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ANGIOGENESIS IN WOUNDS TREATED BY MICRODEFORMATIONAL WOUND THERAPY

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Abstract

Summary Background Data—Mechanical forces play an important role in tissue neovascularisation and are a constituent part of modern wound therapies. The mechanisms by which Vacuum Assisted Closure (VAC) modulates wound angiogenesis are still largely unknown.

Objective—To investigate how VAC treatment affects wound hypoxia and related profiles of angiogenic factors as well as to identify the anatomical characteristics of the resultant, newly formed vessels.

Methods—Wound neovascularization was evaluated by morphometric analysis of CD31- stained wound cross sections as well as by corrosion casting analysis. Wound hypoxia and mRNA expression of HIF-1α and associated angiogenic factors were evaluated by pimonidazole hydrochloride staining and quantitative RT-PCR, respectively. VEGF protein levels were determined by western blot analysis.

Results—VAC-treated wounds were characterized by the formation of elongated vessels aligned in parallel and consistent with physiologically function, compared to occlusive dressing control wounds that showed formation of tortuous, disoriented vessels. Moreover, VAC-treated wounds displayed a well-oxygenated wound bed, with hypoxia limited to the direct proximity of the VAC-foam interface, where higher VEGF levels were found. By contrast, occlusive dressing control wounds showed generalized hypoxia, with associated accumulation of HIF-1α and related angiogenic factors.

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Conclusions—The combination of established gradients of hypoxia and VEGF expression along with mechanical forces exerted by VAC therapy was associated with the formation of more physiological blood vessels compared to occlusive dressing control wounds. These morphological changes are likely a necessary condition for better wound healing.

INTRODUCTION

Angiogenesis, the generation of new blood vessels into a tissue or organ, is controlled by a complex regulatory system and is critical for wound and tissue repair. Alterations of this system lead to uncontrolled angiogenesis, a condition related to tumors. Decreased ability to form normal vessels during repair in diabetic patients leads to delayed healing [1]. Among the variety of factors and pathways involved in the control of angiogenesis, hypoxia seems to play a pivotal role as it regulates the expression and stabilization of HIF-1α and thereby stimulates the expression of VEGF, an important angiogenic factor [2-11] also involved in collagen deposition and wound epithelialization [12, 13]. Directly interconnected with the HIF-1α -VEGF pathway and with hypoxia, the inflammatory microenvironment is another important modulator of wound angiogenesis [14-16]. Starting at day 2, macrophages become the main source of growth factors and cytokines, including angiogenic factors such as VEGF [17-20], whereby hypoxia is recognized as an important condition for the activation of macrophages [21].

Angiogenesis is one of the most crucial processes involved in tissue repair [22] and is considered to be an adaptive response to hypoxia. In skin wounds, the natural course of hypoxia is characterized by an hypoxic peak around day 4 preceded by an absence of hypoxia immediately after wounding and followed by a progressive increase of wound oxygenation [23]. Hypoxia plays a bivalent role in wound healing. In the initial phase of wound healing, it aids in the recruitment and function of inflammatory cells [24, 25] as well as the expression and stabilization of Hypoxia-Inducible-Factor-1 (HIF-1) [2, 26]. HIF-1α expression is regulated by oxygen dependent prolyl hydroxylases and proteosomal degradation mechanisms, whereby an hypoxic environment leads to HIF-1α accumulation [27]. Although hypoxia is beneficial in the initial stage of wound healing, starting in the proliferative phase, it leads to impaired proliferation and migration of endothelial cells and fibroblasts [28], interruption of angiogenesis [29], decreased collagen formation[29, 30] as well as increased risk of infection[31].

Several reports have brought strong evidence of biomechanical forces playing an important role in tissue neovascularisation and we now assist to an increasing interest in trying to understand how this phenomenon is regulated. Recently it was demonstrated that mechanical forces affect neovascularisation not only through a modulatation of the gene expression of angiogenic factors and their receptors [32-36], but also through a nonangiogenic expansion of preexisting vessels and consequent formation of new, fully functional vascular loops [37].

Vacuum Assisted Closure (VAC) is widely used in wound therapies and has been shown to positively affect angiogenesis [38-41]. Research suggests that the combination of a foam interface with negative pressure leads to the application of mechanical microdeformations to wound cells and thereby affects the expression of angiogenic factors [42]. Other essential components of this therapy are the continuous evacuation of wound fluid which leads to the removal of inhibitory angiogenic factors such as matrix metalloproteinases (MMPs) [43, 44] and a modulation of the inflammatory microenvironment of wounds, which finally also controls new vessel formation [44-46]. The foam material interface is critical for the application of micromechanical deformations to wound cells [47] as well as is involved in modulating the migration of inflammatory cells in the treated wounds [46]. Despite the

widespread use of VAC therapy, little is known about the mechanisms of action at the molecular level, and especially about of the mechanisms that regulate neovascularisation in the treated wounds.

With the present study we aimed at identifying the anatomical characteristics of newly formed vessels as well as to investigate VEGF expression profiles under the effect of different treatment patterns of vacuum assisted closure therapy.

METHODS

Fifty adult male wild-type C57Bl6 *db/+* mice (Jackson Laboratory, Bar Harbor, ME) were included in the study. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care certified facility under an approved experimental protocol. The mice are heterozygous for a diabetic gene, but they express a normal wild-type wound healing phenotype [48]. The entire dorsum was clipped and depilated (Nair®, Church & Dwight Co., Princeton, NJ) 24 hours prior to the experiment. Animals were anesthetized with 60 mg/kg Nembutal (Pentobarbital) 15 minutes prior to surgery. After disinfecting the dorsum with alcohol patches, a 1.0 cm^2 area of skin and panniculus carnosus were removed to create a full-thickness wound. To protect wound margins from excess macrodeformations,[49] a 0.5 cm wide Duoderm® (DuoDERM®, CGF®, ConvaTec, Squibb & Sons, L.L.C.) frame was placed around the wound edges. Depending on the group, wounds were covered by a Tegaderm 3M Dressing or by a Vacuum Assisted Closure Device© (KCI Inc., San Antonio, TX) set at continuous 125mmHg negative pressure. The VAC device did not affect ambulation, neither well being of the treated animals. At day 7 a further group of mice was treated with cyclical negative pressure therapy alternating 2 minutes of negative 125mmHg with 2 minutes of negative 50mmHg. The analyzed parameters were monitored at 6 hours (Control Group n= 6, VAC Continuous Group = 6), 3 days (Control Group n=6, VAC Continuous Group n=6) and 7 days (Control Group n=6, VAC Continuous Group n=6, VAC Cyclical Group n=6). At day 7 corrosion casting analysis of the newly built vessels was performed.

Immunohistochemistry

Analysis of wound bed vasculature, cellular proliferation and tissue hypoxia was performed by immunohistochemistry. For hypoxic cell analysis, pimonidazole hydrochloride marker (Hypoxyprobe™-1 Kit, NPI, Burlington, MA) was injected intraperitoneally 90 minutes prior to harvesting. Central wound cross-sections, surrounding skin and underlying muscle tissue were harvested, formalin fixed and embedded in paraffin. Paraffin-embedded sections were rehydrated through a decreasing alcohol chain. Antigen retrieval of CD31 was performed with 40 μg/ml Proteinase K (Roche Diagnostic Corp., Indianapolis, IN) solution in 0.2M Tris-H2O for 20 minutes at 37°C. After application of blocking serum for 1 hour, primary antibodies, namely CD31 (Pharmingen, San Jose, CA) for angiogenesis and anti-Hypoxyprobe™-1 monoclonal antibody (NPI, Burlington, MA) for hypoxia were incubated at 4°C overnight. Biotinylated mouse anti-rat secondary antibodies (Vector Laboratories Inc., Burlingame, CA) for CD-31 and pimonidazole hydrochloride marker were used as secondary antibodies. For CD31, signals were then amplified in association with Tyramide Amplification System® (Perkinelmer, Boston, MA). Activation was performed with NovaRED (Vector Laboratories, Burlingame, CA). Sections were then counterstained with hematoxilin (Hematoxilin 1, Richard-Allan Scientific, Kalamazoo, MI). High power images of stained sections were used to quantify the degree of wound bed angiogenesis. Six digital images of CD-31 stained slides were captured for each sample, 2 in the middle and two on each edge of the wound bed. Blood vessel density was quantified over the entire image and expressed as the ratio of blood vessel number per high-powered field (hpf). A total of 36 microscopic fields were evaluated for each experimental group. Hypoxia was qualitatively

assessed over the entire image. Central wound cross-sections were stained according to routine H&E protocols.

Morphometric Analysis of Newly Formed Vessels

Comparison of CD31 stained slides of animals sacrificed at day 7 and comparison between cyclical VAC therapy, continuous VAC therapy and Occlusive Dressing Control wounds (n=6 per group). Microvessel densities were determined in frozen sections after anti-CD 31 staining. From each wound section, micrographs of representatitve areas were choosen and evaluated with a Weibel grid [50].

Corrosion Casting Analysis

For spatial visualization of granulation tissue microvasculature, 4 mice of each group were euthanized on day 7. Mice were anaesthetised with pentobarbital (0.1 ml, Narcoren, Merial, Germany) and aorta was catheterised with an olive-tipped needle (Acufirm 1428LL; Dreieich, Germany). After systemic heparinisation, 10ml of 2.5% glutaraldehyde in Ringer's saline solution and, subsequently, 40 ml of Mercox CL-2B resin (Vilene Med Co., Tokyo Japan) diluted with 20% methylmethacrylate monomers (Merck Darmstadt, Germany) were perfused. Mouse dorsal back was immersed in a 5% KOH solution to digest all the tissues around the vessel casts. One week later the specimens were dehydrated, mounted on conductive stubs and coated with gold in an SCD 040 sputter-coater (BAL-TEC AG, Leica Microsystems). The specimens were visualised under a Philips XL30 scanning electron microscope (Philips, Eindhoven, The Netherlands), and between 20 and 30 stereo pair images with a 61 tilt angle were recorded per sample. Pairs of stereo images were analysed with the image analysis program, Kontron KS 300 (Carl Zeiss Vision, Eching, Germany). For details of reconstruction and calculations see Malkusch et al (1995).

Western Blot Analysis

While the distal part of the wounds served for immunhistochemistry, the granulation tissue pertaining to the cranial part of wounds of animals sacrificed at day 3 and day 7 was harvested and fresh frozen for western blot analysis of VEGF (n = 4 per group). Tissue samples did not include the surrounding skin and underlying muscle tissue. In VAC treated animals samples were harvested both from the wound bed and from the foam material adjacent to the wound (n = 4 per group). The samples were loaded on 15% SDS–polyacrylamide gel and run at 120V for 90 min. The protein was transferred to PVDF membrane and subsequently blocked for 2 h with 10% milk in TBST (10mM Tris base pH 7.5, 150mM NaCl, 0.1% Tween-20). The membrane was then incubated in primary polyclonal antibody against VEGF. Following three washes with TBST for 10 min each, the membrane was placed in the secondary goat-anti-rabbit antibody (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) for 1 hour. The enhanced chemiluminescence reaction (ECL,Amersham Biosciences, Iscataway, NJ, USA) was then carried out according to the manufacturer's instructions and films were developed for 30 seconds with phosphatase-conjugated secondary antibody.

Real Time RT-PCR Analysis

Real-time RT-PCR expression of hypoxia-induced factor 1α (HIF-1α; 5′-CAAGTCAGCAACGTGGAAGGT -3′; 5′-CTGAGGTTGGTTACTGTTGGTATCA -3′; Gene Bank ID NM_010431), vascular endothelial growth factor-A (VEGF; 5′-CACTGGACCCTGGCTTTACTGC-3′; 5′-CGCCTTGGCTTGTCAC-3′; Gene Bank ID M95200), vascular endothelial growth factor receptor 2 (VEGF-R2; 5′-ACAGACCCGGCCAAACAA-3′; 5′-TTCCCCCCTGGAAATCCTC-3′; Gene Bank ID NM_010612), platelet derived growth factor BB chain (PDGF; 5′-

TGTTCCAGATCTCGCGGAAC-3′; 5′ GCGGCCACACCAGGAAG-3′; Gene Bank ID AF162784) and platelet-derived growth factor receptor beta (PDGF-R; 5′-GTGGTGAACTTCCAATGGACG-3′; 5′- GTCTGTCACTGGCCTCCACCAG-3′; Gene Bank ID BC055311) was performed and compared over the different time points in both the control and study group. At 6 hours, 3 days and 7 days after full thickness skin wounding, fresh samples were taken from the cranial half of the wound bed including granulation tissue and underlying muscle layer (n = 4 per group) and fresh frozen. RNA was extracted using the RNeasy mini kit (Qiagen, Chatsworth, CA) and quantification performed using the NanoDrop (NanoDrop Technologies, Wilmington, DE) method. We synthesized complementary deoxyribonucleic acid (cDNA) using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was mixed with random hexamers and dNTP and then incubated. RT buffer, $MgCl_2$, DTT, RNaseOUT and SuperScript III RT were added, incubated and cooled. Then, *E.coli* RNase H was added and incubated. RT-PCR was performed in an ABI Prism7300 system (Applied Biosystems, Foster City, CA) using RT^2 SYBR Green / ROX qPCR (SA Biosciences, Frederick, MD). Amplification of the cDNA was performed in triplicates and a dissociation curve was generated utilizing fluorescence measurements with 28S ribosomal RNA (28S; 5′- TTGAAAATCCGGGGGAGAG-3′; 5′-ACATTGTTCCAACATGCCAG-3′; Gene Bank ID X00525) as the endogenous control for normalization. 28S has been proved to be the most reliable internal control for comparative analyses of transcription under hypoxic conditions [51]. Change in expression was considered significant when the baseline relative quantity (RQ) minimum/maximum values did not overlap with the sample RQ values at the 95% confidence interval determination.

Wound Closure Analysis

Wounds were photographed immediately after wounding, at each dressing change and at harvest time. Digital photographs captured at the end of the experiment were compared with initial photographs by two independent observers blinded to the treatment mode. Wound closure was calculated as a percentage of the original wound and measured by planimetric analysis (Image J, NIH, Bethesda, MD).

Data Analysis

For comparison of two groups, a two-tailed T-test was used. For greater than two groups, oneway analysis of variance (ANOVA) and *ad hoc* Fischer's LSD tests was used to determine the significant differences between treatment groups if the ANOVA was found to be significant. All statistical analyses were performed using WinStat (R. Fitch Software, Lehigh Valley, PA). A p-value less than 0.05 was considered statistically significant.

RESULTS

VAC treatment was associated with a reduced number of vessels presenting with a more functional anatomical aspect (Figure 2, 3,4)

As a response to boosted expression of angiogenic factors, control wounds stained for CD-31 presented with highly immature tortuous and densely packed vessels. In contrast, VAC treated wounds presented with fewer, more functional looking vessels whose aspect was comparable to those of vessels present in the adjacent non wounded skin. Morphometric analysis of CD-31 stained slides revealed a higher amount of microvessels characterized by fine vessels with small diameters.

Corrosion casting analysis showed how micromechanical forces applied by VAC literally led to the sprouting of new blood vessel pillars directed towards the wound surface. We remarked a directionalization of vessels located at wound margin towards wound center.

These vessel at wound margin showed a shaped specific microvascular archictecture. Tufts of densely packed vessel networks with large caliber at wound margin are building elongations and enlargements as vessel in-growth to wound bed.

VAC treated wounds showed an overall improved wound oxygenation in presence of hypoxic macrophages in direct proximity of the polyurethane foam (Figure 5)

Pimonidazole hydrochloride staining determined absence of hypoxia in both groups at 6h after wounding. The granulation tissue of control wounds presented with a generalized hypoxic peak at day 3 after wounding, while only residues of hypoxia were visible at day 7. While in the control group hypoxia was diffusely localized in all wound layers, especially in the granulation tissue and interstitial spaces, VAC treated wounds presented with an overall improved oxygenation and with hypoxia limited to the area adjacent to the polyurethane foam, where pimonidazole hydrochloride accumulated selectively into macrophages.

In VAC treated wounds Protein Levels of VEGF showed a VEGF gradient towards the wound surface (Figure 5)

Western blot analysis of tissues harvested from the wound bed of VAC treated wounds revealed a lower protein level of VEGF when compared to control wounds. Compared to wound bed protein levels, VEGF was significantly increased in tissues harvested from the wound surface (wound fluids and tissue contained in the VAC foam side adjacent to the wound) thereby leading to a VEGF gradient between wound bed and wound surface.

VAC treated wounds presented with increased VEGF dimers (Figure 5)

We observed that VEGF dimers assessed by western blot analysis were increased in VAC treated wounds when compared to control wounds both in cyclical and continuous therapy patterns.

mRNA Expression of angiogenic factors was decreased in the wound bed of VAC treated wounds (Figure 6)

While at 6h no significant difference among groups was found, starting at 3d after wounding and progressively increasing over time, control wounds showed an increased expression of angiogenic factors related to the HIF-1α – VEGF Pathway. Increased expression of HIF-1α correlated with increased expression levels of VEGF-A and VEGF-R2. At day 7 expression levels of HIF-1α in control wounds were increased of a 100 fold compared to those of tissues harvested at 6 hours after wounding. During the course of wound healing, the expression of angiogenic factors in tissues harvested from the VAC treated wounds was overall reduced, showing an 18 fold increase at day 7. A comparable pattern was found in cyclically treated wounds. Non published data regarding the expression of angiogenic factors in the wound bed of animals sacrificed 2 hours after treatment revealed comparable results to those of 6 hours wounds.

Negative pressure therapy leads to improved wound closure (Figure 7)

During the 7 days follow-up period, the wound area of both groups progressively decreased, whereby the VAC treated group presented with a superior wound closure starting at day 3 and maintaining this trend over the entire observation treatment. In this short term experiment, mean wound area reduction was mainly related to a higher wound edge contraction in the VAC group as measured at postoperative day 7. Cyclically treated wounds did not significantly differ from those treated by continuous VAC therapy.

DISCUSSION

Vacuum assisted wound therapy is widely used to improve wound healing and has been shown to increase wound angiogenesis [38-41]. Mechanical microdeformations, evacuation of inhibitory angiogenic factors (eg. MMPs) and increased expression of proangiogenic cytokines have been hypothesized as possible mechanisms of action. A detailed analysis of the mechanisms controlling neovascularisation in VAC treated wounds and in particular of the molecular changes in the expression of angiogenic factors is missing and limited to the study of the wound secretions collected by VAC devices. While the VAC foam itself seems to affect angiogenesis [47], the specific properties of the foam material including mean pore size, surface tension and surface chemistry have not been addressed by our study and would need to be elucidated in the future in order to better understand the biologic mechanisms of VAC induced angiogenesis.

Both morphometrical studies of CD-31 stained wound cross sections as well as corrosion casting analysis of newly formed vessels demonstrated thatVAC treatment had a regulatory effect on wound angiogenesis. Corrosion casting showed a better hierarchisation of the vascularity coming closer to the normal subcutaneous vessel architecture and distribution patterns. Previous studies of the tumour microvascular architecture have shown that the vascular architecture determines the nutritive blood flow [52]. From this it may be concluded that a normalized vascular architecture implies a better functionality in wound healing. As a possible consequence of a generalized wound hypoxia with consequent excessive expression of angiogenic factors as well as an absence of a VEGF gradient, occlusive dressing control wounds were instead characterized by the uncontrolled formation of tortuous, non directionalized and immature vessels. This chaotic vessel arrangement probably reflects the impaired blood flow in this region.

A recent study found higher VEGF protein levels in VAC secretions in a series of traumatic human wounds and localized VEGF in the nearest proximity of the vacuum polyurethane foam in proximity of macrophages [42]. However, Epigard was used as a control so additional work will need to be done to better define the effect of the interface material and cellular response [46, 53]. The different size and location of the analyzed wounds, difficulty in standardizing the time of tissue harvest, unknown patient's co-morbidities are other issues that make a comparison of highly sensitive chemokine levels difficult. In another clinical study, Grimm et al. found a significant reduction of HIF-1α after VAC treatment of radiated skin wounds [41]. Although the study had some short-comings including a limited number of analyzed patients, unknown co-morbidities and the irradiated condition of the analyzed wounds, it highlighted a possible relationship between VAC treatment and the HIF-1α pathway in wounds and indirectly with the oxygenation of wounds. In effect oxygen regulates both the expression of HIF-1α as well as its degradation. Oxygen inhibits Factor Inhibiting HIF (FIH) and thereby reduces the transcriptional activity of HIF-1α as well as is substrate of proxyl-4 hydroxylase, an enzyme which leads to hydroxylation and rapid degradation of HIF-1α [27]. In our experiments a generalized hypoxia occurring in control wounds induced a high expression of HIF-1α and related angiogenic factors, while these were decreased in well oxygenated VAC treated wounds. In our study hypoxia was measured by pimonidazole hydrochloride staining, a marker that identifies tissue areas with PO2 ranging from 0 to 10mmHg. This is a well validated method which proved to be more accurate than wound oxygenation measurements made by conventional PO2 sensors since these have a limited depth of reading and are strongly influenced by the arterial PO2 levels and vasoconstriction [23, 54]. Our experiments showed that VAC treatment was associated with an overall improved wound oxygenation whereby at both days 3 and 7 wound macrophages located in close proximity to the polyurethane sponge strongly co-localized with pimonidazole staining (Figure 5). We hypothesize that the established hypoxic gradient

and activated hypoxic macrophages were responsible for the formation of a VEGF gradient with high VEGF protein levels in tissues harvested from the foam-wound interface and lower levels in tissues harvested from the wound bed. That oxygen gradients are important for angiogenesis and wound healing is not new and our findings confirm the observations made by Knighton et al. in 1981 [55]. Moreover also VEGF gradients are known to be very important for an effective angiogenesis and directional vessel outgrowth towards higher VEGF concentrations [56, 57]. We also observed a stabilization of VEGF dimers in VAC treated wounds. This process could be mediated by mechanical forces and could be involved in the modulation of neovascularization, as VEGF dimers are known to present with a stronger VEGF receptor affinity and to thereby be able to achieve superior angiogenesis compared to VEGF monomers [58, 59]. Petersen et al. identified that in tendon fibroblasts mechanical forces induce the expression of selected VEGF isoforms [60]. Different patterns and forms of applied mechanical forces could therefore influence angiogenesis through the formation of VEGF forms with different biological function. Although the establishment of a VEGF gradient and the stabilization of VEGF dimers may play an important role in VAC induced angiogenesis, highly directionalized vessel growth demonstrated by corrosion casting images suggests that a direct mechanical action exerted by the combination of a polyurethane foam interface and negative pressure forces might also play an important role in the expansion and sprouting of new vessels under VAC therapies as suggested by Kilarski et al.[37]. Finally, the application of cyclical negative pressure therapy was associated with hierachically built microvascular archictecture. The elongation of preexisting vessels as well intussusceptions enable an improvement of oxygen and nutrient delivery in the granulation tissue. VAC treatment accelarates wound healing not only by a hypoxia and VEGF driven directionalized in-growth of new vessels in the wound bed, but also by a micromechanical forces mediated activation of endothelial cell proliferation.

CONCLUSION

In subatmospheric pressure wound therapies, more importantly than a quantitatively high mRNA expression of angiogenic factors, the establishment of a gradient in hypoxia and VEGF, the stabilization of VEGF dimers as well as the direct exertion of mechanical forces on wound cells leads to a directionalized sprouting of mature and functional vessels, a necessary condition necessary for achieving improved wound healing.

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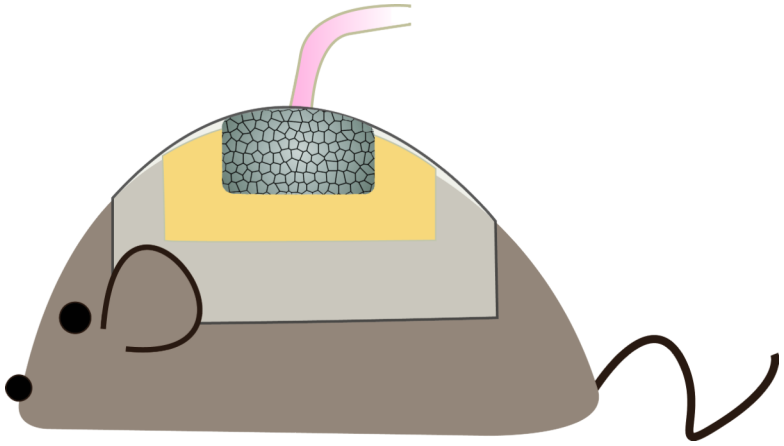

Figure 1.
Diagram of the VAC mouse model used in the current study. The VAC device did not affect ambulation, neither well being of the treated animals.

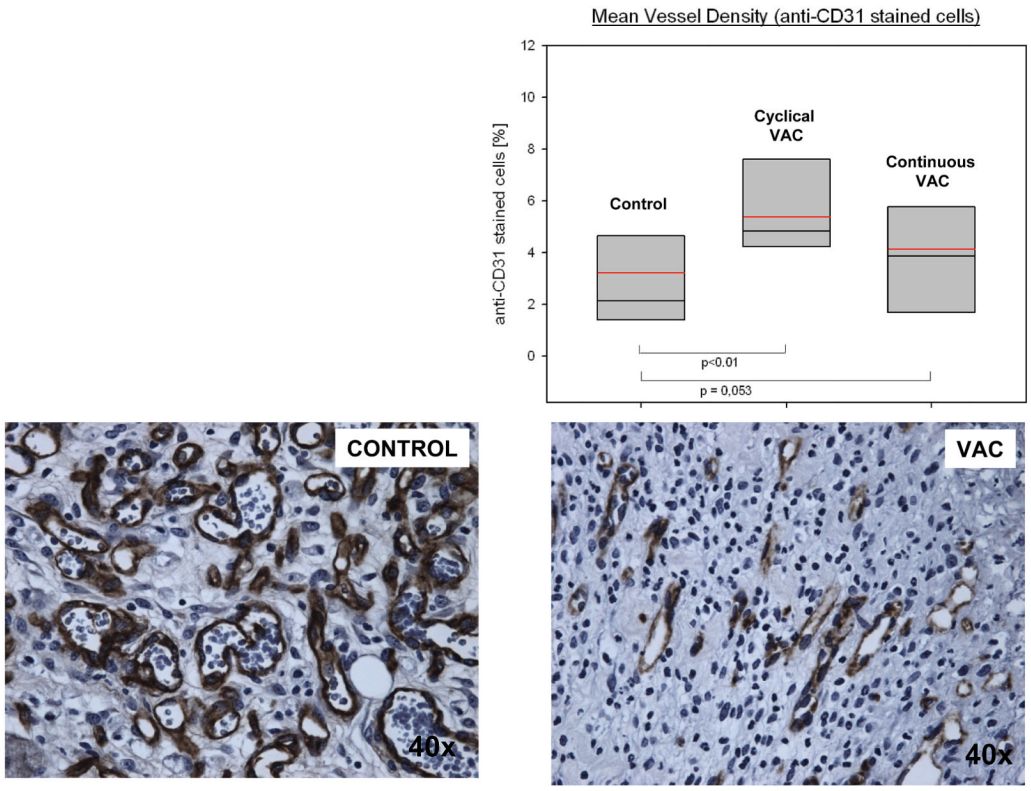

Figure 2.

Morphometric analysis of CD31 stained wound cross sections. VAC treatment is associated with the formation of small endothelial vessels and higher vessel density, whereby occlusive dressing control wounds are associated with large dilatated vessels.

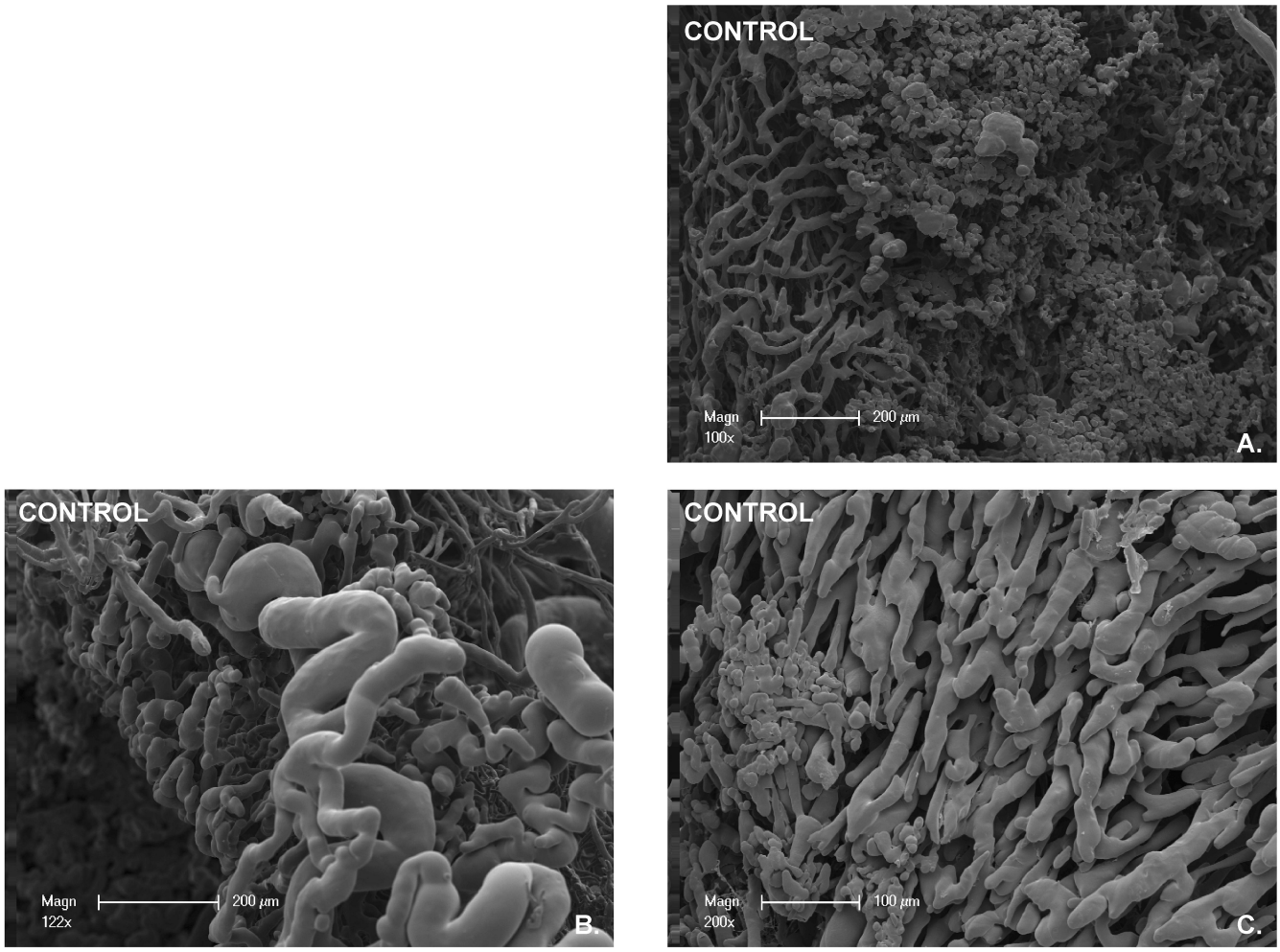

Figure 3.
Corrosion casting analysis of control wounds. A. Change of normal arborized vasculature towards a chaotic bloated, densely packed, tufts of vessels. B. Dilated vessel formations, abnormal tortousity of vessels, wide alterations of vessel diameters. C. Densely packed vessel formations, tumor-like vasculature, deregulation of normal vessel arborization, blind-ending dilated occluded vessels.

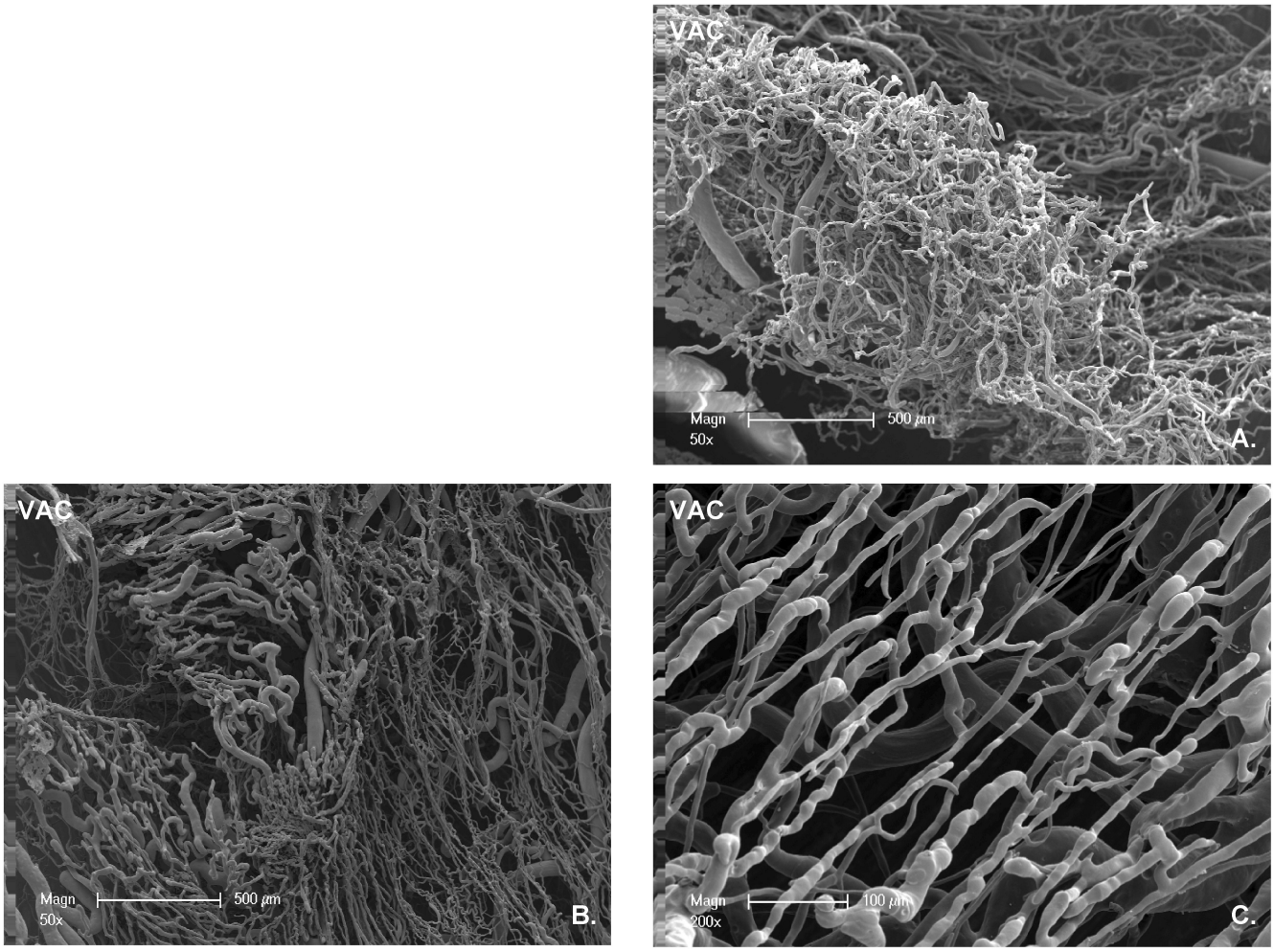

Figure 4.
Corrosion casting analysis of VAC treated wounds. A. Hypervascularized wound margin, vessel wall. B. Directionalization of vessels: from small vessels branches to tortous low densely packed vessel formations that pursuit the wound centre. C. Arborized vessel branches with vessel hierarchy, small vessel diameter in clearly defined vessel network, directionalized.

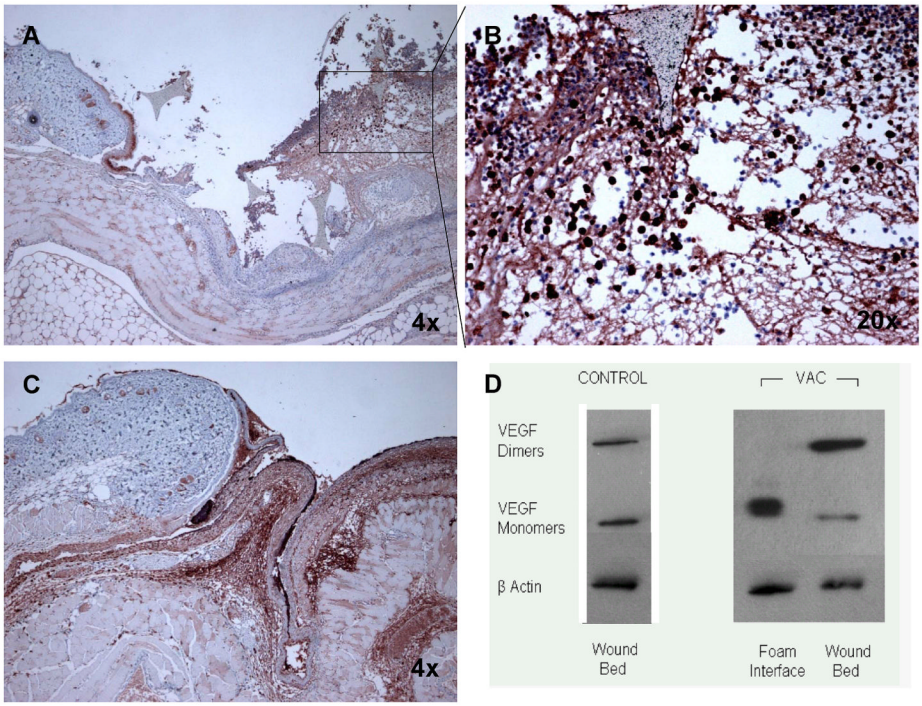

Figure 5.

Pimonidazole hydrochloride staining of wound cross sections of animals sacrificed at Day 3 after wounding shows control wounds (C) presenting with a diffuse hypoxia of the granulation tissue and interstitial spaces. VAC treated wounds (A and B) instead present with a superior oxygenation of the wound bed, with few hypoxic macrophages (B) located at the VAC-Foam interface. This Hypoxic gradient is associated with the formation of a VEGF gradient as assessed by western blot analysis (D). VEGF protein levels are higher at the VAC-Foam interface if compared to the wound bed. VEGF dimers are increased in the wound bed of VAC treated wounds.

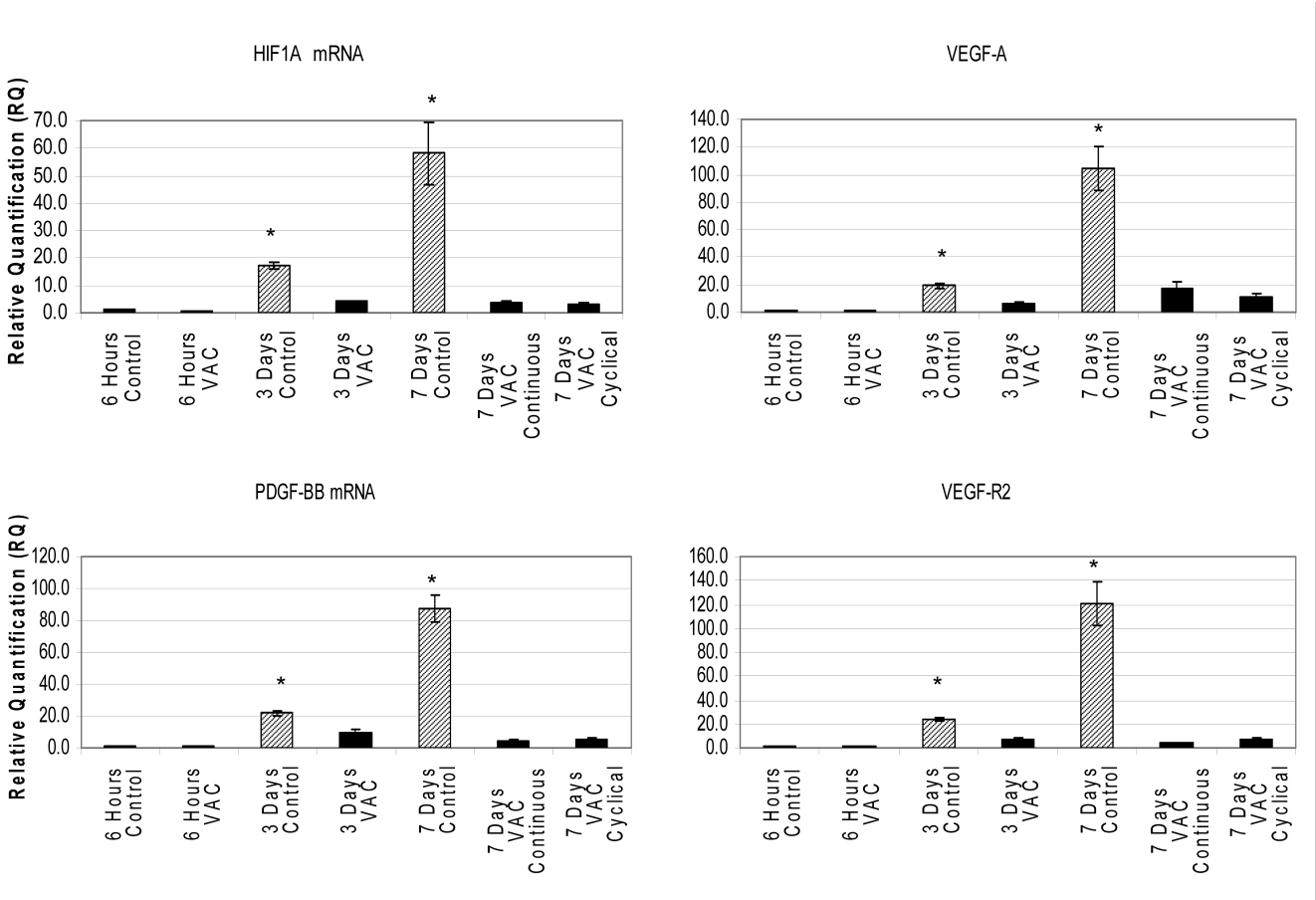

Figure 6.

mRNA profile of HIF-1α and related angiogenic factors and receptors during the time course of wound healing as assessed by quantitative RT-PCR in tissues harvested from the wound bed. In both groups mRNA expression of HIF-1α, VEGF-A, PDGF-BB and VEGF-R2 showed a progressive increase over the observation time, whereby control wounds were associated with a 15 fold increased expression of HIF-1α and 5 fold increased expression of VEGF-A.

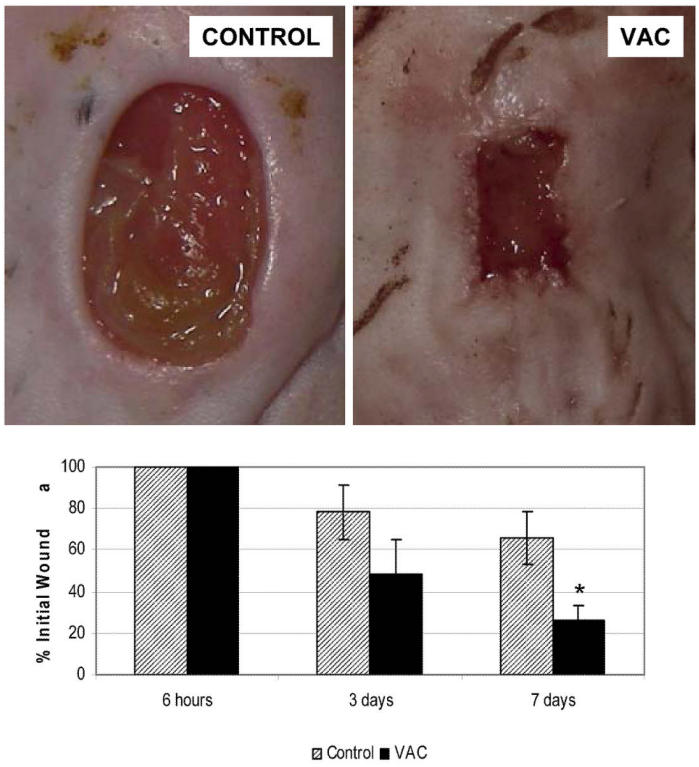

Figure 7.
The formation of a directionalized outgrowth of more functional vessels was associated with improved wound closure in VAC treated wounds.