BRIEF REPORT

An activated factor VII variant with enhanced tissue factor-independent activity speeds wound healing in a mouse hemophilia B model

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Essentials

- Disorders of hemostasis can lead to delayed and defective wound healing.
- In hemophilia B (HB) mice, 7 days of Factor (F)IX or VIIa are needed to normalize wound healing.
- One dose of a highly active FVIIa variant (DVQ) restored normal wound closure time in HB mice.
- Coagulation factors with enhanced activity may acquire biological effects not due to hemostasis.

Summary. *Introduction:* We have previously reported that hemophilia B (HB) mice have delayed healing of cutaneous wounds and alterations in wound histology. Administration of a single dose of either factor IX or recombinant activated FVII (rFVIIa) (NovoSeven) prior to wounding did not improve wound closure time or histology. The **activated** FVII analog DVQ (V158D, E296V and M298Q mutations) was designed to have higher tissue factor-independent activity than rFVIIa. We hypothesized that a single dose of DVQ would be more effective in restoring wound healing in HB mice. *Methods:* Cutaneous punch wounds were made on the backs of HB and wild-type mice, and the time to wound closure was monitored. HB mice were treated with a dose of rFVIIa (10 mg kg⁻¹) or DVQ (1 mg kg⁻¹) that corrected the tail bleeding time. Skin samples were taken at various time points after wounding, fixed, and stained, and the histology was exam-

ined. *Results:* As previously reported, wound closure times in HB mice given one dose of rFVIIa were not improved over those in untreated HB mice. Surprisingly, healing times in HB mice treated with an equally hemostatic dose of DVQ were normalized to that in wild-type mice. However, DVQ did not correct all histologic abnormalities in HB mice. *Conclusions:* As the doses of DVQ and rFVIIa were chosen to support comparable levels of hemostasis, our data suggest that the improved healing seen with DVQ is not solely attributable to its hemostatic activity. It is possible that the improved wound healing arises through the effect of DVQ on cell signaling mechanisms.

Keywords: angiogenesis inhibitors; epithelium; hemophilia; hemostasis; thrombin.

Introduction

We have previously found that healing of cutaneous punch biopsy wounds is delayed in hemophilia B (HB) mice [1]. In addition to the delay in healing, HB mice also show histologic abnormalities of the wound site [1]. These include a delay in the influx of macrophages, increased numbers of angiogenic vessels, and increased iron deposition. In addition, HB mice suffer delayed bleeding, with some hematomas forming even after the surface wound is completely closed. The late hematomas occur in the tissue plane just below the dermis, where blood vessels are located that serve as the source of angiogenic sprouts. A single dose of replacement (factor IX) therapy or a single dose of recombinant activated FVII (rFVIIa) at the time of biopsy wound placement does not normalize healing in HB mice, or prevent late hematoma formation [2]. Hemostatic treatment for at least 5 days is required to normalize healing in this model [3].

DVQ (also called NN1731 and Vatreptacog alfa) is a rationally designed analog of **activated** FVII (FVIIa) in

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which three amino acids are changed in the catalytic domain (V158D/E296V/M298Q) [4]. It has increased proteolytic activity as compared with FVIIa in the absence of tissue factor (TF), but approximately the same activity as FVIIa in the presence of TF [4]. DVQ was developed as a potentially more effective 'bypassing agent' for the management of bleeding in hemophilia patients, based on the hypothesis that the hemostatic effect of high-dose rFVIIa in this setting is primarily attributable to its TF-independent activity on the surface of activated platelets [5]. The DVQ variant was found to have enhanced procoagulant and antifibrinolytic activities in an *in vitro* model of hemophilia relative to wild-type (WT) FVIIa [6], and higher hemostatic efficacy in a mouse hemophilia model [7]. In phase 1 to phase 3 clinical trials, this analog was more potent than rFVIIa [8,9]. However, clinical development was stopped when some patients developed anti-drug antibodies [10]. We hypothesized that DVQ might have a greater effect on wound healing in our HB mouse model than rFVIIa, because of its ability to support a higher level of thrombin generation.

Materials and methods

Animal model of wound healing

Adult WT mice (C57BL/6) and FIX-knockout (HB) mice on a C57BL/6 background were used in these studies [11]. Wound healing studies were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. We used the cutaneous punch biopsy wound model that we have previously reported [1,2]. A three-millimeter biopsy punch wound was made on the back of each WT and HB mouse. For HB mice receiving factor therapy, a dose of either rFVIIa (NovoSeven; Novo Nordisk, Bagsvaerd, Denmark) or the DVQ variant of FVIIa (a kind gift from E. Persson, Novo Nordisk) was administered by tail vein injection 30 min prior to wounding. rFVIIa at 10 mg kg⁻¹ and DVQ at 1 mg kg⁻¹ were the doses required to prevent excess bleeding in a tail clip model [7]. Some mice were also dosed with 10 mg kg⁻¹ DVQ. The plasma half-life of human rFVIIa is ~1 h in mice [7], whereas the plasma half-life of DVQ is probably shorter, owing to its more rapid inhibition by antithrombin [12]. In some experiments, a solution of DVQ (10 µL of 1.8 mg mL⁻¹) was applied topically to the wound site immediately after punch biopsy placement.

Mice were given 480 mg of acetaminophen in 150 mL of water for analgesia until day 4 after wounding. Wound size was recorded daily, and the wound area was calculated. Mice were killed, and wounded skin was collected at selected time points.

Microscopic evaluation of tissues

Collection of skin samples, handling, processing, immunostaining and evaluation of tissue sections were performed as previously described [1]. Briefly, skin from the wound sites was pinned flat in 10% buffered formalin for 12–24 h before being processed into paraffin. Specimens were bisected and embedded with the wound center up. Sections were stained with hematoxylin and eosin or immunostained for vessel counts (anti-CD34; Serotec, Raleigh, NC, USA). Antigen retrieval was performed with DakoCytomation Target Retrieval Solution (Dako, Carpinteria, CA, USA) before immunostaining. Angiogenic vessels were counted as previously described [1].

Measurement of affinity of human FVIIa and DVQ for mouse TF

The cDNA encoding the extracellular domain of mouse TF was amplified from a mouse lung cDNA library (which was purchased ~20 years ago from Stratagene; the product has since been discontinued) with two primers: tataccatggcaggcattccagagaaag and ctaatggatcctcattctcccaggaaactcttcattg. The amplified cDNA was cloned into pET28a (Novagen, now EMD Millipore) through *Nco*I and *Bam*HI sites. The cloned sequence was confirmed by the UNC-CH genome analysis facility. Expression and purification of recombinant soluble mouse TF protein were performed as previously described [13].

FVIIa binding and DVQ binding were assayed as the increase in FX activation when they were bound to TF. Although DVQ has higher activity than FVIIa in the absence of TF, its activity is still increased by binding to TF. Phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were from Avanti Polar Lipids (Birmingham, AL, USA). Large unilamellar vesicles (LUVs) with the composition 15% PS/41% PC/44% PE were prepared as previously described [14]. The assay was performed at room temperature in: 20 mM HEPES (pH 7.5), 150 mM NaCl, 3 mM CaCl₂, and 1 mg mL⁻¹ ovalbumin. The final concentrations of components were: 0.1 nM soluble mouse TF, 60 µM LUVs, 150 nM FX (Enzyme Research Laboratories, South Bend, IN, USA; repurified as in [15]), and 250 µM Pefachrome ~~activated FX~~ (FXa) (Pentapharm, Basel, Switzerland), with varied amounts of FVIIa or DVQ. FVIIa and DVQ were mixed with soluble TF and LUVs for 5 min to allow binding. FX and Pefachrome FXa were added, and FXa cleavage of substrate was monitored.

Statistical evaluation

Wound sizes were compared by the use of Student's *t*-test, with Tukey's correction for multiple comparisons. The percentages of wounds closed at a given time point

were compared by the use of Fisher's exact test [16]. Statistical analysis was performed with GRAPHPAD PRISM 6.

Results and discussion

A single injection of DVQ normalizes wound closure in HB mice

As shown in Fig. 1, a single injection of DVQ at the time of punch biopsy placement normalized wound size (upper left) and time to healing (lower left) in HB mice. HB mice in our previous study were treated with 10 mg kg⁻¹ rFVIIa [2]. We treated HB mice with the same dose of DVQ, and observed that the wound sizes were smaller than those in untreated HB mice, and, surprisingly, that the time to wound closure was normalized. DVQ is significantly

more potent than rFVIIa in a tail clip bleeding model in mice, with 1 mg kg⁻¹ having equivalent hemostatic effectiveness as 10 mg kg⁻¹ rFVIIa [7], so we also tested the lower dose. Wound sizes in the mice given a single injection of either 1 mg kg⁻¹ or 10 mg kg⁻¹ DVQ at the time of wounding were significantly smaller than those in untreated HB mice ($P < 0.05$; Student's *t*-test with Tukey's correction for multiple comparisons) at multiple time points, and were not significantly different from those in WT mice. In addition, the time to complete closure of the skin defect in DVQ-treated mice was significantly improved as compared with untreated mice (by Fisher's exact test [16]), and not different from that in WT mice. A single injection of rFVIIa, as we have previously reported [2], did not significantly improve wound size or time to healing in these experiments (Fig. 1). Thus,

Colour online, B&W in print

Fig. 1. Cutaneous wound healing in hemophilia B (HB) mice is normalized by one dose of DVQ. A punch biopsy wound was made on each mouse. Some HB mice received an intravenous injection of DVQ (1 mg kg⁻¹ or 10 mg kg⁻¹) or activated factor VII (FVIIa) (10 mg kg⁻¹) at the time of wounding. Each point is the mean of measurements from 17–20 HB mice and 11 wild-type mice. For HB mice treated with 10 mg kg⁻¹ DVQ, the numbers of mice were 25 (days 1–4), 23 (days 5–6), 13 (days 7–8), or seven (days 9–12). For 1 mg kg⁻¹ DVQ, the numbers of mice were 15 (days 1–6), 12 (days 7–8), or 10 (days 9–12). For FVIIa, the numbers of mice were 25 (days 1–2), 22 (days 3–4), 19 (days 5–6), 17 (days 7–8), 12 (days 9–10), and seven (days 11–12). The wound areas are plotted as a function of days after wounding in the upper left panel. The bars represent the standard error of the mean. Wound sizes for days 4, 5 and 8 are shown in the upper right panel ($*P < 0.05$ as compared with untreated HB mice; Student's *t*-test). In this panel, the bars represent the standard deviation. The percentages of wounds healed at each day after wounding are plotted in the lower left panel, and the percentages of wounds healed at day 9 are shown in the bar graph in the lower right panel. A significantly greater proportion of wounds were healed in HB mice treated with 1 mg kg⁻¹ or 10 mg kg⁻¹ DVQ at days 9 and 10 than in other HB mice ($*P < 0.05$; Fisher's exact test).

a single dose of DVQ, unlike FIX and rFVIIa, could normalize the time course of cutaneous healing in HB mice.

A single injection of DVQ does not normalize wound histology in HB mice

One of the histologic abnormalities present in HB mice following wounding is the presence of an excess of angiogenic vessels around the wound bed. These vessels predispose the HB mice to episodes of bleeding and hematoma formation for an extended period of time following wounding [17]. Therefore, we tested the hypothesis that a dose of DVQ would also normalize angiogenesis following wounding. However, as shown in Fig. 2, angiogenesis in the wound bed was not normalized by a single injection of DVQ at the time of punch biopsy placement. The vessel counts in HB mice treated with 10 mg kg^{-1} DVQ were not significantly different from those in untreated HB mice at any time point, although both were significantly greater than those in WT mice at 8 days after wounding.

Topical application of DVQ improves wound healing in HB mice

Intravenous infusion of equally hemostatic doses of DVQ (1 mg kg^{-1}) and rFVIIa (10 mg kg^{-1}) had different effects on wound closure; one dose of DVQ normalized wound closure, whereas rFVIIa did not. This could be the result of DVQ producing a more rapid burst of thrombin generation than rFVIIa, leading to more rapid hemostasis [18] and possibly a more favorable fibrin clot structure to support healing. In fact, DVQ was found to

Fig. 2. Angiogenesis in skin wounds is not normalized by one dose of DVQ in hemophilia B (HB) mice. Angiogenesis was assessed by counting vessel profiles in the wound area. Counts are expressed per high-power field (HPF) ($\times 40$). The scores were averaged for two sections of three to seven wounds per time point. Angiogenesis at day 8 was significantly greater in DVQ-treated or untreated HB mice than in wild-type mice ($*P < 0.05$ as compared with wild-type mice; Student's *t*-test). Angiogenesis in DVQ-treated HB mice was not significantly different from that in untreated HB mice at any time point.

produce more rapid hemostasis in a randomized clinical trial [10]. However, an alternative hypothesis is that DVQ does not enhance epithelial wound closure solely because of its hemostatic effect. Even though treatment with DVQ normalized closure of the epithelial defect, it did not normalize the underlying tissue histology – even at a 10-fold higher dose than required to restore surface closure. This suggests that DVQ might have a specific effect on the squamous epithelium.

To begin testing this hypothesis, we examined the effect of the topical application of DVQ to the wound surface. As shown in Fig. 3, topical application of DVQ immediately after wounding of HB mice reduced wound sizes to the point that they were not significantly different from

Fig. 3. Topical application of DVQ improves wound healing in hemophilia B (HB) mice. A single punch biopsy wound was made on HB or wild-type mice. DVQ was applied to wounds on HB mice immediately after wounding. Each point represents the mean of measurements from 11 mice for wild-type mice, 17–20 for HB mice, and seven for HB mice treated with DVQ. The wound areas are plotted as a function of days after wounding in the upper panel, with bars showing the standard error of the mean of each group. Wound sizes in HB mice treated with topical DVQ were significantly smaller than those in untreated HB mice at days 8–9 ($P < 0.05$; Student's *t*-test with Tukey's correction for multiple comparisons), and not different from those in wild-type mice. The percentage of completely healed wounds is plotted in the lower panel. A significantly greater proportion of wounds were closed at days 9–10 in DVQ-treated HB mice than in untreated HB mice ($P < 0.05$; Fisher's exact test).

those in WT controls. In addition, all wounds in DVQ-treated HB mice were closed by day 10, which is the same as what was seen in WT mice. Thus, topical DVQ normalized wound size and the time to complete wound closure in HB mice.

The efficacy of topical DVQ suggests that it might be acting through a receptor-mediated mechanism to enhance squamous epithelial proliferation and/or migration. There are clear precedents for stimulation of cutaneous healing by components of the coagulation system. Topical thrombin has been reported to enhance wound healing via protease-activated receptor (PAR)-1 [19]. Both activated protein C [20] and FXa [21] have been reported to enhance wound healing via PAR-2. FVIIa in complex with TF can activate PAR-2 [22]. FVIIa in complex with the endothelial protein C receptor can activate PAR-1 [23]. Therefore, we hypothesize that the enhanced proteolytic activity of the DVQ variant may enable it to directly cleave one or more PAR in the absence of a cofactor, and thereby enhance epithelial healing.

Different effects of FVIIa and DVQ are not attributable to differences in TF binding

It has previously been shown that human rFVIIa binds extremely poorly to mouse TF [13]. It is possible that (human) DVQ has greater affinity for mouse TF than does the wild type. However, as shown in Fig. 4, we found that this was not the case. FVIIa and DVQ had similarly poor affinity for the recombinant extracellular domain of mouse TF (soluble TF). In contrast, mouse FVIIa bound tightly to soluble TF. Thus, the beneficial effects of DVQ

on cutaneous wound healing are not mediated by binding to TF.

Conclusions

We found that a single injection or topical application of DVQ was able to normalize closure of a cutaneous punch biopsy wound in HB mice. Neither a single dose of WT rFVIIa nor a single dose of FIX could similarly normalize the time to cutaneous wound closure. Even though surface closure was enhanced by DVQ, the histology of the underlying tissues was not normalized. Because DVQ was more effective in normalizing cutaneous healing than an equally hemostatic dose of FVIIa, we conclude that the ability of DVQ to enhance wound healing may not be mediated solely by its hemostatic properties. A number of coagulation proteins modulate other aspects of host defense, such as inflammation, immune responses, and tissue repair. This suggests that novel variants of coagulation proteins can be designed not only to be more effective hemostatic agents, but also to have additional beneficial effects as biological modulators.

Addendum

M. Hoffman and D. M. Monroe: designed the study, collected and analyzed the mouse data, and wrote the manuscript. J.-Y. Chang: cloned and expressed mouse TF, and conducted TF-binding studies. M. Ezban: provided a critical review of the study design, and edited the final manuscript.

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Disclosure of Conflict of Interests

M. Hoffman reports receiving grants from Novo Nordisk during the conduct of the study, and grants from CSL-Behring and Boehringer Ingelheim outside the submitted work. D. M. Monroe reports receiving grants, personal fees and non-financial support from Novo Nordisk during the conduct of the study, and grants from Novo Nordisk outside the submitted work. The other authors state that they have no conflict of interest.

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Fig. 4. Both human activated factor VII (FVIIa) and DVQ bind poorly to mouse tissue factor (TF). The extracellular domain of mouse TF was expressed as described in Materials and methods. FVIIa binding and DVQ binding to mouse TF were assayed as the increase in FX activation when they were bound to TF in the presence of phospholipid vesicles. Although DVQ has much higher activity than FVIIa in the absence of TF, its activity is still increased by binding to TF. The activity of human FVIIa and DVQ, with recombinant mouse FVIIa as a positive control, are plotted as a function of protease concentration. Over the concentration range tested, neither human FVIIa nor DVQ bound significantly to mouse TF. FXa, activated factor X.

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