EDITORIAL

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Ewelina Drela, Barbara Ruszkowska, Arleta Kulwas,
Beata Małecka, Danuta Rość

Angiogenesis in Diabetic Foot Syndrome

Angiogeneza w zespole stopy cukrzycowej

Department of Pathophysiology Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland

Abstract

Postnatal new blood vessel formation occurs via the process of angiogenesis. Under normal conditions, angiogenesis takes part in placenta development, the reproductive cycle, embryo implantation, muscle remodeling after exercise-induced training or wound healing. Angiogenesis is a multi-step process controlled by many cytokines such as VEGF, TGF, FGF or trombospondin. A disturbing balance between cytokines regulates this process or creates new, different, pathological conditions which lead to incorrect angiogenesis or its impairment. Abnormal angiogenesis is observed in patients with diabetic foot syndrome. Impairment of new blood vessel development contributes to the delay of tissue granulation. Incorrect circulation leads to tissues ischemia. The consequence of this long-lasting state is necrosis, leading to amputation. About twenty-five percent of patients with diabetic foot have an amputation. The following fact shows a dire prognosis: about seventy-five percent of patients live no longer than five rears after onset. Angiogenesis and angiogenic factor studies, especially those of vascular endothelial growth, are creating new therapeutic targets. Growth factor treatment is bringing new hope and concerns to the medical world. That is the direction of knowledge about angiogenic factors and angiogenesis that needs to be intensified (**Adv Clin Exp Med 2011, 20, 3, 243–248**).

Key words: angiogenesis, diabetic foot syndrome, vascular endothelial growth factor.

Streszczenie

Powstawanie naczyń krwionośnych postnatalnie zachodzi najczęściej na drodze angiogenezy. W prawidłowych warunkach następuje podczas tworzenia łożyska, implantacji zarodka, remodelingu mięśni lub gojenia ran. Angiogeneza jest wieloetapowym procesem regulowanym przez wiele cytokin, do których należą: VEGF, TGF, FGF, trombospondyna. Naruszenie równowagi między czynnikami inicjującymi i hamującymi proces tworzenia naczyń prowadzi do nieprawidłowej angiogenezy. Zaburzoną angiogenezę obserwuje się u chorych leczonych diabetologicznie z zespołem stopy cukrzycowej. Nieprawidłowe tworzenie nowych naczyń opóźnia proces ziarninowania rany. Wadliwe krążenie przedłuża stan niedokrwienia, a następstwem długotrwałego niedotlenienia jest martwica prowadząca do amputacji. Amputację przeprowadza się u prawie 25% pacjentów z zespołem stopy cukrzycowej. Wykonanie amputacji kończyny dolnej jest obarczone złym rokowaniem: aż 75% pacjentów nie przeżywa kolejnych 5 lat. Badania nad angiogenezą i czynnikami ją kontrolującymi, zwłaszcza *vascular endothelial growth factor*, stwarzają nowe możliwości terapeutyczne. Zastosowanie czynników wzrostowych w leczeniu stopy cukrzycowej niesie ze sobą wielkie nadzieje, ale też obawy. Zasadne jest więc przeprowadzanie badań i zgłębianie wiedzy na temat angiogenezy w zespole stopy cukrzycowej (**Adv Clin Exp Med 2011, 20, 3, 243–248**).

Słowa kluczowe: angiogeneza, zespół stopy cukrzycowej, naczyniowo-śródbłonkowy czynnik wzrostu.

Chronic diabetic complications are established by the general public as a clinical and an economic problem. Type 2 diabetes mellitus (T2DM) is becoming an epidemic of the twenty first century. A significant percentage of patients with T2DM suffer from diabetic foot syndrome. The development of diabetic foot (DF) is contributed to the three major factors: ischemia, neuropathy and the mechanical factor. There are changes in microvessels and the nervous environment that lead to characteristic symptoms. Impaired wound healing in diabetic ulcers is the main cause of amputation

and results from worsening limb blood supply. It is thought that disturbance of the angiogenesis process is the key to impaired wound healing in diabetic foot syndrome.

Angiogenesis and Vascular Endothelial Growth Factor

Angiogenesis is not the only process that leads to new blood vessel formation. The other processes are vasculogenesis and artheriogenesis [1]. The differences between these processes regard the mechanism of vessel formation and the period of their life in which they predominate. During the process of vasculogenesis, the blood vessels create de novo; mesodermal precursor cells administer into angioblast cells and they differentiate into endothelial cells. Vasculogenesis dominates in embryonic life [1, 2]. Artheriogenesis is characterized by a large blood vessel formation with good wall development. This process takes place in adult life and it is induced by ischemic conditions [1]. Angiogenesis is defined as new blood vessel formation based on existing vessels, which plays the biggest role in postnatal development [3]. This very important process for human existence consists of many stages [4]. The first step in new blood vessel creation is increasing permeability, which is possible thanks to vascular endothelial growth factor (VEGF). VEGF increases vascular permeability by tight-junction modulation and vessel fenestration. In this way, water and other molecules leave blood vessels [5]. At this stage, endothelial cells are stimulated by proangiogenic factors, mainly by VEGF. Endothelial cell activation manifest as a decreased adhesion and increased sensitivity to mitogenic factors [5]. The next steps of angiogenesis are extracellular matrix (ECM) degradation and basement membrane deposition. This process requires an enzyme with metalloproteinase activity (MMP). Type I–III collagen is degraded by MMP-1. MMP-2 breaks down the type II collagen which is a primal constructive element of the basement membrane. MMP-3 and MMP-9 also takes part in proteolytic matrix degradation. VEGF induces these enzymes expression by endothelial cells. What is more, vascular endothelial growth factor increases MMP-1, MMP-3 and MMP-9 expression and the factors of coagulation (von Willebrand Factor: vWF) and fibrinolysis (urokinase-type and tissue-type plasminogen activator: u-PA and t-PA) synthesis in vascular smooth muscles cells and the endothelial cells [6].

After matrix degradation, endothelial cells migrate and proliferate [4]. t-PA and u-PA are present in blood circulation and convert plasminogen into plasmin. The plasmin breaks down fibronectin and laminin and activates the basic fibroblast growth factor (bFGF) – another angiogenic factor. The translocation of bFGF into nucleus evokes endothelial cell proliferation [4, 6]. The proangiogenic factor group includes thrombospondin, transforming growth factor beta (TGF-β) and αβ integrin. The result of endothelial cell proliferation is new blood vessel tubiform structure formation.

The last step of angiogenesis is the process of new blood vessel maturation: tubiform structures are surrounded by mesenchymal cells. At this stage, the platelet-derived growth factor (PDGF) plays an important role in inducing mesenchymal cell migration. Additionally, TGF-β and fibroblast growth factors (FGF) induce mesenchymal cell migration and a differentiation into miofibroblast cells. Creation of intracellular connections between endothelial cells and surrounding cells is the final phase of angiogenesis. In this way, there is a possibility of intracellular interactions and signal transduction from an extracellular environment [4].

Full, functional new blood vessel formation is a complex and multi-step process which is regulated by cytokines on each level. One of them and the best known is vascular endothelial growth factor. Investigation of VEGF was conducted by four independent scientist groups. In 1983, Senger et al. isolated a factor that increased vascular permeability. In 1988, Crisculo et al. purified a protein that increased Evans blue dye extravasation. One year later, Ferrara and Henzel published a partial amino acid sequence. In the same year, Connoly et al. showed mitogenic propriety of this protein into endothelial cells [7]. In this way, vascular endothelial growth factor was identified.

The VEGF family is responsible for new blood and lymphatic vessel creation and is composed of VEGF-A, -B, -C, -D, -E and PlGF (placental growth factor). They act via the kinase receptor system [8, 9]. The most heterogenic of the VEGF family is VEGF-A. The results of alternative RNA splicing are isoforms that contain appropriate amino acids: 121, 145, 148, 162, 165, 183, 189 and 206. The biggest expression in physical and pathological tissues is shown in $VEGF_{165}$ and is the most parallel with native VEGF. $VEGF_{121}$ is synthesized in a soluble form and it may be diffused by the cell structures. The biggest isoforms, $VEGF_{189}$ and $VEGF_{206}$, are sequestrated in an extracellular matrix. $VEGF_{165}$ possesses a medial feature: it can diffuse but it is also sequestrated. The sequestrated isoforms are susceptible to plasmin action: the results of a plasmin cut is isoform $VEGF_{110}$ with biological activity. Additionally, $VEGF_{165}$ is susceptible to metalloproteinases action (MMP-3 releases isoform $VEGF_{113}$) [8–10].

The most important moment in signal transduction is the connection of VEGF with its receptor. Actually, three VEGF receptors with tyrosine kinase activity are known, Flt-1, KDR and Flt-4, as well as the receptors from the neuropilins family. Signal transduction via VEGF activates the other molecules that include phospolipase C, thromboxane A2, protein kinase C, protein ras, protein rho or nitric oxide [6, 10]. VEGF is synthesized by endothelial cells, smooth muscles cells, macrophages, thrombocytes, fibroblasts or neutrophiles [6]. Endothelial cells show the highest KDR expression. Binding VEGF with KDR promotes the endothelial cells proliferation and its chemotaxis. Furthermore, binding VEGF with Flt-1 is necessary for endothelial cells for its differentiation. Some authors suggest that Flt-1 takes part in vascular permeability, macrophage chemotaxis or matrix metalloproteinases production by vascular smooth muscle cells [6]. Flt-4 is needed in lymphatic vessel formation.

Vascular endothelial growth factor is a strong mitogen for the cells to descend from the lymphatic and blood systems, thus it plays an essential role in angiogenesis. The processes where angiogenesis occurs are: placenta development, the reproductive cycle, embryo implantation, prenatal development, muscle remodeling after exercise-induced training, hair growth, fat deposition or wound healing [4, 7]. The formation of new blood vessels from pre-existing ones is a physiological process that takes place under specific conditions in tissues. A disturbing balance between cytokines regulates this process or creates new, different, pathological conditions that lead to excessive angiogenesis or its impairment [13].

Impaired Wound Healing in Diabetic Foot

Diabetes mellitus (DM), especially type 2 diabetes mellitus, is characterized as a pathological state with the development of late complications. Excessive blood vessel formation is observed in retinopathy or nephropathy. In these cases the walls of new blood vessels are thin and have a winding course [11, 12]. Non-treated or poorly controlled T2DM contributes to diabetic foot (DF) development. In this diabetic complication, angiogenesis is impaired, in spite of existing conditions to promote this process. Neovascularization is particularly important because of wound healing. It is observed that angiogenic potential is decreased in diabetic foot [14].

It is a fact that diabetes is becoming epidemic in industrial countries [14]. Moreover, diabetic foot is often diagnosed in patients who were not treated for diabetes or among whom diabetes was diagnosed too late. Unfortunately, patients themselves contribute to DF development by taking the medicines irregularly or having an incorrect lifestyle and inappropriate foot hygiene [15]. The most tragic moment in the therapeutic process is amputation. In 2006, the International Diabetes Federation (IDF) announced that four million diabetes foot ulcers are diagnosed annually, worldwide. About twenty-five percent of patients from this four million have an amputation. This fact shows a dire prognosis: about seventy-five percent of patients live no longer than five years after onset [16, 17].

Type 2 diabetes mellitus is almost always accompanied by atherosclerosis which increases the risk of DF. Pathological processes are induced by hyperglycemia (collagen glycation and red blood cell glycation) with oxidative stress (LDL oxidation) and endothelial dysfunction increases development of atherosclerotic plaques. Changes happen in the blood vessel wall which impair microcirculation which, in turn, creates the difficulty in oxygen and nutrient transport into tissues. It contributes to the ischemic state. If it co-exists with neuropathy, the risk of ulceration will increase [16, 18–20]. Hypoxia is the strongest angiogenesis promoter. On the other hand, angiogenesis, which takes part in wound healing, is limited. The underlying basis of the mechanism in this process is still uncertain.

There are four phases of wound healing: coagulation, inflammation, migration with proliferation and remodeling [21, 22], although some authors show only three phases: inflammation, proliferation and remodeling [23]. A wound occurs after injury in healthy people. The breakout continuity of tissues initiates a coagulation cascade. Platelet plug formation with cross-links by fibrin sets back bleeding and closes the opportunity for microorganisms to enter. Platelet activation leads to its degranulation and releases cytokines (such as TGF-β or PDGF) [23, 24] and factors that are needed for inflammatory cell recruitment and extracellular matrix (ECM) postponing. Neutrophiles and monocytes (macrophages) are the major part of the cells' inflammatory phase, plus leucocytes to a lesser extent. The first two steps of wound healing occur in a hypoxia state. This is the result of microvessel damage. Hypoxia increases proangiogenic factor expression: VEGF, TGF-β and PDGF and induces fibroblast proliferation and keratinocyte migration. Angiogenesis is accompanied by ECM production. In the last phase of wound healing, fibroblasts differentiate into myofibroblasts (synthetise actin) and keratinocytes differentiate what is possible in epithelialisation [22, 25–27].

Impaired Angiogenesis in Diabetic Foot

The latest studies show that endothelial progenitor cells (EPCs) take part in angiogenesis during wound healing. Hypoxia promotes VEGF synthesis. Signal transducers by complex VEGF-KDR located on bone marrow stromal cells increase eNOS (endothelial nitric oxide synthase) expression in bone marrow (BM). Nitric oxide (NO) production mobilizes bone marrow-derived endothelial progenitor cells into circulation [23, 24]. Therefore a question is suggested: Why do patients with diabetic foot have impaired angiogenesis despite the conditions that initiate this process? There is still no clear-cut answer, but some hypotheses are suggested.

Chronic hyperglycemia leads to advanced glycation end product (AGE) accumulation in the blood vessel wall which damages endothelium. Endothelium dysfunction is colligated with a limited amount of stromal cell-derived factor-1 alpha (SDF-1α). This is the chemokin that triggers EPCs from BM to sites of injury and aids in angiogenesis. A study conducted by Gallagher et al. demonstrates diminished SDF-1α expression in endothelial cells and myofibroblasts [24]. Moreover, EPC dysfunction is suggested in patients with type 1 and 2 diabetes mellitus [19, 23]. On the other hand, impaired eNOS activation is responsible for decreased EPC recruitment from bone marrow. NO is considered a major inductor of EPCs from BM. eNOS is expressed on the endothelial cell surface and surrounding stroma cells. Impaired eNOS activation in diabetes leads to diminished NO production and in that way EPC recruitment is reduced [23, 24].

A phenotypic change of cells isolated from ulceration and callus was also observed. Keratinocytes demonstrate hyperproliferation, incorrect differentiation and lack of migration. Fibroblasts show decreased migrating and proliferating potential [23, 24, 28]. Besides, fibroblasts isolated from nonhealing diabetic ulcers were characterized by a reduction of sensibility for growth factors [21]. Macrophages demonstrate phenotypic change by IL-1β (Interleukin-1 beta), tumor necrosis factor alpha (TNF-α) and VEGF reduced production. IL-1β is a VEGF gene activator, thus decreased synthesis of this interleukin results in decreased VEGF gene activation.

Fully-functional blood and lymphatic vessel formation needs cooperation between many cytokines. Proangiogenic factors include VEGF, acidic and basic fibroblast growth factor (aFGF and bFGF), TGF, interleukin 2 (IL-2), interleukin 6 (IL-6), insulin-like growth factor-1 (IGF-1), granulocyte colony-stimulating factor (G-SCF), granulocyte-macrophage colony stimulating factor (GM-CSF) and hepatocyte growth factor (HGF). Antiangiogenic factors are: interleukin 12 (IL-12), thrombospondin, fibronectin and endostatin [2, 13].

A literature analysis shows that in hyperglycemic conditions changes are affected by other angiogenic cytokines. In a hypoxia state VEGF gene induction in endothelial cells is supported by bFGF. Labile concentrations of VEGF and bFGF occur with normal blood vessels. When bFGF level decreases, VEGF concentration will rise, which is responsible for early angiogenic stages. VEGF level reduction is simultaneously observed when signals inducing its gene are diminished. Then, bFGF controls later steps of angiogenesis. Chronic hyperglycemia intensifies the glycation processes. AGEs can modify molecules, and bFGF too. Modifying bFGF results in a loss of almost seventy per cent of its activity to endothelial cells. Abnormal bFGF function in relation to VEGF and endothelial cells results in a disturbance of the angiogenesis process [6, 29]. Therefore abnormal bFGF properties and phenotypic changes fibroblasts, which is observed in diabetes. These characteristics contribute to decreased VEGF expression by bFGF and reduced VEGF synthesis by fibroblasts.

Decreased VEGF concentration is not the only reason for impaired wound healing in patients with diabetic foot. Some authors suggest a much faster VEGF degradation [30]. A study that has been conducted by Roth et al. demonstrates increasing isoform $VEGF_{165}$ degradation by plasmin in nonhealing ulcers [31]. The changes in diabetes also include VEGF receptors, especially its receptor Flt-1. Normal Flt-1 binds 10 times higher VEGF than KDR. Decreased Flt-1 expression in endothelial cells in nonhealing wounds has been observed [21, 31].

Diminished TGF-β1 concentration is demonstrated in the endothelium from diabetic ulcers. What is more, bFGF and IGF-1 concentrations do not increase angiogenesis. There is a suggestion that the absence of angiogenic influence on endothelial cells contributes to delaying diabetic wound healing.

In diabetic foot syndrome some paradoxes are observed. Despite existing conditions for angiogenesis induction, this process is impaired. This is the result of a toxic hyperglycemia influence on cells and cytokines involved in new blood vessel formation. Quantitative and qualitative changes of angiogenic factors, phenotypic change of cells, reduction of cell sensitivity to cytokines or disturbance of receptor signal transduction lead to impaired angiogenesis. The consequences of these are

nonhealing ulcers. Limb circulation improvement is probably the only chance to delay amputation.

For treatment, molecular biology and genetic engineering development have made gene therapy possible. In 1996, Isner et al. conducted the first plasmid transfer with a VEGF gene. It was the beginning of a VEGF study in ischemic tissue therapy [13]. The number of amputations in diabetic patients treated with VEGF gene therapy was decreased [5]. PDGF-BB treatment (becaplermin) also contributes to a reduction in amputations [21].

In 1998 Wieman et al. published "Efficacy and safety of a topical gel formulation of recombinant human platelet-derived growth factor-BB (becaplermin) in patients with chronic neuropathic diabetic ulcers", the results originating from a III phase randomized, placebo-controlled, double-blind study. They were carrying out research at 23 medical centers in the United States. Becaplermin gel (concentration 30 and 100 μg/g) and placebo gel were applied to patients with chronic diabetic neuropathic ulcers of the lower limb for 20 weeks. More effective wound healing in patients treated with a greater concentration of gel was observed. Moreover, the essential time for total healing was shorter in the group applying becaplermin gel 100 μg/g [34].

Over 10 years later, a work aggregating the results of the DF treatment with growth factors came into existence. Buchberger et al. noted in their work the results of 25 studies conducted on applying growth factors in treating chronic wounds in patients with diabetic foot. The analysis of results conducted by the authors of the present article shows that treatment with becaplermin, rhEGF (recombinant human epidermal growth factor) and Dermagraft and Apligraf skin implants contributes to faster wound healing and complete wound closure. However, the benefit from bFGF (basic fibroblast growth factor) therapy was not noted. The results reported that becaplermin therapy is cost-effective, while there are no obvious grounds for estimating the profitability of the Dermagraft and Apligraf treatments [35].

However, growth cytokine therapies do not always end successfully. Treatment that uses only VEGF can be insufficient for complete new blood vessels formation. VEGF stimulation contributes to the formation of blood vessels with increased permeability and non-differential. Therapy with more than one proangiogenic factor could be the key to therapeutic success [13, 33]. The results of growth factor therapy are promising, but there need to be more studies. Hyperbaric oxygen therapy (HBO) is the other kind of ischemic tissue therapy. In this therapy, a hyperoxia state leads to EPCs mobilizing from bone marrow into circulation. An increased EPC amount can improve the angiogenesis process [23].

Conclusions

Pathological changes in ulceration tissues are extremely difficult. Coexisting destruction processes and repair processes in tissues during wound healing exclude each other. New blood vessel formation could be the most important point in the whole treatment process. That is the direction of knowledge about angiogenic factors and angiogenesis that needs to be intensified.

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Address for correspondence:

Ewelina Drela
Department of Pathophysiology CM
Marii Skłodowskiej-Curie 9
85-094 Bydgoszcz
Poland
Tel.: 52 585 35 91
E-mail: ewelina.drela@wp.pl

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