Review paper

Chronic inflammation in venous leg ulcer – problems and perspectives

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Abstract

Chronic venous ulceration is highly arduous complication of venous insufficiency. Although its frequency in the whole population is approximately 0.5-1%, nevertheless, in individuals above the age of 65 it increases up to 5-8%. The socioeconomic analysis has revealed that venous leg ulcer deteriorates social and professional activity of individual patient, but also it becomes significant problem for public health care system, with extremely high expenses for governmental budget. Despite numerous studies focusing on presumable mechanisms of chronic venous ulcer development and healing, the current knowledge in this field is still incomplete. Recently, in addition to "mechanistic" point of view, the role of chronic inflammation and various molecular mechanisms has been extensively studied. In this review authors discuss some promising achievements and perspectives of chronic wound management.

Key words: *chronic venous ulcer, inflammation, wound healing.*

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Introduction

Chronic venous leg ulceration (CVU) is, next to the lethal pulmonary thromboembolism, the severest, and certainly the most arduous complication of the venous insufficiency. The frequency of CVU in the whole population does not exceed 0.5-1%, however, it increases significantly in older patients and reaches 5-8% in individuals above the age of 65 [1, 2]. According to statistical data, there are approximately 0.5 million individuals suffering from CVU in the USA. Among these individuals, the CVU-related work absence in employed patients group is estimated to be 6 million working days per year. Thus, in addition to other costs, the CVU-related annual budget expenses reach the level of 1.9-2.5 billion US dollars [1, 3].

Due to a pain, odorous exudate and the extremely long healing process, the chronic leg ulceration impairs business and social activity of a patient. It affects the psyche and self esteem thus significantly decreasing a patient's quality of life (4). It has been found that up to 58% of CVU-suffering patients display symptoms of depression [5]. Therefore, currently, CVU is becoming recognized as a significant public health problem with serious social and economic consequences [4, 6].

Numerous studies have focused on presumable mechanisms of CVU development. It has been found that critical conditions for the wound progression are: significant blood reflux and hypertension in venous circulation of lower extremities, with consecutive tissue hypoxia and ischemia-reperfusion injury [3, 7, 8]. Moreover, new evidences for the key role of microbial wound colonization and biofilm formation in delayed wound healing were provided due to a recent progress in microbiology, especially by the use of genetic methods for bacteria identification [9, 10]. According to current hypotheses, all circumstances mentioned above would induce and/or support the chronic inflammatory process [1, 3, 7, 8]. It may result in impaired regeneration, or even extensive tissue destruction, especially, when associated with overexpression and/or hyper-activation of matrix metalloproteinases (MMPs) [11, 12].

In this review authors focus on selected promising concepts and perspectives in a field of chronic wound management.

Bacterial colonization

The physiological microbial flora of a healthy skin is very complex and varies strongly among individuals [13].

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This normal flora may colonize wound, however, it may not necessarily influence wound healing. Several criteria have been proposed to classify a wound as infected. Apart from the common signs of infection, the number of bacterial cells above 10^5 for 1g of tissue has been proposed as an adequate clinical feature of infection. Nevertheless, this criterion is highly disputable [14]. Various types of bacteria can be found in chronic wounds, however, an active infection should be distinguished from bacterial colonization. The bacterial infection is associated with high amounts of polymorphonuclear neutrophils (PMNs) infiltrating affected tissue. PMNs release cytotoxic enzymes, free oxygen radicals and inflammatory mediators that cause extreme damage in a surrounding tissue [15]. Microscopic analysis of chronic wound specimens revealed that bacteria in this type of wound tend to form colonies coated by biofilms. Such biofilms are common in chronic wounds, however, very rarely present in acute wound specimens [16]. Pathogenic flora, which is very often found in chronic venous ulcers, includes mainly *Staphylococcus aureus* and *Pseudomonas aeruginosa* [17]. Recently, Schierle and coauthors created a murine *in vivo* model of cutaneous chronic wound and established *S. aureus* and *S. epidermidis* biofilm on it. With that model they have clearly demonstrated that bacterial biofilm causes the delay in the wound reepithelialization and thus impairs its healing [9].

It is believed that the ability to form biofilms is one of the main survival strategies of these microorganisms. The bacteria firstly form microcolonies, which then extend into larger structures surrounded by a self-made matrix of biopolymers known as exopolymeric substances (EPS) [15, 16]. EPS may be composed of proteins, lipids and polysaccharides. It may contain alginate, which enhances 3-dimensional structure of the biofilm. Furthermore, the alginate acts as scavenger of free oxygen radicals, inhibits phagocytosis as well as increases tolerance to various antimicrobial treatments [18, 19]. Bacteria living within biofilm communities are protected from immune host response. In biofilm environment PMNs surround *P. aeruginosa* microcolonies, but they cannot penetrate inside them, probably due to the presence of rhamnolipids produced by bacteria [20]. Interestingly, microorganisms living in biofilms can develop resistance to different types of antibiotics, presumably as a result of the cell-to-cell signaling, termed quorum sensing (QS) [21]. Quorum sensing is a type of process used by decentralized bacterial groups to coordinate gene expression according to the local density of their population [22]. It is noteworthy, that biofilm-producing bacterial cells, representing the most primitive form of life, compose a kind of highly specialized tissue-like structure with internal regulation and self-controlling system.

Although previous research concerning anaerobes involvement in delayed wound healing was unsuccessful, recent studies, based on molecular methods rather than traditional culture techniques revealed the presence of anaerobes in chronic wound biofilms [10]. Anaerobic species can avoid contact with oxygen colonizing the internal regions of biofilm, as the oxygen cannot penetrate the surface of biofilm deeper than microns [23]. Also the aerobic species create localized anaerobic environments by consuming oxygen [24]. The pathogenic anaerobic species found in chronic wounds are mainly gram-positive anaerobic cocci (GPAC). GPAC may interfere with the wound healing mainly by producing short-chain fatty acids. These metabolites have been shown to impair PMNs degranulation, lysozyme activity and T-cell proliferation [25].

The increasing knowledge concerning bacterial biofilm formation, as well as quorum sensing mechanisms would create an attractive opportunity to improve the effectiveness of wound healing by more effective treatment of wound infections. Therefore, the current approaches focus mainly on interference with QS signaling to get the control on bacteria metabolism and biofilm formation [26, 27].

Chronic inflammation

The blood reflux with consecutive venous hypertension results in leukocytes accumulation in the venous circulation of lower extremities. This process is known as "leukocyte trapping" [28]. In normal healthy veins circulating leukocytes express L-selectin, adhesion molecule that binds with E-selectin present on endothelial cells. This relatively loose connection allows leukocytes to "roll" along the endothelium and "examine" it more closely [29]. Both, leukocytes and endothelial cells, are activated by hemodynamic forces – mechanical stretching of the venous wall and/or pathological fluid shear stress caused by alternating laminar and turbulent flow in veins [30].

Activated leukocytes shed L-selectin to the surrounding plasma and express on their surface CD11b, an integrin family member [31]. At the same time chemokines released due to the inflammation process activate endothelial cells thus leading to an increase of their adhesion molecules expression. This increase concerns endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) [32]. These receptors act as counter ligands for various leukocyte adhesion molecules (mainly CD11b) [29], and their interaction leads to degranulation and/or extravascular migration [32]. In the experimental model, with venous hypertension induced by 30 min standing in an upright position, a significant fall of L-selectin level on leukocytes surface and increase of its soluble form in plasma was observed [31]. Interestingly, a significant decrease of mean CD11b expression on the surface of circulating leukocytes was observed in that model. That was most probably due to the fact that more activated cells were adhering to the stressed endothelium. These observations were similar in patients with varicose veins and in healthy control group. However, in normal control, but not in venous insufficiency patients group, an increase of mean CD11b receptor level on circulating leukocytes was seen after the hypertensive insult was withdrawn. The most likely

explanation is that in healthy individuals the leukocyte-endothelium adhesion is easily reversible, because there is no endothelial damage; while in patients with chronic venous insufficiency it is largely irreversible due to some pathological changes in endothelial cells and increased leukocyte activation [31]. It is plausible that key role in regulation of leukocyte-endothelial cell adhesion plays glycocalyx. It has been demonstrated that inflammation-induced endothelial cells activation results in shedding of their glycocalyx [33]. Typical endothelial glycocalyx consists of carbohydrates and glycoproteins, such as glycosaminoglycans (GAGs) and glycolipids. These molecules are much longer and larger than ICAM-1, or other adhesion molecules present on endothelial cells. It is postulated that constitutive levels of ICAM-1 are shielded by the glycocalyx from forming adhesive contact with leukocytes. The inflammatory process activates the endothelial cells thus resulting in loss of glycocalyx and its protective function [33].

Venous hypertension and mechanical stretching of the venous wall lead to the extravasation of macromolecules (i.e. fibrinogen and α2-macroglobulin) and red blood cells (RBC) to the blood vessel-surrounding connective tissue with extracellular matrix (ECM). ECM and RBC degradation products act as chemoattractants providing a chronic stimulus for inflammation and leukocyte recruitment [34]. Once leukocytes have migrated to the extracellular space, they localize around capillaries and postcapillary venules that are surrounded by extracellular matrix and create a perivascular "cuff". This "cuff" and fibrin/collagen deposition significantly decreases tissue perfusion and facilitates ischemia-reperfusion injury. This events result in further local accumulation of inflammatory factors thus allowing skin damage and chronic ulcer formation [34]. As found by Jacob and coworkers, CD68+ monocytes/ macrophages were rarely observed in normal veins but frequently in varicose veins. Only did varicose veins demonstrate CD68+ cells expressing TGF-β_1, predominantly in the adventitial tissue layer and regions of intimal fibrosis [35].

TGF-β_1 is a multifunctional cytokine that regulates a wide range of cellular functions, including proliferation, migration, differentiation and extracellular matrix components production. TGF-β_1 released by activated leukocytes stimulates increased collagen production by dermis fibroblasts. This process may be enhanced by nitric oxide (NO) produced by inducible NO synthase (iNOS), an enzyme that is activated in endothelial cells due to stimulation by different inflammatory factors [35-37]. TGF-β_1 also inhibits ECM degradation through its effects on matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), thus leading to tissue fibrosis.

Matrix metalloproteinases (MMPs) constitute a homogenous group of enzymes engaged in ECM remodeling process [38]. Despite a high level of the structure homology, biological properties and functions of various MMP family members differ significantly. Regulation of MMPs gene transcription, pro-MMP activation and/or endogenous inhibition, mainly by plasma proteins or tissue inhibitors (TIMPs), is crucial for ECM homeostasis. Therefore, any abnormalities in this regulation can lead to pathological changes in extracellular space, which have been demonstrated in numerous studies [39-41].

TGF-β_1 regulates the production of some MMPs at the level of gene transcription through the promoter containing a TGF-β_1 inhibitory element (TIE) [41, 42]. TIE was first discovered in MMP-3 gene, and then it has been found in MMP-1 and MMP-9 genes. Binding of the TGF-β_1 to its receptor activates a Smad-dependent signaling pathway, leading to suppression of MMP gene transcription via TIE. Since neither mutation, nor deletion of TIE in MMP-9 gene stopped the inhibitory effect of TGF-β_1 on MMP-9 transcription, it was postulated that TIE is not necessary for inhibitory effect of TGF-β_1. Further analysis has revealed that TGF-β_1 may suppress the MMP-9 transcription via NF-κB site in the promoter of MMP-9 gene [42].

Surprisingly, Saito and coworkers have shown that MMP-1 and TIMP-1 protein levels in patients with different stages of chronic venous insufficiency were not significantly different from controls [41]. It suggests that not only the regulation of transcription, but various post-translational modifications may be crucial in regulation of synthesis and activity of MMPs. On the other hand, high TGF-β_1 levels are probably responsible for the increased levels of MMP-2, found in chronic venous leg ulcers [43]. Unlike the other MMPs, the MMP-2 promoter lacks the TGF-β_1 inhibitory element. Furthermore, TGF-β_1 increases pro-MMP-2 gene expression. Leukocytes that migrated to the extracellular space, as well as fibroblasts stimulated by TGF-β_1 secrete pathological amounts of MMP-2. Its hyperactivity may contribute to impaired ulcer healing by basement membrane degradation [41].

MMP-9 (gelatinase B, or type IV collagenase) is a member of MMPs family. Due to its proteolytic activity against type IV collagen, MMP-9 plays an important role in normal wound healing, especially in remodeling and re-epithelialization of the wound. However, its increased activity can seriously impair wound healing [44]. MMP-9 may be produced by different cell types, in chronic wounds mainly by neutrophils and macrophages. Recent studies have shown that increased MMP-9 activity correlates with the severity of the ulcer [44, 45]. Furthermore, it has been observed that during the healing process levels of MMP-9 in wound fluids decrease to the levels observed in acute wounds [46].

Considering the pivotal role of pro-inflammatory molecules (TGF-β_1, some other cytokines, MMPs, etc.) in chronic wound development and healing one can speculate, that at least some of them could be very attractive targets for molecular treatment approaches. The current arsenal may include synthetic ligands with stimulatory or inhibitory activity, activity-modulating antibodies, as well as gene expression-regulating agents, including antisense oligonucleotides, or RNA interference technology [47].

Extracellular matrix hyaluronan

Synthesis and degradation of extracellular matrix components is a hallmark of tissue injury and repair. A molecule that seems to play an extraordinary role in these processes is hyaluronan/hyaluronic acid (HA) [48]. HA is a glicosaminoglycan present in big amounts in synovial fluid, an eye, cartilage and skin. It is composed of repeating polymeric disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. The number of disaccharide units can reach 10,000 or more. A weight of single molecule of HA reaches approximately 4.0×10^6 Da. It has been found, that in course of inflammation the hyaluronan turnover significantly increases and lower-molecular-weight forms of HA can be found in wound environment [49, 50]. Their appearance is the effect of the activity of different enzymes, especially hyaluronidases [51], reactive oxygen forms [52] and a mechanical damage of high-molecular-weight forms of HA [53]. These small HA fragments seem to have different properties and functions than high-molecular-weight HA. It is postulated that lower-molecular-weight forms of HA accumulate at the site of tissue injury and can stimulate the production of inflammatory mediators, such as chemokines and cytokines, by different types of inflammatory cells which results in a state of unremitting inflammation [54]. Recent studies has shown that small hyaluronan fragments act as ligands for Toll-like receptors (TLRs), which are the main receptors of the innate immune response [55]. The interaction between low-molecular-weight HA and TLR4 can lead to transduction of signal that initiates the pro-inflammatory cascade [56]. According to Fieber and coauthors, low-molecular-weight HA fragments are able to enhance the expression of MMP-9 gene via NF-κB activiation [57]. In contrary to low-molecular-weight fragments, native high-molecular-weight hyaluronan has an anti-inflammatory and immunosuppressive properties. It has been proved that high-molecular-weight HA inhibits phagocytic activity of macrophages and PMN [58], as well as the activity of NF-κB [59]. The last activity could prevent the transcription of numerous pro-inflammatory cytokines and inducible NO synthase (iNOS). Possibly, large HA polymers could also play a role of mechanical barrier for the mediators of inflammation [60].

Summary

Despite numerous studies focusing on chronic venous leg ulcer pathophysiology, the current knowledge concerning this subject remains still incomplete. However, recent progress in that field allows us to expect, that at least some scientific achievements will very soon be introduced into the clinics to further increase the effectiveness of chronic venous leg ulceration treatment.

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