

Changes of bcl-2, bax and caspase-3 expression in the microvascular endothelium of derma in patients with mediterranean spotted fever

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The aim of the present study was to examine the expression of Bcl-2 family of proteins and Caspase-3 in dermal capillary endothelial cells of the skin rash papules of patients with Mediterranean spotted fever (MSF) in order to reveal apoptotic processes in MSF and the time of their onset and progress. Punch-biopsies, obtained from rash papules of patients with MSF, were used to apply immunohistochemical reactions for Bcl-2, Bax and Caspase-3. Weak expression of the immune reaction for Bcl-2 in the capillary endothelial cells of patients was revealed, which did not differ from the healthy controls ($p > 0.05$). The expression of Bax was strongly increased in comparison with the controls ($p < 0.001$). The immune reaction for Caspase-3 also showed increased intensity ($p < 0.001$). The Bcl-2/Bax ratio in the patients was significantly decreased ($p < 0.001$). The changes of the expression of Bax and Caspase-3 are indicators for already initiated apoptotic processes in patients with MSF.

Key words: Mediterranean spotted fever, *Rickettsia conorii*, endothelium, apoptosis, immunohistochemistry

Introduction

In the different stages of the development of infectious processes bacteria are able to either induce or inhibit the apoptotic processes aiming at their own survival [4]. The etiological agent of Mediterranean spotted fever (MSF), *Rickettsia conorii*, is a microorganism with obligate intracellular parasitism and prominent positive tropism to the vascular endothelial cells of the host [8, 9]. The problems of programmed host-cell death in rickettsial diseases are among the not fully un-encoded phenomena of these disorders [9]. The availability of pretty few publications on the problems of apoptosis in micro-vascular endothelial cells in rickettsial diseases and practically missing reports on studies of patients with MSF in vivo was the reason to realize this survey. Our investigations are the first ones in Bulgaria in that relation [1] and we have not found any similar surveys in the available foreign literature.

The aim of the present study was to examine the expression of the Bcl-2 family of proteins and Caspase-3 in the dermal capillary endothelial cells of the skin rash papules

in patients with Mediterranean spotted fever in order to establish whether apoptotic processes occur and the time of their onset and progress.

Materials and methods

In eight patients with a diagnosis of Mediterranean spotted fever capillary endothelial cells of the dermis, taken by punch-biopsy from skin rash papules were investigated. Punch-biopsies from the corresponding skin sections of 4 healthy individuals were used for controls. The biopsy material was processed by a standard method and was embedded into paraffin. Consecutive slices 5µm in thickness were prepared and mounted on microscope slides and immunohistochemical technique was applied. A "Mouse ABC Staining system" (sc-2017, Santa Cruz Biotechnology, Inc.) kit was used as well as the following primary antibodies: Bcl-2 monoclonal antibody (sc-509, Santa Cruz Biotechnology, Inc.), dilution 1:100; Bax monoclonal antibody (sc-7480, Santa Cruz Biotechnology, Inc.), dilution 1:100; Caspase-3 monoclonal antibody (sc-56046, Santa Cruz Biotechnology, Inc.), dilution 1:100. Additionally the slices were processed by staining with Hematoxylin (sc-24973). The so prepared slices were covered with Vector mount (Vector Lab, USA). The applied micro-photographic pictures were taken with the help of a Nikon Microphot-SA (Japan), which was equipped with a Camedia-5050Z digital camera (Olympus, Japan). The expression of the immune reaction for Bcl-2, Bax and Caspase-3 in the dermal vascular endothelial cells was read with the help of the specialized soft ware "DP – Soft" 3.2 (Olympus, Japan). The mean intensity of reaction was analyzed as 50 sections were randomly selected of each slice. The results were processed with the help of the Independent samples *t*-test and $p < 0.05$ was considered significant.

Results

The immune reactions for Bcl-2, Bax and Caspase-3 in the capillary endothelial cells were manifested by slightly expressed to missing brownish colour for Bcl-2 and dark-brown to black colour for Bax and Caspase-3 (Fig. 1), which corresponded to the data of other authors who used the same method [6]. The values of intensity of the immunochemical reaction are presented in relative units (Fig. 2). There was weak expression of the immune reaction for Bcl-2 in the capillary endothelial cells of the skin rash papules of patients with MSF and a very weak expression in the controls, without statistically significant difference ($p > 0.05$). The expression of the immune reaction for Bax in the capillary endothelial cells of skin rash papules was strongly manifested in comparison with the controls ($p < 0.001$). The analysis revealed increased intensity of the immunohistochemical reaction for Caspase-3 in the capillary endothelial cells of skin rash papules in comparison with the control ($p < 0.001$). The analysis of the Bcl-2/Bax ratio revealed that it was significantly decreased in the patients ($p < 0.001$) mainly on the account of increased levels of the pro-apoptotic protein Bax.

Discussion

One of the diagnostic features of MSF is the characteristic papular (boutonneuse) skin rash. There are capillary and lymphatic networks, and nerve endings in the superficial papillary layer of the connective part of the skin – dermis [11]. Searching for signs of

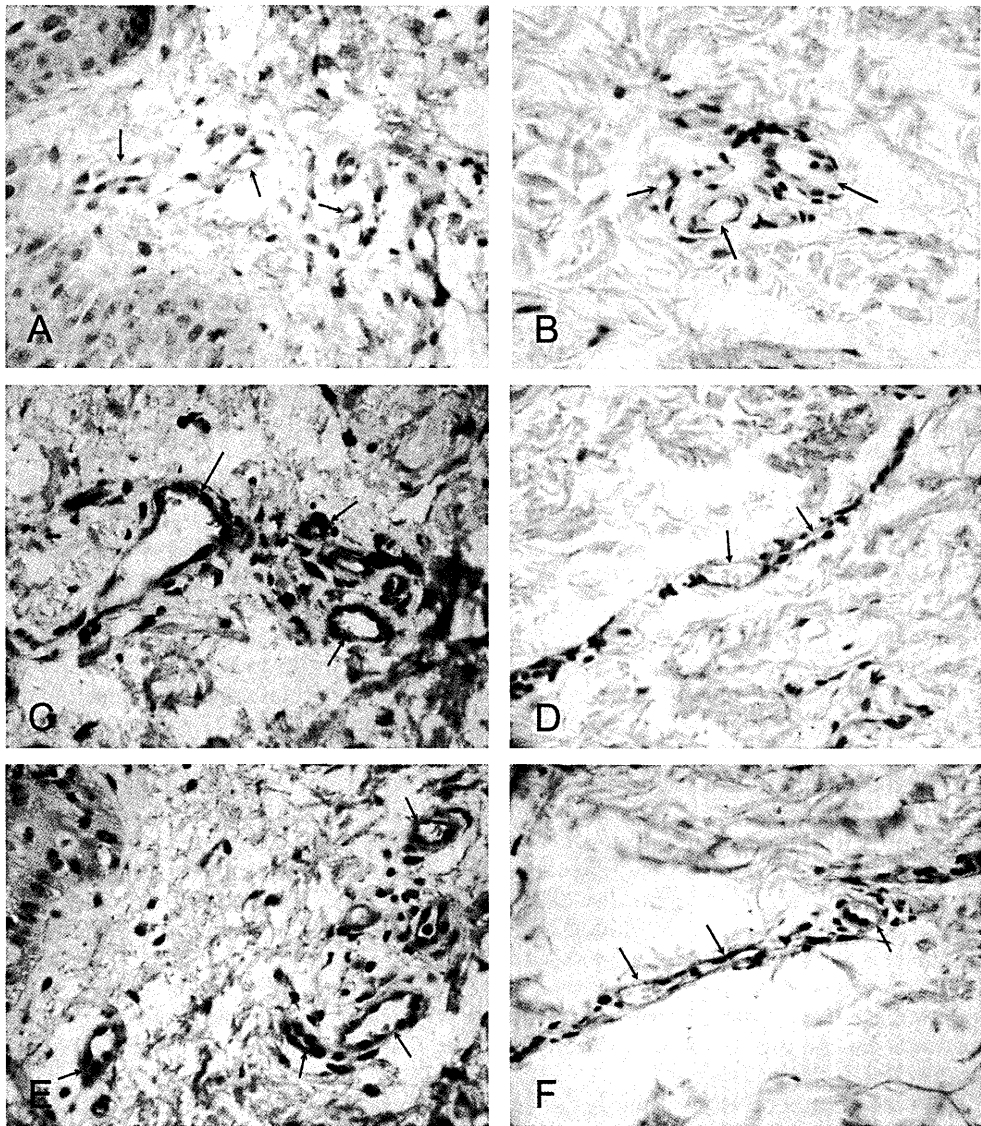


Fig. 1. Immunohistochemical reactions for Bcl-2 (A, B), Bax (C, D) and Caspase-3 (E, F) in the derma of skin rash papules of patients with Mediterranean spotted fever (A, C, E) and controls of healthy individuals (B, D, F). Arrows – reaction in the dermal capillary endothelial cells. Magnification x 400.

apoptosis in the microvascular endothelial cells we selected for biopsy the patient's rash papules as they are an expression of the intracellular pathogen activity i.e. increased capillary permeability, exudation and focal cell infiltration.

In the regulation of programmed host-cell death the B-cell lymphoma-2 (Bcl-2) family of proteins, which includes both pro- and anti-apoptotic factors, plays a critical role by controlling mitochondrial permeability. The anti-apoptotic proteins Bcl-2 and Bcl-xL reside in the outer membrane of mitochondria and they inhibit the release of

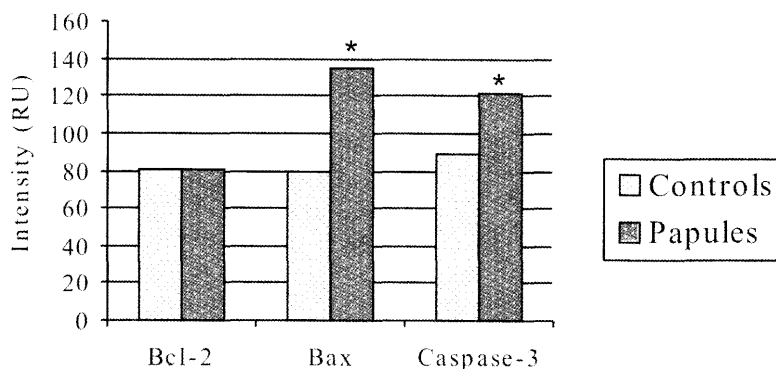


Fig. 2 Intensity of the immune expression of Bcl-2, Bax and Caspase-3 in the dermal capillary endothelial cells of the skin rash papules and controls (in relative units). * $p < 0.001$ in comparison with the control.

cytochrome-C. In contrast, the pro-apoptotic proteins Bad, Bid and Bax are present in the cytosol and their translocation to mitochondria promotes cytochrome-C release [10]. The determination of the effects of Bcl-2 family on nuclear factor NF- κ B reveals significant changes in the expression of various pro- and anti-apoptotic proteins and the ultimate outcome of it is the so called «equilibrium shift» in relation to apoptosis [7, 9]. This was the basis for the origin of the concept of regulation of the mechanisms of programmed endothelial cell death by intracellular *Rickettsia* [2]. The inhibition of apoptosis is necessary in the early stages of disease, when *Rickettsiae* begin to proliferate in the micro-vascular endothelial cells. Later on, when adoptive immunity has fully developed, there is intensification of apoptosis in the infected endothelial cells. The expression of the immune reaction for Bcl-2, Bax and Caspase-3 in skin tissue from papules was examined two days after the onset of the rash. It was estimated that the time period till the second day after the onset of the skin rash (5-6-th day after the initial symptoms of MSF) should be enough for the development of cell-mediated immune response and the effect on *Rickettsia* concerning its anti-apoptotic activity [1]. These presumptions gave us reasons to search for some elements of apoptosis in the endothelial cells of the dermal micro-vessels with the help of immunohistochemical reactions for the pro-apoptotic proteins Bax and Caspase-3 and for the anti-apoptotic protein Bcl-2.

In the dermal capillary endothelial cells of skin rash papules of patients with MSF the expression of Bcl-2 was weak, and the expression of Bax was strongly increased in comparison with the controls. Bax is an essential factor for mitochondria-mediated apoptosis [3]. The decreased Bcl-2/Bax ratio on the account of increased levels of the pro-apoptotic protein Bax is an indicator for intensified apoptotic tendencies and initiation of processes of programmed host-cell death.

While Bcl-2 inhibits apoptosis and Bax promotes it, Caspase-3 is known to be an executioner enzyme in the apoptotic processes. The activation of this executioner protease gives rise to a cascade of proteolytic processes, which lead to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA and phagocytosis of the cell [5]. The increased expression of Caspase-3 revealed in the capillary endothelial cells of the papules in patients with MSF is an indicator for the activation of apoptotic processes and is an initial distinguishing mark for moving to the next final stage of the apoptosis cascade, which leads to cell death.

Conclusion

The decreased Bcl-2/Bax ratio in patients with MSF is related to increased susceptibility to apoptosis, while the elevated levels of Caspase-3 are associated with the apoptosis itself. These markers alone at this stage of the development of the pathological process are not enough to definitely predict the destiny of the endothelial cells, as there are other factors too, which later may have effect on the final result – cell survival or cell death. Nevertheless, the changes of the expression of Bcl-2, Bax and Caspase-3 are indicators for already initiated apoptotic processes, and respectively for the progress of cell-mediated immune reactions in the MSF rickettsiosis within the indicated time limits.

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