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EPO reverses defective wound repair in hypercholesterolaemic mice by increasing functional angiogenesis☆

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Summary This study aims to elucidate the effect of erythropoietin (EPO) on the microcirculation during wound healing in mice genetically depleted of apolipoprotein E ($ApoE^{-/-}$). The skinfold chamber in mice was used for intravital microscopy, whereby an incisional wound was created within the chamber. Animals received Recormon® 1000 U kg^{-1} body weight (BW) intra-peritoneally (i.p.) at day 1, 3, 5, 7, 9 and 11 post-wounding at a concentration of 100 U ml^{-1} ($n = 42$). Normal healing and vehicle-treated wild type animals (WT) served as controls. The microcirculation of the wound was analysed quantitatively in vivo using epi-illumination intravital fluorescence microscopy. Microtomography (micro-CT) analysis of casted wound microvessels was performed allowing three-dimensional (3D) histomorphometric analysis. Tissue samples were examined *ex vivo* for wound scoring and for expression analysis of EPO-Receptor (Epo-R) and endothelial nitric oxide synthase (eNOS). Upon EPO treatment, the total wound score in $ApoE^{-/-}$ mice was increased by 23% on day 3, by 26% on day 7 and by 18% on day 13 when compared to untreated $ApoE^{-/-}$ mice (all $P < 0.05$ vs. vehicle). Improved wound healing was accompanied with a significant increase of functional angiogenetic density and angiogenetic red blood cell perfusion on days 5, 7, 9 and 11 post-wounding. 3D histomorphometric analysis revealed an increase of vessel thickness (1.7-fold), vessel volume (2.4-fold) and vessel surface (1.7-fold) (all $P < 0.05$ vs. vehicle). In addition, improved wound healing was associated with enhanced Epo-R expression (4.6-fold on day 3 and 13.5-fold on day 7) and eNOS expression (2.4-fold on day 7) (all $P < 0.05$ vs. vehicle).

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Our data demonstrate that repetitive systemic EPO treatment reverses microvascular dysfunction during wound healing in hypercholesterolaemic mice by inducing new vessel formation and by providing the wound with more oxygen.

Non-healing wounds represent a considerable socioeconomic burden to society.[1] Erythropoietin (EPO) has gained preclinical acceptance as a protective cytokine in many tissues. Physiologically, EPO is secreted from the kidney in response to hypoxia causing the maturation of erythrocyte progenitors by inhibiting their apoptotic cell death. However, an emerging number of studies demonstrate protective non-haematopoietic effects of EPO treatment during wound healing.[2,3] Wound healing includes the programmed sequence of inflammation, proliferation and reparation.[4] Inflammation is orchestrated by macrophages, which release pro-inflammatory cytokines and promote the transition to proliferation.[5] Chronic wounds often remain in the inflammatory stage and disrupt the linear progression of wound healing. During early inflammation, EPO-mediated improved wound healing was shown to be associated with inhibited lipid peroxidation.[6] EPO was also shown to reduce the release of pro-inflammatory cytokines such as interleukin (IL) 1-β, IL-6 and tumour necrosis factor-α (TNF-α) both *in vivo* in the diabetic mouse and *in vitro*.[7,8] The formation of new blood vessels during wound healing provides a route for oxygen delivery and a conduit for components of the inflammatory response.[9] Using a mouse model of hot water-induced second degree burn, EPO was shown to improve wound healing by increasing vascular endothelial growth factor (VEGF).[10] The same research group demonstrated further that better healing was driven by enhanced angiogenesis in the genetically diabetic mouse.[11] During reparation fibroblasts produce collagen, glycosaminoglycans and proteoglycans, which are major components of the extracellular matrix (EM). EPO was shown to increase the hydroxyproline content in wounds.[6] Regarding the molecular mechanism, improved EM deposition was shown to follow the TGF-β signalling pathway.[12]

This is the first study to explore the *in vivo* effect of EPO on the microcirculation in healing tissues. Cardiovascular disease and peripheral arterial disease represent the most important aetiologic factors for chronic wounds in patients.[13] As hypercholesterolaemia represents a major aetiologic factor for atherosclerosis, we have chosen mice genetically depleted of apolipoprotein E ($ApoE^{-/-}$), which develop atherosclerotic lesions similar to those of humans.[14] Impaired wound healing associated with hypercholesterolaemia is not completely understood but is believed to be caused by endothelial inflammation, impaired neo-vascularisation, decreased synthesis of collagen and increased levels of proteinases.[15,16] We therefore treated $ApoE^{-/-}$ mice and compared them to normal healing, wild type (WT) mice. Further, this is the first study to monitor the microvasculature of the wound *in vivo* by the use of intravital microscopy. We were able to repetitively assess microhaemodynamics *in vivo* exactly in the same vessels of the granulation tissue within the wound margins. *In vivo* assessment of wound microhaemodynamics has been performed in the hairless mouse[17] and in a rat burn wound model[18]; however, no information about the functionality of the newly formed vessels was provided in either study.

Materials and methods

Protocol

A total of 37 wild type (WT) male C57BL/6J and 74 ($ApoE^{-/-}$) (B6.129P2-ApoE/J) mice were used (111 mice, 12–18 weeks of age, 22–25 g body weight; Jackson Laboratories; Bar Harbor, ME, USA). Animals received humane care according to the guidelines of the University Hospital of Zurich; the study protocol was approved by the Federal Veterinary Office. Animals were randomly assigned to following three groups: EPO-treated $ApoE^{-/-}$, $n = 37$, receiving Recormon® 1000 U kg^{-1} body weight (BW) intra-peritoneally (i.p.) on days 1, 3, 5, 7, 9 and 11 at a concentration of 100 U ml^{-1}; vehicle-treated $ApoE^{-/-}$, $n = 37$; and vehicle-treated WT, $n = 37$. Animals were housed one per cage, maintained under controlled environmental conditions and provided with standard laboratory food and water *ad libitum*. Five animals in each group ($n = 15$) were used for intravital microscopy analysis. For histological studies on days 3, 7 and 13 post-wounding, nine additional animals of each group and each timepoint ($n = 81$) were wounded. Five animals in each group ($n = 15$) were used for corrosion casting.

Wound model

The dorsal skinfold chamber in mice was used for intravital microscopy as previously described (Figure 1(A)).[19] Briefly, mice were anaesthetised i.p. with a mixture of 90 mg kg^{-1} BW ketamine hydrochloride (Ketavet®, Parke Davis; Freiburg, Germany) and 25 mg kg^{-1} BW xylazine hydrochloride (Rompun®, Bayer; Leverkusen, Germany), and two titanium frames were implanted to sandwich the extended double layer of the skin. After removing one layer, in the remaining layer a 7-mm incisional wound was created and the wound edges sutured with 9/0 Nylon at 1-mm intervals.

Intravital fluorescence microscopy

An epi-illumination intravital microscope (Leica DM/LM; Leica Microsystems, Wetzlar, Germany) attached to a blue (450–490/ > 520 nm), a green (530–560/ > 580 nm) and an ultraviolet (330–390/ > 430 nm excitation/emission wavelength) filter system was used. Images were captured by a television camera (Kappa Messtechnik, Gleichen, Germany), displayed on a television screen (TrinitronPVM-20N5E; Sony, UK) and recorded on video (50 Hz; Panasonic AG-7350-SVHS, Tokyo, Japan) for subsequent analysis. Wounds were observed