endothelial or epithelial cells, in contrast to the current results utilizing an adenoviral system documented to produce stringent systemic VEGF inhibition. Similar results on wound angiogenesis were reported in studies in which the effect of the synthetic compound SU5416, a selective inhibitor of VEGF type 2 receptor, was analyzed in rodent models of wound angiogenesis after right pulmonary lobectomy or after creation of dorsal skin flaps.[28,29] Of note, these studies primarily focused on the effects of SU5416 on wound vascularity and not wound closure. However, recently it has been demonstrated that SU5416 delays both wound healing and wound angiogenesis in a plexiglass chamber wound model in rats.[30]

In the present study, we particularly focused our attention on the relationship between wound angiogenesis and wound closure after antiangiogenic treatment. Our data indicate that although VEGF function is essential for optimal wound angiogenesis, it is not required for wound closure even under impaired wound healing conditions such as in the diabetic mice. However, our results do not rule out an important role for VEGF-induced angiogenesis on wound tensile strength. Furthermore, it should be noted that adenoviral overexpression of VEGF isoforms (VEGF121 and VEGF165) or topical treatment with VEGF does accelerate wound healing, enhances wound vascularity and increases bursting strength of wounds in mice and rats.[31–35]

The overall small impact of antiangiogenic therapy on wound healing may result from the fact that wound healing is a complex coordinated process encompassing a variety of different cell types (keratinocytes, endothelial cells, fibroblasts, inflammatory cells, epidermal cells) and growth factors other than VEGF such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), keratinocyte growth factor (KGF). Thus, despite an impairment of wound angiogenesis, redundant mechanisms not further analyzed in this study appear to prevent a gross delay in wound closure.

While wound angiogenesis as assessed by fluorescence microscopy and immunohistochemistry was markedly attenuated in Flk-1-treated mice, local VEGF secretion within the wound dermis and hypodermis tended to be more pronounced in Flk-1- compared to Fc-treated animals. Thus, while VEGF function was inhibited by the soluble VEGF receptor, local VEGF release was enhanced. This finding is consistent with a compensatory response to VEGF blockade. In order to determine whether local VEGF blockade inhibited downstream VEGF signaling pathways such as activation of p-Akt, we performed immunohistochemical staining for p-Akt. Interestingly, immunohistochemistry did not reveal significant p-Akt staining in either Fc or Flk1-Fc wound endothelium, despite strong p-Akt immunoreactivity in keratinocytes. Incidentally, we did however observe a marked reduction in p-Akt signal in keratinocytes of Flk1-Fc as compared to Fc wounds (data not shown). This is interesting with regard to potential endothelial–keratinocyte crosstalk as required for keratinocyte Akt phosphorylation, since keratinocytes do not appear to express VEGF receptors.[36] This observation deserves further investigation and goes beyond the scope of the present paper to address in detail.

We conclude that adenoviral gene transfer with soluble Flk-1 ectodomains inhibits wound angiogenesis. Surprisingly, the marked reduction of wound vascularity is accompanied by only a modest reduction in wound closure. Overall, our data is consistent with the notion that VEGF is an essential mediator of wound angiogenesis. However, neither VEGF nor wound angiogenesis are absolutely required for wound closure. The observed reduction in wound angiogenesis after VEGF inhibition is consonant with potent VEGF blockade mediated by Flk1-Fc, and suggests that reduction in wound vascularity could serve as a surrogate biomarker of clinical efficacy. Further, these data suggest that stringent VEGF blockade can be achieved by Ad Flk1–Fc or VEGF-targeting strategies such as monoclonal antibodies or small molecule kinase inhibitors even in the setting of diabetes without significantly compromising gross wound closure.

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