Journal of Cranio-Maxillo-Facial Surgery 42 (2014) 1710–1716

Contents lists available at ScienceDirect

Journal of Cranio-Maxillo-Facial Surgery

journal homepage: www.jcmfs.com

Angiogenic response pattern during normal and impaired skin flap re-integration in mice: A comparative study☆

Christoph Schürmann [a, *], Nadine Schmidt [a], Oliver Seitz [a, b], Josef Pfeilschifter [a], Stefan Frank [a]

[a] pharmazentrum frankfurt/ZAFES (Head: Prof. M. D. Josef Pfeilschifter), Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany
[b] Klinik für Mund-, Kiefer- und Plastische Gesichtschirurgie (Head: Prof. Robert Sader), Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Paper received 14 February 2014
Accepted 4 June 2014
Available online 17 June 2014

Keywords:
Wound healing
Diabetes mellitus
Surgical flaps
Angiogenesis
VEGF
HIF-1α

ABSTRACT

Background: Distal skin flap necrosis represents a severe complication in surgery. This study investigated angiogenic responses in healthy and impaired pedicled skin flap tissue in normal and diabetic mice.
Methods: Histologic, qRT-PCR, ELISA and immunoblot techniques determined expression and localization of angiogenesis-related growth factors, receptors and cell types upon skin flap re-integration.
Results: Skin flap tissue re-integration was severely disturbed in diabetic mice. Impaired skin flap tissue lost early VEGF expression from wound margin keratinocytes and markedly reduced expression of endothelium-specific receptors Tie-2 and FLT-1. Numbers of blood vessels were reduced in impaired flaps. In addition, HIF-1α protein was absent from disturbed skin flap tissue. Reduced VEGF expression and the loss of epithelium in disturbed skin flaps were paralleled by the appearance of VEGF expressing inflammatory infiltrate.
Conclusion: In summary, our data show a dysregulated spatial and temporal pattern of angiogenic processes during skin flap re-integration in diabetic mice. Our data suggest that reduced expression of angiogenic receptors in skin flap tissue might contribute to a loss of VEGF function in impaired tissue.
© 2014 European Association for Cranio-Maxillo-Facial Surgery. Published by Elsevier Ltd. All rights reserved.

1. Introduction

In the clinical setting, autologous skin flaps represent the treatment of choice to cover large and deep tissue defects following excision of tumors, facial reconstructions or severe injury. Partial or complete necrosis of skin flaps (Nakatsuka et al., 2003; Shah et al., 1990; Wei et al., 2001) has been attributed to hypoxia as a consequence of insufficient vascularity, vasospasm, impaired venous backflow or thrombosis (Kerrigan, 1983; Myers and Cherry, 1968). Moreover it has been shown in detail that diabetes mellitus impairs wound healing. Factors that play a decisive role in diabetes-associated wound healing disorders comprise augmented inflammatory responses (Goren et al., 2007; Schürmann et al., 2010), impaired granulation tissue formation (Seitz et al., 2010), reduced growth factors and a disturbed angiogenesis (Blakytny and Jude, 2006; Seitz et al., 2010). Although a series of studies demonstrated a tissue-protective potential of angiogenic growth factors such as vascular endothelial growth factor (VEGF) (Huang et al., 2006; Michlits et al., 2007; Scalise et al., 2004; Zheng et al., 2008), diabetes-associated wound healing disorders represent a still unsolved and serious clinical complication.

Wound angiogenesis is pivotal to wound healing and results from multiple signals acting on endothelial cells. One important inductor of the angiogenic signaling network is hypoxia. The hypoxia inducible factor (HIF)-1 is a key player in translation of local wound hypoxia into a transcriptional cascade that promotes angiogenesis (Semenza, 2010). Hypoxia activates HIF-1 by stabilization of its oxygen-sensitive HIF-1α subunit, which is ubiquitinated and subsequently degraded under normoxic conditions (Covello and Simon, 2004). Active HIF-1 transcriptionally activates VEGF (Forsythe et al., 1996), angiopoietin (Ang)-2 (Simon et al., 2008), stromal cell-derived factor (SDF)-1 (Ceradini et al., 2004) or platelet-derived growth factor (PDGF) (Yoshida et al., 2006) and thereby stimulates proliferation and migration of endothelial cells at the wound site. In particular, VEGF represents a potent factor in

☆ The paper is not based on a previous communication to a society or meeting.
* Corresponding author. Tel.: +49 6301 6958; fax: +49 6301 7942.
E-mail address: Schuermann@med.uni-frankfurt.de (C. Schürmann).

http://dx.doi.org/10.1016/j.jcms.2014.06.004
1010-5182/© 2014 European Association for Cranio-Maxillo-Facial Surgery. Published by Elsevier Ltd. All rights reserved.

the control of vascular permeability (Keck et al., 1989) and angiogenesis (Hoeben et al., 2004). By contrast, HIF-1 activation and subsequent VEGF expression is known to be strongly impaired in diabetic tissue (Botusan et al., 2008; Thangarajah et al., 2010, 2009).

In line with this observation, wound tissue in diabetic mice showed the absence of insulin receptor expression (Goren et al., 2006). This condition contributed to ulceration as insulin was capable to enhance VEGF release from keratinocytes (Goren et al., 2009). To this end, insulin resistance appears to be functionally connected to reduced VEGF expression and thus a low blood vessel density in diabetic wounds (Seitz et al., 2010).

The current study assessed angiogenic response patterns during normal and disturbed skin flap healing. Here we illustrate a decreased blood vessel density in diabetes-associated tissue loss. This condition was paralleled by a loss of HIF-1α, VEGF and the angiogenic receptors tyrosine kinase with immunoglobulin-like and EGF-like domains (Tie)-2 and Fms-like tyrosine kinase (FLT)-1 in diabetes-impaired skin flap tissue in the animals.

2. Materials and methods

2.1. Animals

Female wild-type C57BL/6J and C57BL/6J-*ob/ob* mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and maintained under a 12-h light/12-h dark cycle at 22 °C. Wild-type C57BL/6J mice express a metabolically normal and healthy phenotype. C57BL/6J-*ob/ob* mice are characterized by a functional loss of the cytokine leptin, which is encoded by the *ob* gene (Zhang et al., 1994), leading to the development of a diabetes-obesity syndrome in the animals (Coleman, 1978). The C57BL/6J-*ob/ob* mice were studied at 15 weeks of age, at which time they had a severe diabetic phenotype. Age-matched healthy wild-type C57BL/6J mice were studied as the appropriate control.

2.2. Wounding of mice and generation of caudally based skin flaps

Mice were anesthetized with ketamine (80 mg/kg body weight)/xylazine (10 mg/kg body weight). A full thickness caudally based skin flap (20 mm base of the flap, 40 mm length) was generated on the back of the mice as described in detail previously (Schürmann et al., 2009) (refer also to Fig. 2a). The length of the skin flap (40 mm) comprises the complete back of the animals (Schürmann et al., 2009) and therefore reflects the maximal skin flap length which could be applied to mice. Skin flap tissue biopsies and immediately adjacent (neighboring) skin tissue were isolated from experimental groups (each group consisted of $n = 4$ individual mice) at day 3, 5, 7, 10 and 13 for protein and histology analyses. Left side skin flap biopsies of four animals ($n = 4$) were pooled prior protein analysis. Right side skin flap biopsies were fixed for histology. As a control, a similar amount of skin was taken from the back of four non-operated mice ($n = 4$). Seven additional animals were used at postoperative day 5. Protein ($n = 4$ mice) or RNA ($n = 3$ mice) was isolated from the left and right side of the flap tissue. All animal experiments were performed according to the guidelines and with the approval of the local Animal Ethics Review Board.

2.3. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA isolation was performed as described previously (Chomczynski and Sacchi, 1987). qRT-PCR was performed to assess the expression of VEGF and GAPDH over different transplant sections at day 5 post-surgery. Pre-designed qRT-PCR assays were purchased at Applied Biosystems (Darmstadt, Germany): VEGF (Mm00437304_m1) and GAPDH (4352339E). 0.5 μg of total RNA from day 5 tissue was transcribed using random hexameric primers and Superscript II RT (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. qRT-PCR was performed on 7500 Fast real-time PCR system (Applied Biosystems) as follows: one initial step at 95 °C for 20 s was followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. Expression levels for VEGF could not be normalized to GAPDH, as GAPDH mRNA itself is described to be under regulatory control during wound healing (Kampfer et al., 2005), and thus could not serve as a general marker for constitutive gene expression in wounds. The differential expression of GAPDH was also obvious in normal and disturbed skin flap healing (Fig. 1a) in the presence of equal amounts of total RNA as assessed by RNA gel electrophoresis (Fig. 1b).

2.4. Immunohistochemistry

Skin biopsies were isolated from the back, fixed in formalin and embedded in paraffin. Six-micrometer sections were incubated over night at 4 °C with antisera raised against VEGF (Santa Cruz, Heidelberg, Germany) or α-SMA (DakoCytomation, Hamburg, Germany). Specificity of staining was controlled for all primary antibodies by single incubation with the detection antibody alone. The slides were subsequently stained with the avidin–biotin–peroxidase complex system (Santa Cruz, Heidelberg, Germany) using 3,3-diaminobenzidine-tetra-hydrochloride or Fast Red Substrate-Chromogen System (Dako, Hamburg, Germany) as chromogenic substrates. Finally, sections were counterstained with hematoxylin and mounted.

2.5. Preparation of protein lysates and Western blot analysis

Skin tissue biopsies were homogenized in lysis buffer as described previously (Schürmann et al., 2009). Extracts were cleared by centrifugation. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Inc., Rockford, IL, USA). Specific proteins were detected using antisera raised against HIF-1 (Novus Biologicals, Cambridge, United Kingdom), Tie-2 (Santa Cruz, Heidelberg, Germany), FLT-1 (Epitomics, Burlingame, CA, USA), or signal transducer and activator of transcription (STAT)-6 (Cell signaling, Frankfurt, Germany). A secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system (Amersham, Freiburg, Germany) was used to visualize the proteins. Densitometric analysis of immunoblots was performed using QuantityOne software (BioRad, München, Germany).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Quantification of murine $VEGF_{165}$ protein was performed using the murine Quantikine ELISA kit (R&D Systems, Wiesbaden, Germany) according to the instructions of the manufacturer.

2.7. Statistical analysis

Data are shown as means ± SD. For statistical comparison of two groups, data analysis was performed using the non-parametric unpaired Student's *t* test with raw data. All data were analyzed using the software GraphPadPrismTM version 5.0 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Morphology of normal and disturbed skin flaps

The caudally based skin flaps were generated in wild-type and diabetic *ob/ob* mice as reported previously (Schürmann et al., 2009,

Download English Version:

https://daneshyari.com/en/article/3142733

Download Persian Version:

https://daneshyari.com/article/3142733

Daneshyari.com