Phases of the cutaneous angiogenesis process in experimental third-degree skin burns: histological and immunohistochemical study

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Abstract
Skin burns represent a major problem of public health because of their frequency and because of their seriousness, too. The healing process of the burnt wound is extremely complex, as it requires a well-coordinated collaboration among different tissues and cellular strings. From the morphological point of view, the stages of the repairing process of the skin wounds include processes of inflammation, proliferation and tissular remodeling. Angiogenesis has a role of extreme importance within the healing process of third-degree skin burns. That is because the vascularization remake is necessary for feeding the tissue of granulation with nutritive substances and oxygen. The angiogenesis started relatively fast. Three days after the producing of the burn, there could be identified strings of CD34+ endothelial precursor cells at the edges and deep into the wound, all these having contact with the normal blood vessels or with those less affected by the thermal aggression. After the lumenization of the newly-formed capillary vessels, there appeared the pericytes within their membrane. The CD34+ endothelial precursor cells (EPCs), as well as the pericytes, participate at the synthesis of the base membrane of the angiogenesis vessels. The density of the angiogenesis vessels on the surface unit within the tissue of granulation grew from three to 12 days. After that, they reduced progressively while the tissue of granulation was becoming mature. The angiogenesis vessels go through a process of reshuffling and maturation at the same time with the maturation of the tissue of granulation, but these processes did not appear to be finished when the skin was completely healed, and the epidermis was totally recovered.

Keywords: angiogenesis, precursor endothelial cells, CD34, stem cells, granulation tissue, skin burns.

Introduction
The skin is a complex organ, a connective-epithelial membrane sheath that surrounds the body and continues with mucous membranes of the natural cavities. It is a sensory organ and also a mechanical, thermal, chemical and biological protective barrier (antimicrobial, antiviral and antifungal). Also, the skin protects the body from UV radiation, prevents the loss of body water and electrolytes [1], and participates in thermoregulation and excretion processes.

Being a large organ, the skin is subjected to different types of aggression from the outer environment. Thermal burns are a major health problem [2] as they are found in all parts of the world, have a relatively high incidence and can affect people of all ages, from newborns to the elderly. It is estimated that each year, worldwide, over 6.6 million people suffer from various burns [3]. According to other authors, the incidence of burns is much higher, especially in underdeveloped small countries. Thus, Peck MD (2011) notes that in 2004 there were nearly 11 million people with severe burns requiring medical care worldwide, of which about 300,000 died [4]. The incidence of burns in developed countries is also quite high. Thus, it is estimated that in the U.S. every year over one million people suffer burns that require specialized treatment [5].

The severity of the skin burns and thus the healing possibilities depend on the depth of the lesions and affected skin area. Superficial burns heal with minimal scarring, while second and third-degree burns require both local and supportive intensive treatment. Extensive third-degree burns over large areas require skin graft to achieve wound closure and restore the skin barrier [6–8].

The burned wound healing process is very complex as it requires collaboration between different tissues and cell lines and a variety of intracellular and extracellular signals [9], which ultimately helps to restore the integrity of the skin as well as its main functions. It is known that the burn injuries initiate a cascade of morphological and pathophysiological events, including the processes of inflammation, proliferation and tissue remodeling [10–12], all genetically programmed and implemented in stages. In this process, the restoration of dermal vascular
network, that is neoangiogenesis, is essential for burned wound healing, respectively for the emergence, development, modeling and maturation of granulation tissue as well as for the survival of keratinocytes [13–15].

In this study, we evaluated the main stages of angiogenesis in third-degree skin burns induced on experimental animal model.

Materials and Methods

For this study, we selected 35 adult common Wistar rats, weighing between 280 and 310 g, from the Biobase of the University of Medicine and Pharmacy of Craiova, Romania. The animals were kept before and during the experiment under standard light, temperature and humidity conditions, having permanent access to food and water (ad libitum).

Following the approval of the Ethics Committee of the University of Medicine and Pharmacy of Craiova, in accordance with the European Council Directive of 11.24.1986 (86/609/EEC), the European Convention on the Protection of Vertebrate Animals (2005), and the Government Ordinance No. 37/2.02.2002, the animals received general anesthesia by intramuscular injection of Ketamine hydrochloride (Ketalar®, Parke-Davis), 6 mg/kg. The hair was removed from an area of 1 cm² from the upper portion of the dorsal region of each animal, on which a special cone shaped metal device made of stainless steel, with a diameter of 1 cm, equipped with a control thermometer, heated to 100°C in a pot of boiling water, was applied for five seconds [16–20].

After producing the burns, each animal was bandaged using a dry bandage applied on the wound. The evolution of the burned wound and animal welfare were monitored daily. The animals were randomly divided into seven groups of five animals. Every three, six, nine, 12, 15, 18, and 21 days after the burns, under general anesthesia, from each group of five animals, the lesion area was sampled, with a margin of about 2–3 mm around the lesion, in order to observe the dynamic process of angiogenesis in the burned wound during the spontaneous healing.

Histological study

Fragments from skin burns sampled were fixed in 10% neutral formalin for 72 hours at room temperature (21–23°C) and embedded in paraffin wax. Sections were cut using a rotary microtome (Microm HM350) equipped with a waterfall based section transfer system (STS, Microm). Four μm-thick sections were then stained using the Hematoxylin–Eosin and Goldner–Szekely’s trichrome.

Immunohistochemical study

For the immunohistochemical study, three μm-thick sections were cut using the same equipment and collected on poly-L-lysine coated slides, dried in a thermostat at 37°C for 24 hours in order to obtain a perfect adhesion of the biological material to the surface of the histological slide. For single immunohistochemistry, following deparaffinization and hydration, after antigen retrieval, sections were cooled down to room temperature and were incubated for 30 minutes in a 1% hydrogen peroxide solution. The sections were next washed in PBS, followed by a blocking step of 30 minutes in 2% skim milk. Next, the slides were incubated with the primary antibodies overnight at 4°C, and the next day, the signal was amplified for 30 minutes using a peroxidase polymer-based secondary detection system (EnVision, Dako). The signal was detected with 3,3’-diaminobenzidine (DAB) (Dako) and the slides were coverslipped in DPX (Fluka) after Hematoxylin counterstaining.

The antibodies used for the immunohistochemical study are presented in Table 1.

Table 1 – Antibodies used in the study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host / Target</th>
<th>Clone</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Manufacturer</th>
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<tr>
<td>Anti-CD34</td>
<td>Rabbit / rat</td>
<td>EP373Y</td>
<td>1:100 (histochemistry)</td>
<td>Sodium citrate buffer, pH 6</td>
<td>Epitomics</td>
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<td>1:300 (fluorescence)</td>
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<td>Sodium citrate buffer, pH 6</td>
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<td>1:100 (fluorescence)</td>
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<td>ab19808</td>
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<tr>
<td>Anti-fibronectin</td>
<td>Mouse / mouse, rat, chicken, cow, dog, human, pig, monkey</td>
<td>IST-9</td>
<td>1:50 (histochemistry / fluorescence)</td>
<td>Sodium citrate buffer, pH 6</td>
<td>Abcam</td>
</tr>
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Fluorescent immunohistochemistry

For the immunofluorescence study, we performed double fluorescence stainings using the following combinations: CD34 + SMA, CD34 + fibronectin, SMA + collagen IV, SMA + laminin.

In the case of the double immunohistochemistry for anti-CD34 (1:300, rabbit, Epitomics) in combination with anti-SMA antibody (1:50, mouse, Dako), the immunodetection was a sequential protocol that began with the overnight incubation in the anti-CD34 antibody, amplification with a goat anti-rabbit HRP secondary (1:100, Dako) for 30 minutes, followed by a 30 seconds precipitation step of fluoresceinated tyramide (Perkin Elmer, Medicalkit, Romania). Next, the anti-SMA antibody was added in another overnight incubation, this being detected with a goat–anti-mouse Alexa 596 antibody (30 minutes, 1:400, Invitrogen, Medicalkit, Romania). After DAPI counterstaining, the sections were coverslipped in anti-fading fluorescent mounting medium (Dako).
In the case of the double immunohistochemistry for anti-CD34 (1:300, rabbit, Epitomics) in combination with anti-fibronectin antibody (1:50, mouse, Abcam), the immunodetection was also a sequential protocol that began with the overnight incubation in the anti-CD34 antibody, amplification with a goat anti-rabbit HRP secondary (1:100, Dako) for 30 minutes, followed by a 30 seconds precipitation step of fluoresceinated tyramide (Perkin Elmer, Medicalkit, Romania). Next, the anti-fibronectin antibody was added in another overnight incubation, this being detected with a goat–anti-mouse Alexa 596 antibody (30 minutes, 1:400, Invitrogen, Medicalkit, Romania). After DAPI counterstaining, the sections were coverslipped in anti-fading fluorescent mounting medium (Dako).

In the case of the double immunohistochemistry for anti-SMA (1:300, mouse, Dako) in combination with anti-collagen IV antibody (1:100, rabbit, Abcam) and anti-laminin antibody (1:100, rabbit, Abcam), the immunodetection begun with the overnight incubation in a cocktail of antibodies (SMA + collagen IV, SMA + laminin). Then, the incubation with a cocktail of goat–anti-rabbit Alexa 596 antibody (45 minutes, 1:150, Invitrogen, Medicalkit, Romania) and the biotinylated goat anti-mouse (45 minutes, 1:400, Dako) followed. The signal was visualized with Alexa Fluor Streptavidin 488 (30 minutes, 1:300, Invitrogen, Medicalkit, Romania). After DAPI counterstaining, the sections were coverslipped in anti-fading fluorescent mounting medium (Dako).

Fluorescently-labeled sections were imaged with an Eclipse 90i microscope (Nikon, Apidrag, Romania) equipped with a QImaging Rolera cooled CCD camera and with narrowband fluorescent filters centered for Alexa 594, Alexa 488 and DAPI excitation and emission wavelengths. Images were captured and analyzed using the Image ProPlus 7 AMS software (Media Cybernetics Inc., Buckinghamshire, UK). For fluorescence, images were obtained by sequential scanning of each channel with the specific pair of filters to eliminate the cross-talk of the fluorophores, and to ensure a reliable quantification. Next, the fluorescent images were subjected to a blind deconvolution algorithm based on a multi-pass, adaptive point spread function (PSF) subtraction of diffracted light (Sharp Stack, Media Cybernetics Inc.).

The sections were imaged with a Nikon Eclipse 55i microscope (Nikon, Apidrag, Romania) equipped with a 5-megapixel cooled CCD camera. Images were captured and analyzed using the Image ProPlus 7 AMS software (Media Cybernetics Inc., Buckinghamshire, UK).

Results

In our research, we intended to emphasize the way of appearance of the component elements of the angiogenesis vessels, respectively of the endotelial cells, of the pericytes and of the biochemical elements that compose the base membrane of the capillary vessel: collagen IV, laminin and fibronectin, during the skin reparatory processes. We have noticed, from the previous researches [16–20] that, by placing a metallic object heated at 100°C for five seconds on the animal skin (rat), there happens a thermal transfer large enough to determine necrosis of coagulation in all the histological structures of the skin: epidermis, dermis and hypodermis.

In the present research, by producing third-degree burns on the skin, we have destroyed the entire vascular system on the dermis level through coagulation necrosis and we have created experimental conditions for the appearance of the angiogenesis vessels within the reparatory process.

The healing process of the cutaneous lesion has started rapidly, even from the very first day of its producing, being obvious, macroscopically, the appearance of a brownish crust having the diameter of about 15 mm, limited at its edge by a hyperemia zone and edema of about 2 mm.

By correlating the macroscopical aspects with the microscopical ones, three days after the burn was produced, there were obvious three distinct lesional zones:

- the zone of the necrosis of coagulation (the central lesional zone), where the tissues within the skin structure were completely altered;
- the erythematous-exudative zone at the edge of the necrosis zone, where there were histological structures partially deteriorated, partially viable;
- the erythematous one that continued with the normal tegument, where the thermal lesions were minor.

The angiogenesis process began in the erythematous zone where the thermal transfer was of low intensity and altered only partially the vascular system of the skin. In this zone, there were identified remaining vessels, congested, some of them even amputated, others having discontinuous membrane or intravascular thrombosis and multiple hematic extravasation within the conjunctive matrix. In this zone, three days after the producing of the burn, there have been identified capillary vessels of angiogenesis, having the membrane formed of endothelial precursory cells CD43+. The capillary vessels of angiogenesis appeared with ramifications of the preexistent vessels, in the form of full (without lumen) or with low lumen cellular strings, formed of CD34+ cells (Figure 1, a and b), with abundant cytoplasm, slightly basophilic, with large, ovalar, hypochrome, nucleolate nucleus (Figure 2, a and b), all these aspects showing the character of young cells, with a higher capacity of synthesis for diverse biochemical components. There were, also, identified numerous CD34+ isolated cells at a distance from any vascular components. There were, also, identified numerous CD34+ isolated cells at a distance from any vascular components, having the origin in the hematogenous medulla, some of which forming capillary excrescences starting from the preexistent vessels, and others being non-uniformly spread within the conjunctive matrix.

The membrane of the capillary vessels of angio-
genesis most often appeared as being delimited by two CD34+ endothelial precursory cells which were correlated; sometimes, we found a single endothelial cell that delimited the lumen of the capillary vessel (Figure 2, a and b; Figure 5, a and b).

The process of angiogenesis was preceded by an intense response of the immune system cells, and there appeared numerous granulocyte, lymphocytes and macrophages cells at the edge and deep into the burnt wound. The recovering of the vascular system at the level of the burn wound was accompanied by a rapid proliferation of the young conjunctive cells of fibroblastic type that synthesized the components of the extracellular matrix and, together with the vascular excrescences, have generated a young tissue of granulation under the crust provoked by the thermal agent.

The pericytes, the second type of cells that are present in the membrane of the vessels of angiogenesis, were specifically emphasized by using the anti alpha-SMA (α-Smooth Muscle Actin) antibody by using the immunohistochemistry techniques as well as the immunoflorescence techniques. These cells appeared at the edge of the capillary vessels of angiogenesis after these had got lumen (Figures 6 and 7). Their distribution was discontinuous in the membrane of these capillary vessels. We believe that they represent an element of functional and structural stabilization of the newly-formed capillaries. The growing diameter of the vessels of angiogenesis and their transforming in arterioles and venules was associated with the growing of the positive alpha-SMA cells, their concentrically organization and the forming of a distinct muscle tunic. We consider that the pericytes have the capacity of turning into smooth muscle cells in the membrane of the vessels.

In the zones where the granulation tissue became mature, and the collagen fibers from the deep dermis were well-structured, we noticed immature capillary vessels of angiogenesis, formed only by CD34+ endothelial precursory cells, without pericytes at the edge, an aspect that suggests the fact that the capillary membrane can be stabilized directly by the conjunctive fibers, mainly by the collagen fibers that can overtake, partially, the functions of the pericytes.

The collagen IV from the structure of the base membrane of the capillary vessels of angiogenesis was precociously synthesized by the CD34+ endothelial precursory cells. In the young tissue of granulation, the first positive cells at the anti collagen IV antibody were endothelial precursory cells (Figure 8, a and b), before other cells from the conjunctive matrix. Collagen IV was, also, synthesized by the pericytes, but in small quantity and even by the fibroblasts from the stroma of the tissue of granulation.

For the laminin and fibronectin, the immunohistochemistry reaction was positive at the same time at the endothelial precursory cells CD34+ as well as at the conjunctive cells from the conjunctive matrix, especially in fibroblasts and pericytes, this confirming the capacity of these cells of synthesizing the essential elements of the base membrane in the structure of the vessels of angiogenesis (Figures 9 and 10).

As far as the density of the vessels of angiogenesis on the surface unit in the tissue of granulation is concerned, this grew from three to 12 days. Then, it progressively reduced while the tissue of granulation was becoming mature. Simultaneously with the growing number of the vessels of angiogenesis, there was obvious their functional coupling, thus forming a vast system of capillary vessels of angiogenesis that assured the recovering of the skin conjunctive tissue.

The forming and maturing of the vessels of angiogenesis developed from the edge of the wound towards its centre, and from its depth towards its surface. Still, there has to be mentioned that the aspect of these vessels is extremely varied, as we can identify, in the same area, immature vessels of angiogenesis, mature vessels and CD34+ cells disseminated in the conjunctive matrix (Figures 11 and 12). This histological aspect proves the fact that the process of angiogenesis is extremely complex, being coordinated by multiple factors, some of them less known, while others unknown.

Figure 1 – (a) Angiogenesis vessels as cellular cords CD34+ (EPc), partially lumenized, observed in the granulation tissue six days after burning onset. Anti-CD34 immunostaining, ×200. (b) Partially lumenized angiogenesis capillary. Detail from preceding figure. Anti-CD34 immunostaining, ×400.
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Figure 2 – (a) Angiogenesis vessels marked off by CD34+ cells presenting a large, ovalary nucleus and abundant cytoplasm. Anti-CD34 immunostaining, ×1000. Microscopic image evidentiating CD34+ cells organized within angiogenesis capillaries, and also CD34+ cells, diffusely disseminated within the granulation tissue matrix. Anti-CD34 immunostaining, ×1000.

Figure 3 – Young granulation tissue, presenting multiple angiogenesis capillaries and CD34+ cells, diffusely disseminated within the conjunctive matrix. Anti-CD34 immunostaining, ×200.

Figure 4 – Positive (green) CD34 cells, with large, round, ovalary (blue) nuclei, disseminated within the granulation tissue. Anti-CD34 immunostaining, ×200.

Figure 5 – (a) Angiogenesis capillaries in various formation stages. Endothelial preceding cells (green) CD34+ initially form the capillary wall by themselves, where red blood cells may be seen (yellow). After vascular lumen formation, the pericytes appear (red) at vessel fringe, playing the role of vascular wall stabilization. Anti-alpha-SMA (red), CD34 (green), blue nuclei (DAPI) triple immunostaining, ×1000. (b) Detail from preceding figure. Anti-alpha-SMA (red), CD34 (green), blue nuclei (DAPI) triple immunostaining, ×1600.
Figure 6 – Lumenized angiogenesis vessels with a wall made up of two rows of cells: internal EPC row and external pericyte row (brown). Anti-alpha-SMA immunostaining, ×400.

Figure 7 – Granulation tissue nine days after burning onset, where multiple angiogenesis vessels in various evolution stages are evidenced: young capillaries presenting a lumen limited only by EPC (green) and mature capillaries presenting a lumen limited by preceding endothelial and pericyte cells (red). Anti-alpha-SMA (red), CD34 (green), blue nuclei (DAPI) triple immunostaining, ×200.

Figure 8 – (a) Angiogenesis capillaries six days after burning onset, limited only by one row of EPC, intensely positive at anti-collagen IV antibody. Anti-collagen IV immunostaining, ×100. (b) Detail from preceding figure where there may be observed that EPC are the first cells within the granulation tissue matrix that synthesize collagen IV. Anti-collagen IV immunostaining, ×200.

Figure 9 – Microscopic image of granulation tissue nine days after burning onset where there may be observed the fact that the lamina is synthesized both by EPC and by other cells within the conjunctive matrix. Anti-laminin immunostaining, ×200.

Figure 10 – Similar to laminin, fibronectin has also been synthesized both by EPC and by other conjunctive cells. Anti-fibronectin immunostaining, ×200.
Discussion

The healing of the skin burns is a very complex and vital process as the body recovers its main protection and external contact barrier [21]. The complexity of the inflammatory, proliferative and remodeling processes at the wound level, depend on the gravity of the burning and on the ability of the skin to recover its histological structures. The serious burnings provoke, at the level of the wound, an important capillary leaking that determines liquid derangements, electrolytic unbalance, loss of proteins and, as far as the deep and large wounds are concerned, circulatory scarcity [22].

The angiogenesis is an essential process in the healing of the skin burns because it assures a provision of the tissue of granulation with nutritive substances and oxygen, and it allows the elimination of the residual products towards the excretion passages of the body [23–25]. The angiogenesis favors the remaking of the derma structure as well as the reparatory processes at the epidermis level.

In our research, we noticed that, in a first stage, the angiogenesis vessels are formed of CD34+ endothelial precursory cells strings that start from intact vessels or vessels a little affected by the thermal agent, at the edges of the wound. The fact that these cells proliferate from the pre-existent blood vessels raise the question that these cells originate in the vascular endothelial cells or originate in some cells that are present in the peripheral blood.

According to some authors [26], the angiogenesis are due to a subset of precursor endothelial cells that are present in the peripheral blood, because most of the endothelial cells from the pre-existent blood vessels remain passive to aggressions.

The theory that the cells derived from the bone marrow (BMDCs) contribute to the after-birth neoangiogenesis through endothelial difference, was proposed, for the first time by Asahara T et al. (1997) [27]. They identified, in the peripheral blood, a population of cells derived from the bone marrow that had the capacity of differentiating into endothelial cells, that they called endothelial progenitor cells (EPCs). Since then, the differentiation of the endothelial cells from BMDCs has been widely accepted as a substantial mechanism for the remaking of the vascular structures in ischemic tissues [28, 29].

The migration of the endothelial precursory cells from the blood into the conjunctive matrix of the granulation tissue seems to be directed by some proangiogenic factors. According to some authors [30], the angioblasts situated at the edges of the vascular excrescences, form the cellular prominences or filopods that direct their migration towards a source of angiogenic growth factors. The proangiogenic factors are secreted, mainly, by the cells of the inflammatory infiltrate from the conjunctive matrix.

In our research, we identified CD34+ endothelial precursory cells at a distance from the vessels of neoformation, isolated in the matrix of the granulation tissue. We consider that these cells are able of multiplying and of forming new capillaries that couple, afterwards, with the rest of the vascular system. We can say this, because through studies of immunofluorescence, we identified endothelial precursory cells capable of generating isolated cell-strings in the conjunctive matrix. We also believe that the CD34+ endothelial precursory cells can differentiate into other types of cells, because through immunofluorescence, we identified a great number of isolated CD34+ cells that seem not to form angiogenesis strings.

Some authors argued that the endothelial precursory cells (EPCs) are capable of differentiating into vascular smooth muscle cells [31].

The pericytes are adventicial cells situated in the base membrane of the capillaries and in the post-capillary venule, having the role of stabilizing the membrane of the vessel [32].

In our research, the pericytes were identified in the structure of the angiogenesis vessels by using the anti-alpha-actin specific antibody on the smooth muscle (α-SMA). They appeared at the edges of the endothelial precursory cells (EPC) after the angiogenesis vessels got lumen.
According to other researchers [33], the in vitro as well as the in vivo pericytes differentiate from the mesenchymal cells. Recent studies argue that some cells that are derived from the bone marrow generate mesenchymal stem cells that represent precursors cells of the pericytes [34]. Other authors consider that the pericytes are recruited in the structure of the angiogenesis capillaries through the differentiating from the mesenchymal precursors cells that are positioned close to these vessels, or through the migration from the membrane of the adjacent vessels [35]

It appears that the pericytes are among the first cells that invade the newly vascularized tissues. They locate in the area of the growing endothelial excrencences, determining the location of the forming of the vascular excrencences and of the guiding of the newly formed vessels [36, 37]. Their role seems to be in the stabilization of the capillary membrane and in the regulation of the local blood flow. It appears that there is a well-controlled balance through paracrine factors between the number of endothelial cells and the pericytes in the angiogenesis vessels [32].

In our research, the biochemical components of the base membrane in the capillary structure (collagen IV, laminin, fibronectin) were synthesized, mainly, from the endothelial precursors cells and pericytes. However, these biochemical elements were synthesized by other conjunctive cells, as well, especially by the fibroblasts of the tissue of granulation.

After the forming of the blood vessels, the angioblasts express most of the markers of the endothelial cells [38]. They go through a process of reshuffling and tissular maturation, just like the entire tissue of granulation. Thus, most part of the angiogenesis vessels disappeared when the granulation tissue became mature.

We consider that angiogenesis is a dynamical process that has special implications in the healing of skin burns.

Conclusions

The cutaneous angiogenesis within the third-degree burns started with the appearance of CD34+ endothelial precursors cells at the edges as well as deep within the burnt wound. These cells generated strings of angioblasts, capable of lumening and producing new capillary vessels. These cells are capable of synthesizing the biochemical elements within the structure of the base membrane. The angiogenesis vessels go through a process of reshuffling and maturation at the same time with the maturation of the tissue of granulation. The reshuffling of the angiogenesis vessels was not finished when the cutaneous wound was completely healed and the epithelium was totally recovered.

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Contribution Note

All authors have equally contributed to the manuscript.

References


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