Biochemical and Biophysical Research Communications 421 (2012) 197–202

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Vascular endothelial insulin/IGF-1 signaling controls skin wound vascularization

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ARTICLE INFO

Article history:
Received 23 March 2012
Available online 4 April 2012

Keywords:
Impaired wound healing
Diabetic foot ulcers
Vascular endothelia
Insulin/IGF signaling

ABSTRACT

Type 2 diabetes mellitus affects 6% of western populations and represents a major risk factor for the development of skin complications, of which impaired wound healing, manifested in e.g. "diabetic foot ulcer", is most prominent. Impaired angiogenesis is considered a major contributing factor to these non-healing wounds. At present it is still unclear whether diabetes-associated wound healing and skin vascular dysfunction are direct consequences of impaired insulin/IGF-1 signaling, or secondary due to e.g. hyperglycemia. To directly test the role of vascular endothelial insulin signaling in the development of diabetes-associated skin complications and vascular function, we inactivated the insulin receptor and its highly related receptor, the IGF-1 receptor, specifically in the endothelial compartment of postnatal mice, using the inducible Tie-2CreERT (DKO^{IVE}) deleter. Impaired endothelial insulin/IGF-1 signaling did not have a significant impact on endothelial homeostasis in the skin, as judged by number of vessels, vessel basement membrane staining intensity and barrier function. In contrast, challenging the skin through wounding strongly reduced neo-angiogenesis in DKO^{IVE} mice, accompanied by reduced granulation tissue formation reduced. These results show that endothelial insulin/IGF signaling is essential for neo-angiogenesis upon wounding, and imply that reduced endothelial insulin/IGF signaling directly contributes to diabetes-associated impaired healing.

1. Introduction

Type 2 diabetes mellitus represents the most frequent endocrine disease, affecting more than 6% of western populations [1]. Thereby, it represents a major socio-economical health burden. Lethality results largely from diabetic complications including micro- and macrovascular diseases [1]. Moreover, diabetes is associated with a plethora of skin diseases. One hallmark of diabetes is impaired wound healing [2]. It is now well established that ulcerations and subsequent amputation events represent serious complications of diabetes mellitus and are associated with significant mortality as well as socio-economical costs [3–5]. The lifetime risk for any diabetic patient to develop such a complication ranges around 15% [6]. It is not resolved yet whether diabetic skin complications arise in response to metabolic alterations such as hyperglycemia or directly from impaired insulin action in skin [7]. A severe impairment of insulin signaling in wounds of diabetic mouse models has been described [8], suggesting a potential direct role for altered insulin signaling in impaired healing.

A range of physiological changes can contribute to diabetes-associated wound healing deficiencies, such as impaired responsiveness to growth factors, macrophage function, collagen accumulation, and altered granulation tissue formation [2,9]. Vascular insufficiency is thought to be one major driver of impaired healing [9–12]. Vascular endothelial cells express functional insulin receptors and even higher levels of the highly related IGF-1 receptors [13,14]. Importantly, endothelial dysfunction is observed early upon systemic insulin resistance [15,16] and is associated with a blunted insulin/IGF signaling response in endothelial cells [17,18]. A direct function for cell-autonomous insulin/IGF signaling in endothelial dysfunction associated with diabetes was further

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http://dx.doi.org/10.1016/j.bbrc.2012.03.134

confirmed using transgenic mice. Vascular endothelial specific deletion of the insulin receptor (IR) reduced basal expression of the vasoactive mediators eNOS and ET-1 [19], whereas endothelial expression of a dominant-negative IR mutant resulted in reduced phosphorylation of eNOS accompanied by a blunted vasodilation response upon insulin stimulation [20]. In both cases global glucose homeostasis was unaltered, suggesting that dysfunctional vascular endothelial IR signaling does not directly contribute to overall insulin resistance. Perhaps more importantly in the light of diabetic wound healing, either IR or IGF-1 receptor inactivation reduced neo-angiogenesis upon hypoxia-induced retinopathy, although the effect was less dramatic than loss of IR [21].

To address the role of vascular endothelial insulin/IGF-1 signaling in the homeostasis of the skin vasculature and in neovascularization in the skin in wound healing, we inactivated both the IR as well as the IGF-1R in adult vascular endothelia using inducible Cre-LoxP technology. We show that endothelial insulin/IGF-1 signaling upon wounding regulates angiogenesis and granulation tissue formation.

2. Material and methods

2.1. Mice

Floxed IR and IGF-1R alleles and tamoxifen inducible Tie2-CreER^{T2} mouse lines and their genotyping have been described previously [22,28]. All mice used in experiments were derived from at least 5th generation C57BL/6 crossings. For induction of Cre activity, adult 4–5 weeks old mice carrying the Tie2-CreER^{T2} transgene and their Cre-negative littermates were fed a tamoxifen-containing diet (400 mg/kg tamoxifen citrate, 5% sucrose, 95% Teklad Global, 16% Rodent Diet) from Harlan Teklad[22,23]. All animal procedures were conducted in accordance with European, national, and institutional guidelines, and protocols and were approved by local government authorities. PCR was used to detect deletion efficiency for *Insr* or *Igf1*r using the following primers: *Igf-1r*. 5′-TTATGCCTCCTCTCTTCATC-3′ (sense) and 5′-CTTCAGCTTTGC AGGTGCACG-3′ (antisense) resulting in products of 1165 bp for wt, 1350 bp for floxed and 491 bp for deleted alleles. *Insr*. 5′-ACGCCTACACATCACATGCATATG-3′ (sense) and 5′-CCTCCTGAATA GCTGAGACCACAG-3′ (antisense) resulting in products of 2048 bp for wt, 2188 bp for floxed and 249 bp for deleted alleles on DNA isolated from lung, heart and on occasion endothelial cells.

2.2. Skin irritant response, wounding and preparation of wound tissues

Irritant response was elicited and measured as previously described [30]. Wounding and preparation of wound tissue for histology was performed as recently described [29]. Briefly, mice were anesthetized by i.p injection of Ketanest/Rompun (Ketanest S: Park Davis GmbH; Rompun 2%: Bayer), the back was shaved and four full thickness punch biopsies were created. For histological analysis wounds were excised at indicated time points post injury, bisected in caudocranial direction and the tissue was either fixed overnight in 4% formaldehyde or embedded in OCT compound (Tissue Tek). Histological analysis was performed on serial sections from the central portion of the wound.

2.3. Histology and immunohistochemistry

For histology paraffin skin or wound sections were stained for hematoxilin/eosin (H&E) as described [28]. Immunohistochemistry was performed on either cryosections (10 μm) or paraffin sections fixed with 4% PFA or acetone, blocked (10% NGS, 1& BSA, 0.02% Tween-20), and incubated with the appropriate primary antibodies followed by Alexa Fluor 488- or 594- conjugated (Invitrogen) secondary antibodies. Nuclei were counterstaining with DAPI (Invitrogen). Primary antibodies used were rabbit antibodies to collagen IV (Progen), laminin411 (a kind gift of Lydia Sorokin, University of Munster, Germany) and mouse mAbs, Desmin (DakoCytomation), rat mAbs to CD31 (BD pharmingen), VE-cadherin (BD Biosciences) as well as the appropriate isotype-matched negative control antibodies. Histological images were generated using a LEICA microscope equipped with a digital DZM1200 camera. Fluorescent analysis and images were done either with a Nikon Microscope Eclipse 800E or with an Olympus IX81 microscope with a digital cool snapTM HQ2 camera (photometrics) or an Olympus Fluoview 1000 laser scanning microscope. Autoquant software was used for the high magnification vessel images.

2.4. Morphometric analysis

Morphometric analysis was performed on digital images using the Imaging Software Lucia G 4.80 (Laboratory Imaging Ltd., Prague, Czech Republic). The extent of epithelization and granulation tissue formation was determined on hematoxylin/eosin (H&E)-stained paraffin tissue sections as previously described [29]. The length of the epithelial tongue was determined as the distance between the epithelial tip and the margin of the wound as defined by the presence of hair follicles in non-wounded skin. This parameter reflects the formation of neo-epithelium. In addition, the width of the gap between the epithelial tips reflects wound closure. Granulation tissue was defined as the cellular and vascular tissue that formed underneath the neo-epithelium and between the wound margins and above the subcutaneous fat tissue. Images were processed with Adobe Photoshop 7.0. Staining for CD31, desmin, collagen IV and VE-cadherin was quantified in high magnification fields ($7000 \times 5500\ \mu m^2$) using ImageJ software. Histomorphometric analyses were performed in a blinded manner by two independent investigators. Statistical analyses were performed using GraphPad Prism5 (GraphPad Software). Significance of difference was analyzed using Student's unpaired two-tailed *t*-test. All data presented as mean ± SD, a p value of <0.05 was considered significant.

3. Results

3.1. Vascular homeostasis is not obviously affected by loss of endothelial IR/IGF-1R

To examine the role of Insulin and its closely related growth factors IGFs in the adult vasculature in the skin, mice floxed for both the IR and IGF-1R were crossed with mice carrying Tie2-CreER^{T2}, which induces Cre activity specifically in vascular endothelial cells (IVE) upon feeding mice tamoxifen for 5 weeks [22,23]. In general, after 5 weeks of tamoxifen feeding, efficient deletion of the insulin and IGF-1R alleles was induced (Fig. 1A) in mice carrying the Tie2-CreER^{T2} allele (hereafter referred to as DKO^{IVE}). As controls we used mice carrying either floxed alleles only or the Tie2-Cre^{ERT2} allele with wt IGF-1R and IR alleles. DKO^{IVE} mice were viable and showed no obvious defects, even upon prolonged deletion (not shown). Histological analysis of the skin did not reveal any major alterations (Fig. 1B, upper panel) and vessels appeared microscopically similar when compared to controls (Fig. 1B lower panel).

To address if loss of endothelial insulin/IGF signaling affected vessel density in the skin, sections were stained for VE-cadherin (Fig. 2A) and CD31 (not shown). No obvious changes in localization were observed between control and DKO^{IVE} for either endothelial specific marker. Further quantification did not reveal any obvious changes in VE-cadherin in the area stained positive for VE-cadherin