ARTICLE — CLINICAL STUDY

Inflammatory mechanisms involving neutrophils in chronic venous insufficiency of lower limbs

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Abstract

Background: **It is supposed that an inflammatory reaction is one of the major factors responsible for the chronic venous insufficiency (CVI) of lower limbs which cause leg ulcers.**
Obj*ectives:* **The main objective of the present study was to determine the differences in the levels of typical inflammatory mediators and markers produced by neutrophils of patients with CVI and normal control subjects.**
Subjects and methods: **26 patients with CVI and 39 clinically healthy subjects were included in the study. In peripheral neutrophils of both groups the production of superoxide, total reactive oxygen intermediates and activities of lysosomal enzymes were measured together with the expression of 8 adhesion molecules.**
Results: **Increased formation of superoxide by patient neutrophils and activities of elastase in both neutrophils and serum of patients were demonstrated. On the contrary, activities of myeloperoxidase and beta-D-glucuronidase were decreased in patient neutrophils. Comparing to control group adhesion molecules CD11b, CD18, CD31, CD49d, CD54 and CD62L were increased on the surface of patient neutrophils whereas no differences were observed in the expression of CD11a abd CD15.**
C*onclusion:* **The neutrophils of patients with CVI are primed and/or activated because they are able to release higher amount of superoxide, lysosomal enzymes and express elevated number of adhesion molecules. It may serve as one of the important evidences of an inflammatory mechanism imvolved in the pathogenesis of chronic venous insufficiency.** ***(Tab. 3, Ref. 27.)***
Key words: neutrophils, superoxide, lysosomal enzymes, elastase, adhesion molecules.

Neutrophils are important professional phagocytes that provide the host with a first line of defense against acute bacterial and fungal diseases, but they are also pivotal effector cells in numerous inflammatory conditions. To fulfill the defensive role, intravascular neutrophils need to sense the focus of infection, slow down and adhere to the endothelium of capillaries and venules adjacent to the inflammatory focus, migrate to the infectious site, phagocytose, kill and digest the invading microorganisms. Although destruction of infectious agents occur intracellularly, release of cytotoxic molecules into the extracellular milieu can damage body tissues. There is several lines of evidence that the inappropriate neutrophil-mediated tissue damage is involved in the pathogenesis of conditions such as acute respiratory distress syndrome, septicemia with multiorgan failure, ischemia-reperfusion injury and rheumatoid arthritis.

Neutrophils are heterogenous. Subpopulations exist in various stages from dormant to primed to fully activated. The most cytotoxic substances are released by activated neutrophils. This process is regulated locally in microenvironments and systemically by a plethora of mediators including cytokines, chemotactic factors, bioactive lipids, adhesion molecules, reactive oxygen and nitrogen intermediates and neuroendocrine hormones (Smith, 1994; Ainsworth et al., 1996; Ferenčík et al., 1997, 2001).

The chronic venous insufficiency (CVI) of lower limbs is one of the most prevalent chronic conditions in the industrialized countries (Štvrtinová et al., 1991). In spite of the fact, the underlying cause of CVI is not precisely known. CVI is characterized by dysfunctional, incompetent venous valves and venous hypertension

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with significant consequences to the small venules and capillaries. In the last ten years it became more and more clear, that white blood cells may play an essential role in the etiopathogenesis of CVI. It is supposed that the main cause of CVI is a derangement of venous function, which leads to the increased ambulatory venous pressure. It causes a reduction in capillary perfusion pressure and hence the capillary flow rate, resulting in trapping of the white blood cells in the leg (*trapping hypothesis* — Coleridge Smith et al., 1988). These cells may then cause plugging of the capillaries, resulting in areas of localized ischemia, and become activated, releasing toxic oxygen metabolites, proteolytic and other lysosomal enzymes, chemotactic substances and other mediators of inflammation (Shields and Saharay, 1998; Coleridge Smith, 1999 a, b).

The purpose of this study was to verify whether the typical markers of neutrophil inflammatory activation, as are superoxide production, lysosomal enzyme release and expression of adhesion molecules, are elevated in a group of patients with CVI comparing to a group of control subjects.

Subjects and methods

The *patient group* comprised 26 patients (10 men, 16 women) with a mean age of 52.4 years (range 33—79) with primary varicose veins in clinical stages 2, 3 and 4 according to the CEAP classification (Beebe et al., 1995). Analysis of adhesion molecules was performed only in 18 of them (6 men and 12 women; mean age of 50.8, range 32—68 years).

The *control group* consisted of 39 healthy subjects (18 men and 21 woman) with a mean age of 49.3 years (range 29—76). Adhesion molecules on granulocytes were analysed only in 29 of them (13 men and 16 women) with a mean age of 47.4 years (range 31—70).

All patients and control subjects were investigated with Doppler ultrasound and digital photoplethysmography (Blažek and Schulz-Ehrenburg, 1996). In none of them damage of arterial system was found (ankle-brachial index >0.9) or any other condition which might result in leucocyte activation, including diabetes, connective tisue disorders, infection within the previous six weeks or any medication known to alter white cell activity. The mean value of venous refilling time T_0 measured by digital photoplethysmography in the patient group was 17.9 sec and in the group of healthy volunteers (control subjects) was 32.1.

Neutrophils for the determination of toxic oxygen intermediates and lysososomal enzyme activities were separated from heparin (20 U/ml) anticoagulated blood with the use of Dextran T-500 (Pharmacia, Uppsala). Contaminating red blood cells were removed by hypotonic lysis. Differential cell counts with Wright-Giemsa stain showed that at least 95 % of the cells were neutrophils.

Toxic oxygen intermediates were measured in the final suspension of neutrophils (10^7/ml) in phosphate-buffered saline (pH 7.4) containing 5 U/ml heparin and 0.1 % glucose as the reduction of 3-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT, Sigma, St. Louis) and/or superoxide production. The ability to reduce INT was assayed by determining the colored formazan at 485 nm after incubation of pure neutrophils or neutrophils with zymosan opsonized by autologous serum (ZO) and/or phorbol-12-myristate-13-acetate (PMA, Sigma) on microplates (Ferenčík et al., 1988). The production of superoxide was also estimated on the principle of INT reduction to formazan by neutrophils incubated in the presence and in the absence of superoxide dismutase (Sigma). Both INT reduction and superoxide production were expressed as fmol of formazan released by a single neutrophil during 30 min incubation with INT.

Total *activities of lysosomal enzymes* were estimated on microplates in the presence of 0.1 % Triton X-100 using suspensions of neutrophils (10^7 cells/ml) obtained from individual subjects.

Elastase activity was determined spectrophotometrically at 410 nm with the use of N-succinyl-(L-alanyl)$_3$-p-nitroanilide (Štvrtinová and Ferenčíková, 1992). It was expressed as pcat per 10^6 neutrophils or per one ml serum.

Lysozyme was also assayed spectrophotometrically by the lysis of *Micrococcus lysodeicticus* (lysozyme substrate, Difco). The activity was expressed as µg per 10^6 neutrophils or per one ml serum (Štvrtinová and Ferenčíková, 1992).

Determination of *myeloperoxidase* (MPO) activity was made by the o-dianisidine method (Ferenčík et al., 1982) and the activity was calculated as ncat per 10^6 neutrophils.

Beta-D-glucuronidase activity was measured using phenolphtalein glucuronide (Sigma) as substrate. Data are expressed as pcat per 10^6 neutrophils.

Adhesion molecules CD11a, CD11b, CD15, CD18, CD31, CD49d, CD54, CD62L were quantified by flow cytometry with the aid of monoclonal antibodies conjugated to fluorescein isotiocyanate (FITC) or phycoerythrin (PE) from Becton Dickinson. Ten microliters of FITC-conjugated or 10 µl of phycoerythrin conjugated antibodies were added to 50 µl of whole blood and incubated for 15 minutes at room temperature. Then 250 µl of lysing solution OptiLyse C was added and incubated for 10 min. After lysing of red blood cells, 250 µl of PBS was added followed by brief vortex mixing. After 20 min preparation, the cells were analysed by using an COULTER EPICS XL cytometer. Granulocytes were separated on the basis of their forward and side scatter pattern. The cells were gated using forward versus side scatter to exclude dead cells and debris. Non-specific immunofluorescence was determining using isotype-control.

Statistical analysis. All values obtained in this study are expressed as means ±SEM. The data were analyzed using one-way analysis of variance (ANOVA). Statistical analysis of changes in the expression of adhesion molecules was done using Statgraph version 5. Normality was verified using the Kolmogorov-Smirnov test. Data were compared using one way and multifactorial analysis of variance (MANOVA) for significant differences between groups.

Results

Superoxide production and INT reduction by peripheral neutrophils of patients with chronic venous insufficeincy of lower limbs (uncomplicated varicose veins) are in Table 1. The results are expressed as the ratio of superoxide production or INT reduction by neutrophils incubated in the presence of opsonized zymosan (ZO) or phorbol myristate acetate (PMA) and without these stimulatory agents (C — controls). Comparing control subjects and patients with CVI, significantly increased values were obtained only in the production of superoxide by neutrophils isolated from patients after

their *in vitro* stimulation by both ZO and PMA and no differences were found out in the INT reduction.

Activities of lysosomal enzymes in peripheral neutrophils and serum of patients and control subjects are shown in Table 2. Elastase, lysozyme, myeloperoxidase (MPO) and beta-D-glucuronidase (BDG) activities were determined in neutrophils whereas those of elastase and lysozyme also in serum. Mean activities of elastase were elevated in patient neutrophils and serum. In the case of lysozyme, increased mean values were determined only in the patient serum. On the other hand the mean activities of MPO and BDG were decreased in neutrophils of patients with CVI.

The mean values of eight adhesion molecules on the surface of granulocytes of patients with CVI and control subjects are reported in Table 3. The results represent the means ±SEM of cell fluorescence in arbitrary units. In comparison to control subjects, significantly increased expression of CD11b, CD18, CD31, CD54, CD49d, and CD62L were ascertained in patients with CVI. No differences were determined in the expression of CD11a and CD15.

Discussion

Neutrophil margination is a normal event in the microcirculation, occuring most frequently in the post-capillary venules. This phenomenon is thought to be also important in many pathophysiological vascular events including CVI (Sullivan et al., 2000). In this case neutrophils, particularly those attached to capillary endothelium, become primed or activated. The mechanism of priming and activation is poorly defined but it is widely accepted that the optimal activation of both neutrophils (Coffer and Koenderman, 1997) and endothelial cells (Štvrtinová et al., 1998) by different inflammatory mediators requires priming by chemotactic factors or cytokines (Peschen, 1999; Koenderman et al., 2000).

Only activated and/or primed neutrophils are able to fulfil their role of regulatory and effector cells in inflammatory processes. This is orchestrated by the activity of changing series of released and displayed mediators. They include cytokines, tissue-destructive proteases, lysosomal enzymes, reactive oxygen intermediates released during a non-mitochondrial respiratory burst and increased expression of adhesion molecules on neutrophils and underlying vascular endothelium.

Our results shown in Table 1 demonstrate that the neutrophils of patients with noncomplicated varicose veins have an increased capability to produce superoxide after both corpuscular (ZO) and soluble (PMA) stimuli. Therefore it is possible to conclude that these neutrophils are activated or at least primed by an inflamma-

Tab. 1. Superoxide production and INT reduction by peripheral neutrophils of patients with chronic venous insufficiency (CVI) of lower limbs and control subjects.
Tab. 1. Produkcia superoxidu a redukcia INT periférnymi neutrofilmi pacientov s chronickou venóznou insuficienciou (CHVI) dolných končatín a kontrolných osôb.

Parameter		Patients with CVI Pacienti s CHVI	Control subjects Kontrolné osoby
Number of investigated Počet vyšetrených		26	39
Superoxide production Produkcia superoxidu	— ZO/C — PMA/C	11.23±0.43* 11.91±0.45*	9.42±0.24 10.18±0.27
INT reduction Redukcia INT	— ZO/C — PMA/C	7.83±0.30 7.71±0.27	7.40±0.19 7.23±0.21

Data are calculated as mean values±SEM, *p<0.05.
Údaje sú vyjadrené ako priemerné hodnoty±stredná chyba priemeru.

Tab. 2. Activities of lysosomal enzymes in peripheral neutrophils and serum of patients with CVI of lower limbs and control subjects.
Tab. 2. Aktivity lyzozómových enzýmov v periférnych neutrofiloch a sére pacientov s CHVI dolných končatín a kontrolných osôb.

Parameter	Patients with CVI Pacienti s CHVI	Control subjects Kontrolné osoby
Number of investigated Počet vyšetrených	26	39
Elastase — neutrophils Elastáza — serum	71.8±2.76* 29.3±1.13**	54.3±1.40 21.4±0.55
Lysozyme — neutrophils Lyzozým — serum	0.48±0.02 8.66±0.33*	0.43±0.02 7.21±0.18
Myeloperoxidase — neutrophils Myeloperoxidáza — neutrofily	1.67±0.06*	1.96±0.05
Beta-D-glucuronidase Beta-D-glukuronidáza — neutrophils	5.19±0.20**	6.36±0.16

Data are calculated as mean values±SEM, *p<0.O5, **p<0.01.
Údaje sú vyjadrené ako priemerné hodnoty±stredná chyba priemeru.

Tab. 3. Adhesion molecules on peripheral granulocytes of patients with CVI of lower limbs and control subjects.
Tab. 3. Adhezívne molekuly na periférnych granulocytoch pacientov s CHVI a kontrolných osôb.

Parameter	Patients with CVI Pacienti s CHVI	Control subjects Kontrolné osoby
Number of investigated Počet vyšetrených	18	29
Adhesion molecules:		
CD11a ($ß_2$-integrin α-chain)	3.66±0.81	3.56±0.98
CD11b ($ß_2$-integrin α-chain)	99.90±0.11***	77.70±2.96
CD18 ($ß_2$-integrin ß-chain)	97.95±1.01**	88.75±2.07
CD15 (Lewis' antigen X)	95.44±3.26	98.28±2.02
CD31 (PECAM-1)	62.84±4.11*	51.21±4.69
CD54 (ICAM-1)	0.53±0.06**	0.18±0.02
CD49d (α-chain of VLA-4)	1.77±0.08***	0.58±0.02
CD62L (L-selectin)	42.95±4.13***	16.42±3.11

Data are expressed as the percentage of positively staining neutrophils (mean±SEM), *p<0.05, **p<0.01, ***p<0.001. PECAM — platelet-endothelial adhesion molecule, ICAM — intercellular adhesion molecule, VLA — very late antigens.
Údaje sa vyjadrujú ako percento pozitívne označených neutrofilov (priemer±stredná chyba priemeru), *p<0.05, **p<0.01, ***p<0.001.
PECAM — trombocytovo-endotelová adhezívna molekula, ICAM — medzibunková adhezívna molekula, VLA — veľmi neskoré antigény.

tory process. The increased production of reactive oxygen intermediates by neutrophils of patients with CVI was also found out by other authors (Whiston et al., 1993; Pearson, 1999).

There are several lines of evidence that neutrophils taken from patients with CVI released an increased amount of lysosomal enzymes, namely of elastase (Shields and Saharay, 1998; Coleridge Smith, 1999 a, b). It follows from our results summarized in Table 2, that the mean activities of elastase are elevated not only in neutrophils but also in sera of investigated patients. In serum but not in neutrophils of patients, also higher activities of lysozyme were ascertained. On the contrary, the mean activities of myeloperoxidase and beta-D-glucuronidase were decreased in patient neutrophils. In our precedent work (Štvrtinová and Ferenčíková, 1992) we demonstrated that the mean activities of beta-D-glucuronidase together with the mean activities of acid phosphatase and N-acetyl-beta-D-glucosaminidase were elevated in sera of patients with CVI. All these results indicate the increased degranulation of neutrophils in patients with CVI which is one of important markers of leucocyte activation.

Another important marker of neutrophil pro-inflammatory activation is increased expression of leucocyte adhesion molecules (Weyl et al., 1996; Coleridge Smith, 1999 a, b). Such increased expression of several adhesion molecules have been found also on peripheral granulocytes of our patients with CVI (Tab. 3). These molecules include L-selectin (CD62L), integrins (CD11b/CD18, CD49d) and members of immunoglobulin superfamily (CD31, CD54). No changes in the expression of $ß_2$-integrin α-chain (CD11a) and Lewis' antigen X (CD15) were observed in comparison between patient and control granulocytes. The CD54 molecule was expressed only on a very low percentage of granulocytes because it is mainly localized on the surface of endothelial cells and monocytes. Therefore, one can suppose that CD54 is not an integral part of granulocytes but a few of them were probably able to bind soluble CD54 molecules released from endothelial cells and monocytes. The ligand for CD54 is CR3 molecule which is composed from CD11b and CD18 chains. The strong increase of CD11b/CD18 molecule expression is in conformity with amplification of neutrophil adhesion to endothelial cells during an inflammatory process (Rosales and Juliano, 1995). The CD31 molecule is expressed on the surface of different cells including neutrophils and endothelial cells. It is involved in the transendothelial migration of leucocytes from postcapillary venules during inflammation (Panes et al., 1999; Horváthová and Ferenčík, 2000). Therefore its elevated expression on neutrophils may facilitate their inflammatory action also in areas behind the endothelium.

In patients with uncomplicated varicose veins or ulcus cruris we observed increased expression of several adhesion molecules also on peripheral monocytes and lymphocytes. Besides it, in patient lymphocytes significant changes in CD profil were found in the time of diagnosis (Štvrtinová et al., 2001). The higher level of circulating adhesion molecules has been demonstrated in sera of patients with CVI as well (Ciuffetti et al., 1999; Smith et al., 1999; Štvrtinová et al., 2001).

All these results cleary suggest that in patients with CVI there are important changes in the levels of various inflammatory markers such as increased production of reactive oxygen intermediates, increased release of lysosomal enzymes from neutrophils and increased expression of adhesion molecules on leucocytes. Therefore, there is a great probability that in the etiopathogenesis of chronic venous insufficiency of lower limbs inflammatory and immune mechanism are involved in an important way.

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Abstrakt

Štvrtinová V., Jahnová E., Weissová S., Horváthová M., Ferenčík M.:
Zápalové mechanizmy s účasťou neutrofilov pri chronickej venóznej nedostatočnosti dolných končatín
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Pozadie problému: Predpokladá sa, že zápalová reakcia je jedným z hlavných faktorov zodpovedných za chronickú venóznu insuficienciu (CHVI) dolných končatín vyúsťujúcu do vredov predkolenia.
Cieľ: Hlavným cieľom bolo určiť rozdiely v hladinách typických zápalových mediátorov a markerov produkovaných neutrofilmi pacientov s CHVI a kontrolných klinicky zdravých osôb.
Vyšetrované osoby a metódy: Do štúdie bolo zahrnutých 26 pacientov s CHVI a 39 klinicky zdravých dobrovoľníkov. V periférnych neutrofiloch obidvoch skupín vyšetrovaných osôb sa zisťovala tvorba superoxidu, celkových intermediátov kyslíka a aktivity štyroch lyzozómových enzýmov spolu s expresiou ôsmych adhezívnych molekúl.
Výsledky: V neutrofiloch pacientov s CHVI sa zistila zvýšená tvorba superoxidu a zvýšené aktivity elastázy, ktoré boli zvýšené aj v ich sérach. Naproti tomu aktivity myeloperoxidázy a beta-D-glukuronidázy boli v neutrofiloch pacientov znížené, čo naznačuje zvýšené extracelulárne uvoľňovanie týchto enzýmov. V porovnaní s neutrofilmi kontrolných osôb sa na neutrofiloch pacientov dokázala významne zvýšená expresia adhezívnych molekúl CD11b, CD18, CD31, CD49d, CD54 (ICAM-1) a CD62L (L-selektín), kým expresia CD11a a CD15 bola bez zmeny.
Záver: Neutrofily pacientov s CHVI sú primované alebo aktivované, lebo sú schopné uvoľňovať zvýšené množstvá superoxidu a lyzozómových enzýmov a exprimovať zvýšené počty adhezívnych molekúl. To možno považovať za dôležitý dôkaz o účasti zápalového mechanizmu v patogenéze chronickej venóznej insuficiencie. (*Tab. 3, Ref. 27.)*
Kľúčové slová: neutrofily, superoxid, lyzozómové enzýmy, elastáza, adhezívne molekuly.

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