Angiogenesis assessment in experimental third degree skin burns: a histological and immunohistochemical study

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Abstract
In the past 30 years, after the discovery of vascular proliferation factors, angiogenesis is one of the most intensively studied fields. Restoring the vascular network after burn injury is essential for healing, as it brings oxygen and nutrients to injured tissues, improves the contribution of inflammatory cells and prepares the damaged area for repair and tissue regeneration. To study the process of angiogenesis we used seven groups of five animals, each of adult Wistar rats, which were inflicted with third degree skin burns. From each group of animals, we sampled at successive intervals of three days the entire burnt wound with a ring of surrounding normal skin. Sampled skin fragments were processed for paraffin inclusion, sectioned with a microtome and stained with Hematoxylin–Eosin or Masson trichrome. The samples were also analyzed using single chromogenic immunohistochemistry or double immunofluorescence for the presence of CD34 and alpha smooth muscle actin (α-SMA). Angiogenesis process started at about three days after the burn infliction, with the appearance of tubular structures lined by CD34-positive cells. Subsequently, these cells showed intense proliferative activity that generated a network that included progressive neovascularization around the wound surface. Maximum vascular proliferation occurred at 9–15 days after injury, when the number of capillaries reached 229/mm², and the total area of capillary angiogenesis at 100.27 μm² (about 10% of the section area). Subsequently, the process of angiogenesis was gradually reduced, but remained at moderate levels after wound healing. During the process of angiogenesis, there was a very close relationship between CD34-positive cells and pericytes (as α-SMA-positive).

Keywords: angiogenesis, skin burns, endothelial progenitor cells, pericytes.

Introduction
Worldwide, each year, millions of patients present with skin burns. Post-burn complications represent one of the most difficult pathologies in the whole medical field, considering the vital importance of skin, as a barrier structure that protects the organism from external insults, especially of infectious nature. Moreover, the yearly cost of burns in general, is very high, being estimated around 1000 US dollars per patient per day, in order to provide satisfactory care and support [1]. More than 90% of the fatal burns produced by open fire are registered in developing countries, most frequently in social groups with low and medium incomes. Mortality induced by burns, is appreciated at approximately 265 000 deaths per year worldwide [2].

Some researchers [3] have reported an overall mortality of 31% for burnt patients over 65 years old in southern France. Burns in the elderly have always been a particular problem in terms of therapeutic outcomes and recovery. Restoring the vascular networks in the burned wound, a process called angiogenesis, is essential for healing, as it brings oxygen and nutrients to injured tissues and/or infection, and improves the intake of inflammatory cells, preparing the damaged area for repair and tissue regeneration. In other words, the vascular system plays a crucial role in restoring and maintaining local homeostasis.

In this paper, we aimed to quantify the angiogenesis in the third degree skin burns, on an animal model.

Materials and Methods
The study was performed on seven groups, each of five adult male Wistar rats, weighing between 290 and 340 g, kept under surveillance in the animal facility of University of Medicine and Pharmacy of Craiova, under standard conditions of light, temperature, food and water (ad libitum), both before and after the burns.

The experimental protocol was approved by the
Evolution of the damage was monitored daily for three weeks by assessing macroscopic signs of inflammation (swelling, redness and re-epithelialization). Judging after the appearance of macroscopic lesions, animals in all groups had third degree burns: necrosis of the epithelial and of the connective tissue underneath, until the muscle layer, edema of about 4 mm, and peri-lesional intense redness.

**Histological study**

From each group of animals, under general anesthesia, burnt skin was later on sampled (with approx. 3 mm perilesional areas), at intervals of 3, 6, 9, 12, 15, 18 and 21 days, to track in dynamic the evolution of the angiogenesis process.

Immediately after sampling, the burnt skin fragments were fixed in 10% buffered neutral formalin for 72 hours at room temperature (23–28°C) and included in paraffin.

To compare the results, we have also collected normal skin from five rats (the control group), which were also processed for paraffin inclusion.

For the histological study, 4-µm thick serial sections were cut using a rotary microtome (Microm HM350) equipped with a waterfall based section transfer system (STS, Microm). Sections were routinely stained with Hematoxylin–Eosin or Masson trichrome, after which they were examined using the microscope.

**Immunohistochemical study**

For the immunohistochemical study, sections were cut using the same equipment, but with a thickness of 3 µm for chemical staining, and 15 µm for the fluorescence study. Sections were collected on poly-L-lysine slides, dried in a thermostat at 37°C for 24 hours, and then they were subjected to different techniques as it follows.

For single immunohistochemistry (antibodies showed in Table 1), after antigen retrieval, sections were incubated for 30 minutes using a peroxidase polymer-based secondary detection system (EnVision, Dako, Bucharest, Romania). Finally the signal was detected with 3,3’-diaminobenzidine (DAB) (Dako) and the slides were coverslipped in DPX (Fluka) after Hematoxylin staining.

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<th>Antibodies used for immunohistochemistry</th>
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Fluorescent double immunohistochemistry was performed for anti-CD34 and anti-α-SMA antibodies. After endogenous peroxidase blocking and skim milk incubation, the anti-CD34 antibody was added on the slides in a 1:400 concentration, overnight, at 4°C. Next day, the signal was amplified for 30 minutes using another peroxidase polymer-based secondary detection system (Nikirei-Histofine, Medikit, Craiova, Romania) and visualized with fluorescein-tyramide (TSA Plus kit, Perkin Elmer, Medikit, Craiova, Romania), with a development time of 1 minute. After thorough washing in PBS, the slides were incubated with anti-α-SMA for another overnight incubation step. In the third day, an anti-mouse Alexa Fluor 596 (Invitrogen, Medikit, Craiova, Romania) was brought in the system for a 30 minutes incubation, after which the slides were washed and incubated for 15 minutes in DAPI (Invitrogen). Finally, the slides were coverslipped with an anti-fading mounting medium (Dako). Each experiment included negative controls where the first primary antibodies were skipped.

All intermediate washing steps were performed in 0.1 M PBS, pH 7.2, and all antibodies were diluted in PBS with 1% bovine serum albumin (Sigma-Aldrich, Medikit, Craiova, Romania). All incubation times were kept constant for each protocol.

**Microscopy and image acquisition**

The sections were imaged with an Eclipse 90i microscope (Nikon, Apidrag, Romania) equipped with a 5-megapixel cooled CCD camera and with fluorescent filters centered for Alexa 594, Alexa 488 and DAPI excitation and emission wavelengths. Both light and fluorescent images were captured and archived using a Nikon frame grabber and the Nikon NIS-Elements software.

In order to quantify the vascular areas and densities in the post-burn granulation tissues, images of vessels stained for anti-CD34 were collected with a 20× objective and archived. All the vessel contours were delineated manually as regions of interests (ROIs) in these images, and total vessel areas and numbers were calculated after the images were scaled down to the correct pixel-µm ratio (Image Por Plus, Media Cybernetics, Bethesda, MD). All the values originating from images of the same slide were averaged, and the results were expressed as average...
vascular areas / 1000 µm², and average vessel numbers / 1 mm² respectively.

All graphing was done in Microsoft Excel and statistics in SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

For fluorescent-labeled slides, images were obtained by sequential scanning of each channel with the specific pair of filters to eliminate the cross-talk of chromophores and to ensure a reliable quantification. To ensure that the analysis was performed on signals coming from the same optical planes, the fluorescent images were subjected to a blind deconvolution algorithm based on a multi-pass, adaptative point spread function (PSF) subtraction of diffracted light (AutoQuantX2, Media Cybernetics).

**Results**

In our study, we aimed to evaluate the process of angiogenesis at the site of coagulation necrosis induced by heat, because in the surrounding areas of hypoxia and hyperemia there are functional changes of remnant blood vessels that would have not allowed an accurate quantitative assessment of the changes in the newly formed vessels. Applying a temperature of 100°C for 5 seconds led to a complete destruction of the epidermis and dermis, with tissue necrosis and reaching to the muscle planes (Figure 1). It was also observed that the entire vascular network of the dermis had been destroyed by the thermal aggression.

In the cross-sections stained with Hematoxylin–Eosin and Masson trichrome, at the periphery of tissue necrosis areas there was a progressive reduction in intensity of the lesion, which extended for about 2–3 mm of skin. In this area, congested blood vessels, with discontinuous walls, with numerous perivascular petechial hemorrhages or intravascular thrombi stood out.

On the histological samples obtained from Group 1, at 3 days after the burn, we noted that the process of angiogenesis in the burned wound was absent.

However, at the periphery of the wound, in the hyperemic area as well as in the area of hypoxia, blood vessels with large lumens were present, bordered by a thickened endothelium formed of cells with rich moderately basophile cytoplasm, enlarged and less dense nuclei, compared to normal endothelial cells, which corresponded to the appearance of neovascularization capillaries (Figure 2).

![Figure 1 – Rat skin burn lesion at three days: coagulation necrosis, which involves the whole thickness of the skin, towards the muscle plane (HE stain, ×100).](image1)

![Figure 2 – Rat skin burn lesion at three days: weak immunostaining of rare vessels for anti-α-SMA, ×100.](image2)

In the immunohistochemical study, we evaluated the labeling of anti-CD34 and anti-α-SMA antibodies at the level of endothelium and at the level of smooth muscle cells in the vascular walls respectively. Thus, by examining the sections under a low power objective, we observed 1–2 angiogenesis capillaries on the cut surface of lesion, with small diameters, consisting only of a few CD34-positive or α-SMA-positive cells (Figures 3 and 4). On the other hand, at the edge of the wound, the angiogenesis process was well developed as compared to the center of the wound; and it consisted of capillaries with variable lumen sizes, bordered by two rows of cells, one internal layer (CD34+ cells), and one external layer of α-SMA+ cells. Based on these data, we could state that the process of angiogenesis begins relatively fast after the burn on the outskirts or in the depth of the
wound, consisting of capillaries whose wall is made of two concentric layers of cells, an external one, α-SMA-positive, and a CD34-positive internal one. After analyzing the slides of the next groups, sampled at 9, 12, and 15 days after the burn, we found a massive and progressive increase in the number and areas of angiogenic capillaries in the burned wound (Figures 5 and 6).

Figure 3 – Rat skin burn lesion at six days: superficial zone of a necrotic area showing acute inflammatory infiltrate together with the occurrence of newly formed vessels in the depth of the wound (HE stain, ×100).

Figure 4 – Rat skin burn lesion at six days: α-SMA-positive vessels, ×100.

Figure 5 – Rat skin burn lesion at 12 days: CD34-positive endothelial cells, ×200.

Figure 6 – Rat skin burn lesion at 12 days: α-SMA-positive pericytes, ×400.
Thus, at six days post-burn, the average number of capillaries increased to approximately 119/mm², and the average area was of 59.92 µm², and at day 9 the capillary number reached 229/mm² with a total area of 100.27 µm², almost double from the previous group (Figures 7 and 8). For the Groups 3, 4 and 5, the total area of angiogenic capillaries reached approx. 10% of the cut section, a morphological feature which denotes the capacity of the vascular system for a fast rehabilitation of the oxygen and nutrient intake in the lesion (Figure 9).

The average number of angiogenic capillaries and their total areas remained at high levels also in Groups 4 and 5 also, i.e. at 12 and 15 days after the burn, and then they had a strong decrease on Groups 6 and 7, i.e. at 18 and 21 days after the burn (Figure 10).

Overall, this dynamic development of the angiogenic process is consistent with the development of granulation tissue, and with fibroblast growth respectively, and the occurrence of myofibroblasts, macrophages, mast cells, lymphocytes and plasmocytes. We believe that during the wound healing process, inter-cellular or cell–stromal connections occur, and this coordinates and shapes neo-angiogenesis.

Regarding the diameter of angiogenesis capillaries, on the same microscope field the capillary diameters found varied from 4 to 38 µm. This great variability can be explained by the fact that in the granulation tissue of the burned wound, angiogenesis capillaries are formed in different time steps. The general orientation of these neoformation capillaries was from the depth of the wound to its surface.

In our study, we observed by double immunofluorescence (Figures 11 and 12), the existence of a close relationship between endothelial progenitor cells (EPC, CD34+) and pericytes (P, SMA+). Capillaries with lumens over 10 µm showed that the wall consisted of two concentric different cell phenotypes. Immunohistochemically, they were expressing CD34 in the inside layer, the outside layer being formed of α-SMA-positive cells. Capillaries with smaller diameter had only a single row of CD34-positive cells. This microscopic appearance allowed us to conclude that first EPCs are formed, followed by pericyte differentiation at their periphery of the inner EPCs layer, during the genesis of neoformation capillary. It is possible that the differentiation of pericytes from mesenchymal stromal cells of the burned wound could be a result of various factors released from CD34-positive cells. Due to the relatively rapid differentiation and arrangement on the periphery of newly formed capillaries, we consider that pericytes serve as a stabilizer for capillary angiogenesis.

Another role of pericytes seems to be the genesis of the smooth muscle cells from the walls of arterioles and venules, because we observed a multiplication of pericytes in the deep of the burnt wound, in Groups 5–7, the formation and stratified settlement of a structure related with the tunica media of arterioles and venules.

Figure 7 – (a) and (b): Descriptive statistics revealed that the normalized average vascular areas and numbers increased from Group 2 to Group 5, with a drastically decrease for the Group 6 and Group 7.

Figure 8 – We have also noted a good correlation between the average vessel area and the average vessel counts, r(45) = 0.678, p<0.01.
Analysis by deconvolution suggested that there are at least two ways of developing angiogenesis capillaries. The first way occurs by multiplying the EPCs integrated in the structure of a neoformation capillary, followed by “sprouting capillary” occurrence. Thus, a network of capillaries is formed; which is tributary to a larger vessel (Figures 13 and 14).

A second way is represented by the migration of a single CD34-positive EPC into the mesenchymal stroma of granulation tissue, multiplying and forming cellular cords which later on become canalized and give rise to new capillary that later connects to the vascular network. This second method was suggested by the identification of some isolated CD34-positive cells in the stroma of granulation tissue at distances of about 90–120 µm from other capillaries, and that become canalized giving rise to new vascular structures.
Discussion

Because of increased morbidity and mortality, high social costs, but also the physical and psychological sequelae, burns are an important public health problem for all countries. Therefore, numerous studies of cellular and molecular biology seek to unravel the pathophysiological mechanisms that disrupt local homeostasis and can generate “burn disease”.

One of the most studied processes occurring in the burned wound is angiogenesis. In recent years, the study of this process has allowed at least partial decoding of the cellular mechanisms involved leading to the shaping of new therapeutic options. Despite these efforts, most of the molecular mechanisms responsible for blood vessel formation are not well-understood [4].

In our study, the heat destroyed the entire vascular network from the area subjected to the experiment, as well as the epithelial and connective tissue of the skin. The reaction of the animal body was swift and intense with the purpose of restoring damaged tissues and local homeostasis.

The angiogenesis process started relatively quickly, about 2–3 days after injury by the formation of neo-vascularization capillaries at the periphery of the burned wound, consisting of CD34-positive cells, which were morphologically different from endothelial cells. Numerous studies have emphasized the role of these cells in restoring damaged vascular networks following trauma, burns or tumors.

By the late 1990s, it was considered that adult neo-vascularization occurs through a process of angiogenesis which meant the proliferation and migration of differentiated endothelial cells [5]. This dogma (theory) was challenged first by Asahara T et al. (1997), which managed to isolate from the peripheral blood of the adult some particular cells called angioblasts, which differentiated in vitro into endothelial cells and contributed to the formation of new vessels in vivo, in response to tissue ischemia [6]. These cells express various surface markers, the most important being the hematopoietic surface marker CD34 and the vascular endothelial growth factor receptor-2 marker (VEGFR2). They were later called endothelial progenitor cells EPCs [7, 8]. The origin of EPCs is therefore the bone marrow pluripotent stem cell.

The predominant signal that induces the mobilization of EPCs from the bone marrow parenchyma (MB) and their passage in the peripheral blood vessels would be hypoxia [9].

We believe that the mechanism leading to the presence of EPCs in the peripheral blood is more complex and not completely understood, because the burns induced to the experimental animal caused only local significant ischemia, in the burned wound. In support of this assumption come the investigations of other researchers [10], which showed that a cytokine, the granulocyte monocyte-colony stimulating factor increases EPC levels and contributes to better neovascularisation of the ischemic tissue.

Other studies indicated that many cells are able to differentiate into endothelial cells. Thus, Capobianco S et al. (2010) mentions that cells of the myelo-monocyte line derived from the red bone marrow, mononuclear cells derived from the spleen, mononuclear cells derived from the umbilical cord blood, adventitial stem cells and skeletal muscle progenitor cells, make up a pool of cells from which endothelial cells derive [9].

In our opinion, local hypoxia in the burned wound is the determining factor in the emergence of EPCs in the wound and the onset of angiogenesis. It is well known that most cells under hypoxia respond by secretion of a pro-angiogenic key growth factor called vascular endothelial growth factor (VEGF). Hypoxia caused by an imbalance between oxygen supply and consumption stimulates embryonic and adult tissue vasculogenesis [11], induces synthesis of VEGF, which in turn stimulates the migration of EPCs into the region to be vascularized [12, 13].
In our study, the first CD34-positive cells appeared in the transition zone from coagulation necrosis to that produced by the heat in the healthy dermis, where normal vascular structures existed allowing a free local blood flow, which was the vehicle for endothelial progenitor cells (EPCs). EPCs are cells equipped with the ability to move through the connective matrix of the granulation tissue. Therefore, in our study, they were highlighted in both the wall of angiogenesis capillaries and distant from any vascular structure. The movement of EPCs is facilitated by their ability to form long cytoplasmic extensions called filopodia through which they release in the connective matrix a series of proteolytic enzymes that degrade the matrix and create tunnels for future movement [14, 15]. When reaching a particular area, EPCs multiply, create cellular cords, which quickly develop into tubes with lumena, thus generating a new capillary. Pericytes differentiate at the periphery of EPCs serving as stabilizers for the newly created vascular structure. Angiogenesis capillaries unite to form vascular plexuses through which blood flow is resumed.

Aside from hematopoietic cells, another source of EPCs appears to be the endothelial cells in blood vessels from the wound edge. Normally, the endothelial cells (EC) are fixed in a monolayer by both intercellular junctions and junctions with the capillary basal membrane. Experimental studies have shown that VEGF-induced migration leads to intercellular junction degradation by endothelial cells before the onset of migration by VE-cadherin internalization. This degradation process is directly induced by VEGF. EC react and migrate with a delay due to the degradation processes of intercellular junctions [16].

Other studies have shown that mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into a variety of cell types both in vitro and in vivo, such as osteoblasts, chondrocytes, myocytes, adipocytes, pancreatic islet β-cells, and endothelial cells [17]. These cells are also present in granulation tissue in burned wounds. Therefore, EPCs are a heterogeneous population of cells of the adult tissue originating from the BM or other sites, who share a common phenotype and which, when properly stimulated in vivo and in vitro, give rise to endothelial cells.

In our study, the great migration and proliferation capacity of CD34-positive cells lead to a rapid increase of the number of angiogenesis capillaries and their total cross section area, reach very high values nine days after the burn. After this time point, the number and total sectional area of angiogenesis capillaries remained relatively constant at high values, up to 15 days after the burn, followed by a rapid decrease of the their number and sectional area. We believe that the angiogenesis process is strongly inhibited after the re-epithelization of the burned wound and the disappearance of the dermal hypoxia from the dermal connective tissue. Factors that determine the inhibition of the angiogenic process are less known. According to some authors [18] the disappearance of hypoxia and the installation of normoxia or a hyperoxic state would result in blocking of angioblast and EPC proliferation and would induce apoptosis and even necrosis of these cells.

Part of EPCs becomes mature endothelial cells through intercellular junction development. The main elements of the endothelial junctions are the platelet-derived endothelial adhesion molecule (PECAM) (CD31) and the vascular endothelial (VE)-cadherin [19]. This phase of endothelial junction formation can be defined as a transition step from angioblasts to endothelial cells (EC) and may also be defined as the transition between vasculogenesis and angiogenesis. As they mature, the migration ability of EC progressively decreases, but they retain a reduced migratory capacity even when they became fully mature [20]. With the functional maturation, endothelial cells become “dormant” in the sense that they reduce their ability to multiply, lose some surface antigens and strengthen the intercellular junctions. Much of the angiogenic vessels regress and disappear if no longer needed [21].

In our study, for the Group 7, at 21 days after the burn, even if the wound was macroscopically healed and the surface epithelium was completely restored, the process of angiogenesis was still present, witnessing the fact that vascular remodeling is not completed with the healing of burn injury.

Mechanisms that characterize angiogenesis are highly dynamic processes, modulated by intercellular (cell–cell), and cell–extracellular matrix (ECM) interactions in the presence of growth and morphogenesis factors [22]. For decades, great attention was paid to the role of vascular endothelial growth factor (VEGF) and its influence on EC migration and proliferation [23]. However, recent studies indicate a number of new factors involved in the mobilization and orientation of endothelial precursor cells. These signaling molecules include EPH-ephrins, bone morphogenetic proteins (BMP), transforming growth factor beta (TGF-β), Notch ligands, Ras protein family members and many others [4].

Like others [24, 25], we also believe that the process of angiogenesis is part of the local and general processes of wound healing which, in addition, include connective cell proliferations, migration and activation of immune cells, connective matrix deposition, nerve tissue proliferation and remodeling of granulation tissue.

**Conclusions**

The process of angiogenesis in experimental skin burns of the third degree started relatively quickly after the injury, with the occurrence of endothelial progenitor cells (EPCs, CD34-positive) at the edge of tissue necrosis areas.

Their rapid proliferation lead to a rapid increase in the number of angiogenesis capillaries and their total areas in the granulation tissue, reaching a maximum development between days 9 and 15, after which they incompletely regressed, remaining at moderate levels after wound epithelization. Therefore, vascular remodeling process continues after the restoration of epithelial integrity.

Altogether, this works stands as a proof of the very close relationships that exist between EPCs and pericytes during angiogenesis, proliferation or involution of EPCs being followed by a parallel evolution of pericytes.
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Contribution Note
All authors have equally contributed to this work.

References

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