Original paper

Overexpression of cathepsin K and vascular endothelial growth factor in chronic venous ulcerations

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Abstract

Introduction: Chronic venous disease (CVD) is a disabling condition affecting about 1% to 3% of the general population. Besides varicose veins, CVD can result also in the formation of severe skin lesions, especially venous ulcerations (VU). The exact mechanism of VU is still unknown.

Aim: To evaluate immunoexpression of vascular endothelial growth factor (VEGF) and cathepsin K in healthy individuals and patients with VU.

Material and methods: The study included 12 patients with venous ulcers and 10 healthy individuals who served as controls; both groups were sex- and age-matched. Biopsy samples were obtained from lower leg areas and submitted to histochemical analysis.

Results: There was a significant difference between the study group and the control group in cathepsin K expression (1.007 ±0.3 vs. 0.22 ±0.2, respectively, $p < 0.001$) and VEGF expression (1.17 ±0.59 vs. 0.27 ±0.19, respectively, $p < 0.001$). Additionally, the microvessel density (per mm^2) differed significantly between the study group and the control group (97.6 ±28.81 vs. 59.32 ±12.71, respectively, $p < 0.001$). We found no correlation between cathepsin K and microvessel density, and cathepsin K and VEGF in both groups, but there was a significant correlation between microvessel density and VEGF immunoexpression in the study group ($r = 0.82$, $p = 0.002$).

Conclusions: Increased immunoexpression of VEGF and cathepsin K suggests that both of these proteins may play a role in VU development.

Key words: venous ulceration, immunohistochemistry, cathepsin K.

Introduction

Chronic venous disease (CVD) is a disabling condition affecting about 1% to 3% of the general population [1]. Besides varicose veins, CVD can result also in formation of severe skin lesions, especially venous ulcerations (VU). Venous ulcerations are considered as one of the most common consequences of CVD with a prevalence of about 0.3% of the adult population [2] thus making it a problem for healthcare providers. Prognosis for the patients with venous ulcerations is usually poor due to the issues with its recurrence, delayed healing and superinfections. Additionally the socioeconomic impact of VU seems to be destructing because the patients are unable to engage in occupational and social activities, which results in decreased quality of life, social isolation and loss of self-esteem and may eventually lead to anxiety and depression [3]. Taking the socioeconomic impact and the high prevalence of VU into account, an understanding of the underlying mechanism of VU is highly anticipated. In recent years a lot of risk factors for VU/CVD have been described however the exact pathomechanism, especially the role of angiogenesis and proteolysis has not been evaluated thoroughly. The disturbance of homeostasis between deposition and synthesis of extracellular matrix proteins is one of the key points in terms of VU development [4]. Recent studies have shown that VU progression is highly correlated with the presence and expression of active matrix metalloproteinases (MMPs) which are deranging extracellular

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matrix (ECM) properties [5]. MMPs are produced and secreted by several cells (i.e. keratinocytes, leukocytes, fibroblasts) and are implicated in excessive ECM degradation in VU that is resistant to heal. However some recent research has suggested that aside from MMPs there may be another protein involved in VU development, cathepsin K, which is emerging as an important regulator of wound microenvironment. Cathepsin K is a member of the cysteine protease family, which is responsible for maintaining the homeostasis of extracellular matrix. So far cathepsin K is one of the most potent elastases described in the literature [6]. It is well known that cathepsin K is expressed predominantly in osteoclasts and is involved in bone resorption and remodelling. Cathepsin K mutations in humans lead to pycnodysostosis, a disease characterized by abnormally dense bones [7] (osteopetrosis), but it seems that the role of cathepsin is not limited to bones only. Recent research has provided some new discoveries about the role of cathepsin K in progression of several tumours including malignant melanoma [8], basal cell carcinoma [9] and glioblastoma [10], lung fibrosis [11], lymphangioleiomyomatosis [12], cardiac hypertrophy [13] and coronary artery disease [14]. The main feature of cathepsin K is ability to degrade collagen I, elastin and gelatine, thus it is also involved in lung elasticity loss and recoil in emphysema [15]. Yet, there is still lacking information about the role of cathepsin K in the skin. It is suggested that cathepsin K is involved in skin aging and photodestruction [16, 17], scar formation [18] and development of psoriasis-like lesions [19]. Taking those facts into consideration we are hypothesizing that cathepsin K, due to its collagen-degrading ability, may also play a role in venous ulcer development.

On the other hand, it has been proven that abnormal microcirculation in patients with CVD is correlated with an increased expression of vascular endothelial growth factor (VEGF) [20]. VEGF is both a potent chemotactic agent and cell mitogen as well as an inductor of vascular permeability [21]. VEGF stimulates tumour growth in breast, colon and non-small cell lung cancers [21], controls angiogenesis in healing wounds [22] and is one of the major regulators in diabetic retinopathy [23]. Until now the role of VEGF in the development of VU has been well established, however there are still some gaps in current knowledge including the relation between cathepsin K and VEGF, and the exact impact of VEGF on treatment and prognosis. It is known that inflammation, which is present at VU sites can create hypoxia in tissue due to elevated metabolic activity and increased demand for oxygen. Low oxygen levels could activate hypoxia inducible factors (HIFs) and eventually VEGF which are orchestrating the process of angiogenesis. On the other hand there is evidence showing that hypoxemic injury leads to cathepsin K dependent Notch1 activation, which is stimulating the process of angiogenesis [24]. According to these findings, it seems that cathepsin K may regulate development of venous ulcers.

Aim

The aim of the study was to assess the expression of cathepsin K and vascular endothelial growth factor in chronic venous ulcerations.

Material and methods

Study design

The study included 12 patients with venous ulcers and 10 healthy individuals who served as controls; both groups were sex- and age-matched (mean age: 73 ±4.9, 6 males, 6 females vs. 71.9 ±4.44, 5 males, 5 females). All patients have the ulceration in the same site (left medial malleolus) and similar area affected. The mean duration of disease was 4.22 ±3.52 years and all skin lesions were classified according to CEAP (clinical signs, aetiology, anatomic distribution, pathophysiological dysfunction) classification as C6 E_C $A_{S,D}$ $P_{R,O}$ (skin changes with active ulceration, known secondary aetiology, superficial and deep veins affected with reflux and obturation). All patients were referred from the surgery department with a confirmed diagnosis of CVU. The study was approved by the local Ethics Committee and conducted according to the Declaration of Helsinki principles. All study subjects signed an informed consent form before enrolment into the study. Biopsy samples were obtained from lower leg areas (left medial malleolus) and submitted to histochemical analysis. 4-mm punch biopsies were taken from the border between unchanged skin and ulceration in local anaesthesia.

Immunohistochemistry

Paraffin-embedded, 3-µm tissue sections were mounted onto SuperFrost slides, deparaffinised in xylene and ethanol of graded concentrations. For antigen retrieval, the slides were treated in a microwave oven in a solution of TRS (Target Retrieval Solution, High pH, Dako, Denmark) for 30 min (2 × 6 min 360W, 2 × 5 180W, 2 × 4 min 90 W). After cooling down at room temperature, they were transferred to 0.3% hydrogen peroxide in methanol, for 30 min, to block endogenous peroxidase activities. Sections were rinsed with Tris-buffered saline (TBS, Dako, Denmark) and incubated with rabbit primary antibodies against: cathepsin K (Abcam, UK; ab 19027, dilution 1 : 200), and with mouse monoclonal antibody against VEGF (Dako, Denmark, clone VG1, dilution 1 : 300), CD34 (Dako, Denmark, clone QBend 10, dilution 1 : 50). Immunoreactive proteins were visualized using adequate EnVision-HRP kit (Dako, Carpinteria, CA, USA) according to the instructions of the manufacturer. Visualisation was performed by incubation of the sections in a solution of 3,3'-diaminobenzidine

(Dako, Denmark). After washing, the sections were counterstained with Mayer's haematoxylin and mounted.

For each antibody and for each sample, a negative control was processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

Semiquantitative evaluation

In each specimen, staining intensity of VEGF and cathepsin K was recorded semiquantitatively by two independent observers in 5–7 adjacent high power fields and graded from 0 (staining not detectable), 1 (weak immunostaining), 2 (moderate immunostaining) and 3 (strong staining). The mean grade was calculated by averaging grades assigned by the two authors and approximating the arithmetical mean to the nearest unit.

Morphometry

Histological morphometry was performed by means of the image analysis system consisting of a PC computer equipped with a Pentagram graphical tablet, Indeo Fast card (frame grabber, true-colour, real-time), produced by Indeo (Taiwan), and colour Panasonic (Japan) TV camera coupled to a Carl Zeiss microscope (Germany). This system was programmed (MultiScan 8.08 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects (automatic function with manual correction, as needed). The coloured microscopic images were saved serially in the memory of a computer, and then quantitative examinations were carried out. CD34 immunostaining was evaluated in the vessels with lumen only (not in the individual cells), in the most vascular areas. The microvessel density was measured in ten monitor fields (0.0205 mm^2 each), and results were expressed as the number of the microvessels per mm^2.

Statistical analysis

All values were expressed as the mean ± SD (standard deviation). The differences between groups were tested using Student *t*-test for independent samples preceded by evaluation of normality and homogeneity of variances with Levene's test. Additionally the Mann-Whitney *U* test was used where appropriate. Correlation coefficients were calculated using Spearman's method. Results were considered statistically significant if $p < 0.05$.

Results

In the study group and controls the cytoplasmic staining of cathepsin K in dermal fibroblasts, inflammatory infiltrates and some keratinocytes was observed (Figure 1), however in controls this immunoexpression was very weak (Figure 2). The VEGF protein was detected in epithelial and endothelial cells as well as in some cases on inflammatory infiltrates (Figure 3). In controls the immunoexpression of VEGF protein was found in keratinocytes (Figure 4) of normal epidermis and in endothelial cells. There was a significant difference between the study group and the control group in cathepsin K expression (1.007 ±0.3 vs. 0.22 ±0.2, respectively, $p < 0.001$) and VEGF expression (1.17 ±0.59 vs. 0.27 ±0.19, respectively, $p < 0.001$). Additionally, the microvessel density (per mm^2) differed significantly between the study group and the control group (97.6 ±28.81 vs. 59.32 ±12.71, respectively, $p < 0.001$). We found no correlation between cathepsin K and microvessel density, and cathepsin K and VEGF in both groups, but there was a significant correlation between microvessel density and VEGF immunoexpression

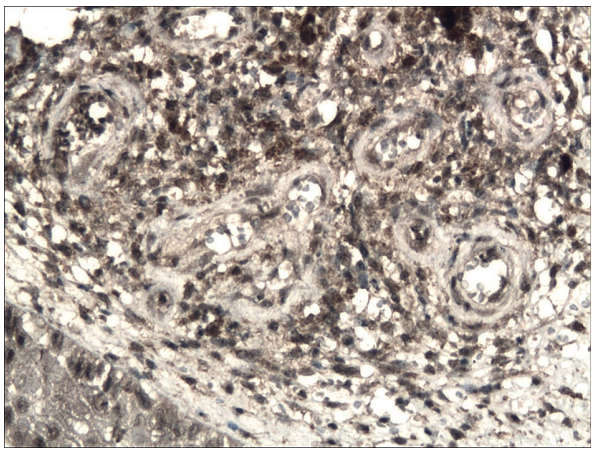

Figure 1. Cytoplasmic staining of cathepsin K in dermal fibroblasts, inflammatory infiltrates in the patient from the study group. The immunohistochemical staining was performed as described in the Material and methods section. Magnification 200×

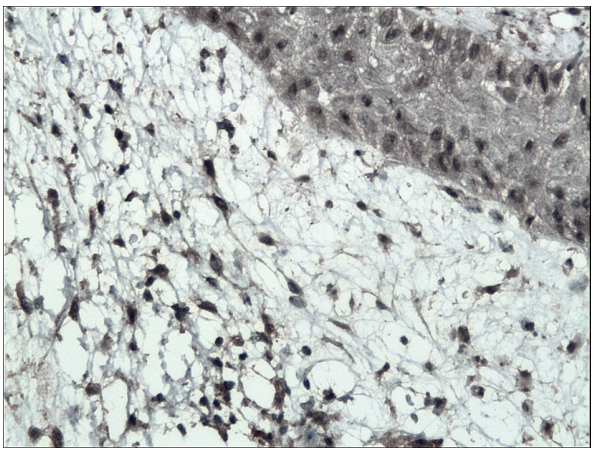

Figure 2. Weak immunoexpression of cathepsin K in the control patient. The immunohistochemical staining was performed as described in the Material and methods section. Magnification 200×

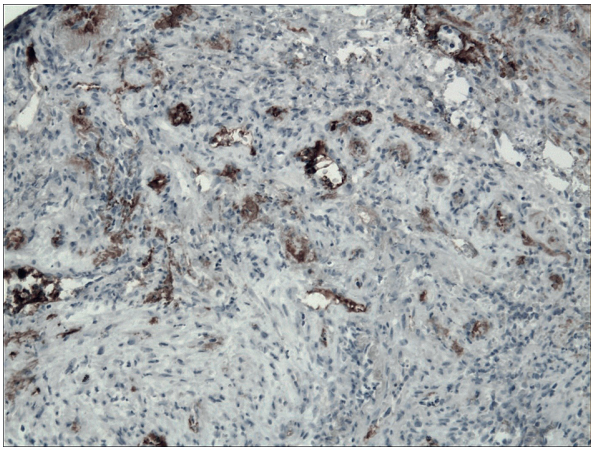

Figure 3. The VEGF protein immunoexpression in epithelial and endothelial cells as well as in some inflammatory infiltrates. The immunohistochemical staining was performed as described in the Material and methods section. Magnification 200×

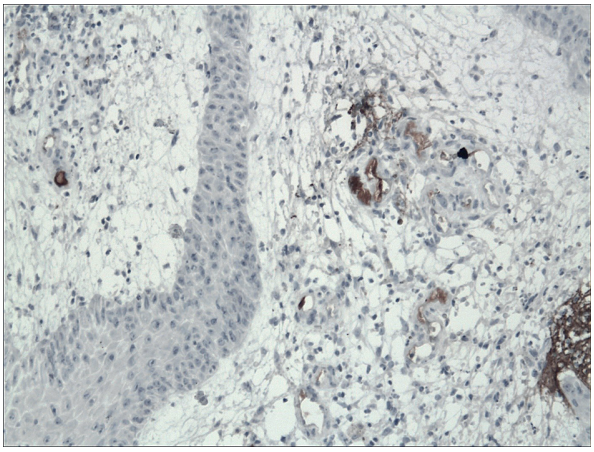

Figure 4. In controls the immunoexpression of VEGF protein was found in keratinocytes (of the normal epidermis) and in endothelial cells. The immunohistochemical staining was performed as described in the Material and methods section. Magnification 200×

Table 1. Comparison of semiquantitative and quantitative data of VEGF and cathepsin K immunoexpression as well as microvessel density in the study group and controls

Parameter	Study group (n = 12)	Controls (n = 10)	P-values
Cathepsin K (mean score)	1.007 ±0.30	0.22 ±0.20	< 0.001
VEGF (mean score)	1.17 ±0.59	0.27 ±0.19	< 0.001
Microvessel density (per mm^2)	97.6 ±28.81	59.32 ±12.71	< 0.001

Table 2. Correlation between selected parameters in the study group and controls

Pair of variables	Study group (n = 12)	Control group (n = 10)
Microvessel density vs. VEGF	r = 0.82, p < 0.002	r = 0.53, p = 0.11 (NS)
Microvessel density vs. cathepsin K	r = 0.06, p = 0.84 (NS)	r = –0.14, p = 0.69 (NS)
VEGF vs. cathepsin K	r = 0.34, p = 0.26 (NS)	r = 0.11, p = 0.76 (NS)

in the study group (r = 0.82, p = 0.002). Detailed results are shown in Tables 1 and 2.

Discussion

Cathepsins belong to the cysteine protease family and are playing various roles in extracellular matrix turnover [25]. So far involvement of cathepsins has been described in lung parenchyma homeostasis and in bone resorption, however their role in the skin still remains elusive. For the time being the research is focused on cathepsins B, D and G, but data on cathepsin K are unequivocal. It has been proven that cathepsin K is one of the critical regulators of extracellular matrix homeostasis ensuring balance between ECM protein synthesis and lysis [25]. It should be also taken into account that cathepsin K may play a role in carcinogenesis and metastasis of several tumours including oral squamous cell carcinoma [26] and neovascularization in response to hypoxia [24]. Expression of cathepsin K is elevated in damaged skin. In the current study, we found that cathepsin K was upregulated in the venous ulceration lesions compared with normal control skin. In the physiologic state any disruption of the dermis could start the wound healing process. During wound healing the equilibrium between synthesis and degradation of extracellular matrix proteins is shifted to the profibrotic state. Rünger *et al.* [25] found that proteolytic activity of cathepsin K helps in rebalancing the profibrotic state and affects scar remodelling. Furthermore, in undamaged skin the expression of cathepsin K is somewhat weak, however any dermal damage stimulates its expression in dermal fibroblasts [25]. Sage *et al.* [16] studied the relationship between age and cathepsin K expression in Caucasian women and concluded that photoprotection leads to decreased abundance of cathepsin K in the skin in comparison to unprotected females. In one of our previous studies [17] we found that exposure to ultraviolet radiation leads to an increased expression of cathepsin K suggesting that even low doses of UVR may be sufficient to start ECM remodelling and also may be leading to skin damage. Thus far the role of cathepsin K in development of venous ulcers has not been studied extensively. Quintanilla-Dieck *et al.* [27] investigated possible pathways that regulate cathepsin K

expression. They found that cathepsin K activity may be induced by interleukin-1α which is also abundant in the wound fluid from venous leg ulcers [28].

Although lacking statistical significance, a weak correlation between VEGF and cathepsin K expression may suggest that there is a possible relationship existing, however this hypothesis has several limitations; our study was performed on a small group and we did not analyse the expression of the proteins using more sophisticated methods. Interaction between VEGF and cathepsin may create a positive-feedback loop which could be responsible for some treatment failures in venous ulcers therapy, however this fragile relation has to be investigated in further *in-vitro* studies. An increased expression of VEGF in venous ulcer lesions is not surprising at all. It is well established that venous ulcers in patients with CVD are a result of disrupted microcirculation. This microcirculatory dysfunction eventually leads to hypoxia, which is one of major stimulators of VEGF expression. In that case it is important to determine the exact role of VEGF in venous ulcers formation. Hypoxia in VU wounds results in stimulating both cathepsin K-mediated Notch1 activation and VEGF activation, eventually leading to increased neoangiogenesis. In our study we found a very strong correlation between microvessel density and VEGF expression which supports those theories.

Additionally, an increased VEGF expression has been reported in non-healing ulcers suggesting that VEGF may disturb wound healing [29]. The possible explanation of that phenomenon was proposed by Birkenhauer and Neethirajan [30] by studying Pseudomonas aeruginosa chemoattraction by VEGF. They found that VEGF may attract opportunistic pathogens thus resulting in impaired healing.

There is still a controversy regarding the use of VEGF as a prognostic factor in venous ulcers treatment. Shoab *et al.* [31] suggested that VEGF could be employed as such based on observation of serum VEGF in correlation with dermatological signs of venous ulcerations. Opposite results were obtained by Gohel *et al.* [32] who performed an interesting study regarding VEGF serum concentration in venous ulcers. They found that VEGF correlates negatively with the patients' age, however it does not correlate with the ulcer size. They also found no correlation between serum levels of VEGF and ulcer healing which implicates that VEGF should not be considered as a marker of therapeutic response. Study findings are limited due to a small sample size, however to maintain population homogeneity we decided to include only the patients with the same location of the lesion.

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Conflict of interest

The authors declare no conflict of interest.

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