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Apoptosis of T Lymphocytes in Systemic Sclerosis

M. Szymanek, G. Chodorowska, A. Pietrzak and D. Krasowska
Department of Dermatology, Venereology and Paediatric Dermatology,
Medical University of Lublin, Lublin, Poland

1. Introduction

Systemic sclerosis (SSc) is a systemic, autoimmune, chronic inflammatory disease affecting the connective tissue. SSc is mainly characterised by progressive fibrosis of the skin, subcutaneous tissue and internal organs, leading to their failure (1). In the majority of cases, lesions involve the osteoarticular, gastrointestinal or cardiovascular system, lungs, kidneys, and nervous system (2-4). The disease occurs in all ethnic groups and mainly affects women; its peak incidence is observed in the 5th and 6th decade of life. Occasionally, lesions develop in childhood (about 3% of cases) (5).

The aetiology and pathogenesis of SSc have not been fully elucidated. The immune system activation appears to be essential for the development of disease (6-8). By releasing cytokines and growth factors, the immune response markedly affects the growth and differentiation of fibroblasts as well as synthesis of collagen (9). The study findings demonstrate that the extent of lymphocytic infiltrates in the affected skin of SSc patients correlates with the severity and degree of skin hardening (10). In the early stages of SSc, inflammatory infiltrates in the skin composed of T lymphocytes, macrophages, mast cells, eosinophils, basophils, and, although less frequently, of B lymphocytes, precede the histological features of fibrosis (7,11). With the progression of fibrosis, inflammatory infiltrates tend to regress (12).

2. The role of T lymphocytes in SSc

T lymphocytes are essential for the pathogenesis of immunological abnormalities in systemic sclerosis. CD4+ T lymphocytes and macrophages are most abundant in the skin whereas CD8+ T lymphocytes are abundant in the lungs (13). The total number of lymphocytes in the peripheral blood is normal or slightly decreased; however, the ratio of circulating CD4/CD8 lymphocytes and the percentage of CD4+25+ T cells are increased while the number of CD8+ T lymphocytes is reduced. Additionally, increased concentration of the soluble CD8 molecule (sCD8) in peripheral blood is suggestive of enhanced activation of lymphocytes in systemic sclerosis (14-16). In the inflammatory stage of SSc, the activated T lymphocytes induce fibrotic processes through the production of cytokines or through direct contact with fibroblasts. The mediators secreted by Th1 lymphocytes (IL-2, IL-12, IL-18, IFN-γ), Th2 lymphocytes (IL-4, IL-5, IL-6, IL-10, IL-13, IL-17) and macrophages are of
particular importance (17-19). Serum levels of IL-4, IL-10, IL-13, IL-17 secreted by Th2 lymphocytes are elevated. IL-4 appears to be essential for fibrosis. It increases the synthesis of collagen in fibroblasts and induces the production of TGF-β, which stimulates the synthesis of various types of collagen, proteoglycans and fibronectin, and inhibits their synthesis by increasing the production of a tissue inhibitor of matrix metalloproteinases. Moreover, a negative correlation between the serum concentration of IL-10, severity of skin lesions and duration of vasomotor disorders has been demonstrated (20). Through the inhibition of IFN-γ and TNF activities, IL-10 is most likely to stimulate indirectly the processes of tissue fibrosis (16), because both IFN-γ and TNF are important SSc mediators. IFN-γ is secreted by Th1 lymphocytes and, to a lesser degree, by NK cells, CD8 lymphocytes, macrophages and dendritic cells. IFN-γ is one of the key inhibitors of collagen synthesis. It decreases the levels of procollagen I, II and III, inhibits proliferation of fibroblasts and stimulating effects of TGF-β. Its involvement in the pathogenesis of systemic sclerosis is supported by significantly lower levels of this cytokine in serum of patients compared to controls (19). TNF, on the other hand, affects directly and indirectly the growth of fibroblasts, synthesis of collagen and activation of the endothelial cells. Increased concentrations of the soluble CD30 molecule (sCD30), belonging to the TNF receptor family, are suggestive of activation of Th2 cells and are directly proportionally correlated with the severity of skin lesions (16).

The involvement of T lymphocytes in the pathogenesis of systemic sclerosis is also confirmed by changes in concentration of these mediators secreted by immune response cells. Increased levels of IL-2 were found in serum of SSc patients, which correlated with the extent of skin involvement and progression of the disease, as IL-2, a pro-inflammatory cytokine, decreases the levels of procollagen I, II and III, inhibits proliferation of fibroblasts and stimulating effects of TGF-β. Its involvement in the pathogenesis of systemic sclerosis is supported by significantly lower levels of this cytokine in serum of patients compared to controls (19). TNF, on the other hand, affects directly and indirectly the growth of fibroblasts, synthesis of collagen and activation of the endothelial cells. Increased concentrations of the soluble CD30 molecule (sCD30), belonging to the TNF receptor family, are suggestive of activation of Th2 cells and are directly proportionally correlated with the severity of skin lesions (16).

Elevated levels of a soluble IL-2 receptor (sIL2R) were observed; the relation between the duration of Raynaud’s phenomenon and sIL2R concentrations in patients with ISSc was found to be inversely proportional whereas in dSSc patients directly proportional (10). Elevated levels of IL-1, IL-6, IL-13 and the connective tissue growth factor (CTGF) were detected in serum and tissues of SSc patients. IL-17 was found to be overexpressed in the peripheral blood and skin of SSc patients. IL-17 is synthesized by Th1 and Th2 lymphocytes. It induces the endothelial cells to produce IL-1, IL-6 and stimulates the expression of adhesive molecules ICAM-1 and VCAM-1 (12,21). Moreover, it stimulates proliferation of fibroblasts and activates macrophages to produce TNF and IL-1, which in turn induces fibroblasts to produce collagen, IL-6 and the platelet-derived growth factor (PDGF) (7).

Since cytokines are essential for the activation of mediators and humoral immune response, their impaired production by Th1 and Th2 lymphocytes may be the key factor for the development of systemic sclerosis. Noteworthy, cytokines secreted by Th2 cells stimulate whereas those secreted by Th1 cells inhibit the synthesis of collagen. However, some studies demonstrate the inhibiting effects of Th2 cells on synthesis of type I collagen (14). Furthermore, the most recent reports indicate significant involvement of B lymphocytes in the pathogenesis of systemic sclerosis (18). The activation of B lymphocytes in SSc is manifested by hypergammaglobulinemia, presence of autoantibodies, stimulation of polyclonal B cells and overexpression of CD 19 molecules on naive and memory B lymphocytes (22,23). Noteworthy, homeostasis of the peripheral B lymphocyte...
subpopulation is impaired in systemic sclerosis. Increased activity of naive B lymphocytes and decreased numbers of memory cells as well as plasmoblasts are observed. Despite their reduced numbers, memory lymphocytes are activated continuously, which is most likely associated with CD 19 overexpression (13). Overexpression of CD 19 appears to be specific for systemic sclerosis (24). It has not been demonstrated in other autoimmune diseases, such as systemic lupus erythematosus or dermatomyositis (18). The detection of autoantibodies in over 90% of SSc patients is a relevant diagnostic and prognostic marker of internal organ involvement and severity of disease (25). In systemic sclerosis, antinuclear antibodies react mainly with the nucleolar antigens and are directed against one antigen (26). A close genetic relationship of autoantibodies with the HLA system suggests the involvement of immunogenetic mechanisms in the development of SSc (1). T lymphocytes have been shown to affect the synthesis of anti-DNA topoisomerase antibodies, other autoantibodies and accumulation of B lymphocytes in skin lesions. This confirms the hypothesis that interactions between T and B lymphocytes are likely to play a significant role in the pathogenesis of systemic sclerosis (7,27).

The cause of lymphocyte activation in systemic sclerosis is not known. Genetic predisposition (haplotypes DR3, DR5, DRw52) and environmental factors are considered (28). Moreover, microchimerism, exposure to organic solvents and toxins (toluene, benzene, xylene, aliphatic hydrocarbons, epoxy resins), infective factors, particularly human cytomegalovirus, some drugs, including bleomycin, vitamin K, penicillamine, beta-blockers, pentazocine, and genetically-determined individual susceptibility to oxidative stress, combined with secretion of free radicals, are also implicated (1,29-32). Recent studies stress the role of impaired or deregulated apoptosis in the pathogenesis of SSc immune changes regarding compromised ability to eliminate autoreactive T or B lymphocytes (2,13,33). According to the recent findings, the abnormal ratio of CD4/CD8 lymphocytes, associated with excessive loss of CD8+ T lymphocytes, may result not only from the activity of lymphocytotoxic antibodies and anti-lymphocyte antibodies blocking determinants but also from enhanced apoptosis of CD8+ T cells (34). Noteworthy, inhibition of apoptosis in systemic sclerosis leads to excessive activation of T and B lymphocytes, contributing to overproduction of antibodies (35).

The objective of the present review is to discuss the selected parameters of T lymphocyte apoptosis in patients with systemic sclerosis.

3. Apoptosis – genetically programmed cell death

Apoptosis (from Greek – “dropping off” of leaves) is an active, programmed process of morphological and biochemical changes determined by the expression of appropriate genes leading to cell death. It enables the elimination of cells without inducing inflammation and damage to the surrounding tissues (36). Apoptosis always involves single cells although their overall number may be high. As a genetically programmed cell death, apoptosis plays a key role in maintaining proliferation and homeostasis of multicellular organisms. It counteracts excessive proliferation and ensures the choice of cells with an optimal set of receptors in the immune system. Moreover, it conditions the precise control of the number and type of cells during ontogenesis and organogenesis, and eliminates excessively produced embryonic and damaged cells, whose survival would not be beneficial for the organism (37-39). The process of apoptosis was discovered
by Alastair Currie, John Kerr and Andrew Wyllie in 1972 (40). Morphologically, apoptosis is characterized by shrinkage of the cytoplasm, condensation of the cell nucleus followed by its fragmentation (41); due to such changes, the microvilli are lost and apoptotic bodies formed, composed of morphologically intact nuclear fragments or other cell organelles. Finally, the apoptotic bodies are phagocytised by the adjacent scavenger cells (Fig.1) (42). The biochemical changes observed during apoptosis involve a decrease in mitochondrial potential, release of cytochrome c from mitochondria, an increase in intracellular concentration of calcium ions, formation of free radicals, activation of caspases, loss of asymmetric distribution of phospholipids in the cell membrane and enzymatic degradation of DNA. Due to gradual suppression of metabolic activity and increased permeability of the cell membrane, the cell, whose nucleus shows apoptotic changes, dies within several hours (43). Normal apoptosis neither impairs the tissue structure and function nor generates the immune response (44).

Fig. 1. Morphological cell changes during apoptosis (42)
There are several stages of programmed cell death: initiation, effector, and destruction (45). The initiation stage involves cell damage in response to a death signal, which leads to critical DNA damage, metabolic stress or activation of programmed cell death receptors. A relevant element of initiation is protein p53, which decides whether the signal received is strong enough to initiate apoptosis or if there is still a possibility to inhibit the cell cycle at phase G1 and activate the repair mechanisms. When the signal is strong enough, the cell enters the effector stage, which determines the irreversibility of changes. At this stage, however, internal regulation (e.g. mediated by Bcl proteins) is possible. The activation of caspase cascade initiates the destruction stage – irreversible structural and functional changes leading to cell death. The remaining parts of a damaged cell are phagocytised, most commonly by tissue macrophages (44,46).
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Fig. 2. Pathways of apoptosis induction (based on literature data)

Apoptosis may be induced by direct DNA damage caused by intrinsic (e.g. cytokines) or extrinsic factors (e.g. hyperthermia, ionizing radiation). The physiological activators of apoptosis are considered to be the tumour necrosis factor (TNF), transforming growth factor \( \beta \) (TGF-\( \beta \)), some neurotransmitters (e.g. dopamine), calcium, glucocorticosteroids, NK cells or cytotoxic T lymphocytes. Moreover, apoptosis is induced by loss of cell-extracellular matrix contact. The pathological factors inducing apoptosis include some bacterial toxins, free radicals, metabolites and some viruses. Apoptosis can also be triggered by physical factors, e.g. ultraviolet radiation, gamma radiation, thermal shock or hypoxia (43,47,48). The pharmacological inducers of apoptosis include chemotherapeutics such as cisplatin, doxorubicin, bleomycin, cytosine arabinoside, methotrexate, vincristine, inhibitors of DNA
topoisomerase I (from the camptothecin family) and inhibitors of DNA topoisomerase II (etoposide, teniposide) (49).

Apoptosis can be induced via the intrinsic (mitochondrial) or extrinsic (receptor) pathway. The dominating mitochondrial pathway is connected with caspase cascade activation. The permeability of the outer mitochondrial membrane is increased resulting in translocation of proteins from the perimitochondrial space to the cytoplasm. The mitochondria release the programmed cell death-inducing factors: cytochrome c, apoptosis-inducing factor (AIF), the second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pi (Smac/Diablo), Omi/HtrA2 serine protease (high temperature requirement) and endonuclease G. This results in decreased mitochondrial potential – the marker of early apoptosis, autocatalytic activation of pro-caspase 9 and effector caspases, which induces proteolysis of various nuclear and cytoplasmic proteins (44,50,51). The extrinsic (receptor) pathway induces apoptosis through binding of a specific ligand by the receptor on the cell surface. The receptors in question are the TNF receptors (TNF-R, Fas), binding TNF and FasL, respectively. The ligands are protein death signals sent by other cells. The activated ligand-bound receptor binds adaptor proteins, which results in autocatalytic activation of pro-caspase 8 and other effector caspases, ultimately leading to cell death (Fig.2) (44,52).

4. The role of Bcl in SSc apoptosis

The best-known products of cellular oncogenes regulating apoptosis are Bcl proteins. The family includes both proteins inhibiting (Bcl-2, Bcl-X, Bcl-w, Mcl-1, BAG-1) and initiating (Bax, Bcl-x, Bak, Bik, Bad, Bid, Bim, NOXA) apoptosis (40). The basic functional elements of Bcl proteins are p26, responsible for binding the protein with intracellular membranes, and at least one of the four Bcl-2 homology domains (BH 1-4). The BH1 subunit determines the regulation of apoptosis, BH2 is responsible for formation of homo- or heterodimers with other Bcl proteins, BH3 occurs also in other proteins regulating the process of programmed cell death whereas BH4 enables the anti-apoptotic action (53,54). According to the function and structure of Bcl-2 constituents, the proteins can be divided into three groups: 1- proteins with all four domains and anti-apoptotic effects (e.g. Bcl-2, Bcl-X); 2 – pro-apoptotic proteins (e.g. Bax, Bak), deprived of the BH4 domain (except for Bcl- x); and 3 - pro-apoptotic proteins containing only the BH3 domain (e.g. Bim, Bid, Bik, Bad) (46,55).

B cell lymphoma/leukaemia 2 (Bcl-2) is the product of bcl-2 gene localized on chromosome 18. It is detected in the inner mitochondrial membrane, endoplasmic reticulum and nuclear membrane, albeit in smaller amounts. Bcl-2 shows the anti-apoptotic action; therefore, under physiological conditions, its expression is observed in the cells of all three embryonic germ layers, non-renewable cells (e.g. neurons) and epithelial basilar cells (56,57). Bcl-2 acts anti-apoptotically thanks to formation of heterodimers with the molecules enhancing apoptosis (Bax) (46). In addition to blocking pro-apoptotic proteins, Bcl-2 stabilizes the cell membranes contributing to increased membranous potential, increased adenosine triphosphate (ATP) synthesis and inhibition of calcium ion escape. Moreover, it activates the regulatory proteins of G1 phase (including p53) (58).

In systemic sclerosis, the effects of Bcl-2 on T lymphocytes are regulated by various cytokines, such as IL-2, IL-4, IL-7, IL-13, and IL-15 (59). The study conducted by Stummvoll
et al. in 39 SSc patients, demonstrated significantly higher expression of Bcl-2 in CD4+ lymphocytes compared to the control group of 47 healthy individuals. There were, however, no significant differences in the expression of Bcl-2 in CD8+ lymphocytes, which suggests that increased expression of Bcl-2 exerts protective effects on CD4+ lymphocytes, hence promotes increased loss of CD8+ lymphocytes and increased ratio of CD4+/CD8+ (in favour of CD4+) (60). Kessel et al., who studied 27 SSc patients, did not find significant differences in Bcl-2 expression in CD8+ lymphocytes compared to the control group (28 healthy individuals), which strongly suggests that anti-apoptotic effects of Bcl-2 do not involve CD8+ lymphocytes (61). Furthermore, Czuwara et al. observed increased apoptosis and impaired expression of Bcl-2 in mononuclear cells of peripheral blood in SSc patients as well as reduced response to camptothecin. They demonstrated that camptothecin, an inhibitor of topoisomerase I, stimulated the process of programmed cell death resulting in decreased expression of Bcl-2. In mononuclear cells of peripheral blood of SSc patients, this effect was markedly lesser compared to the control group of healthy individuals (62).

Bcl-2 is an anti-apoptotic protein, which prevents programmed cell death both via the intrinsic pathway, inhibiting the release of pro-apoptotic particles from the mitochondria and via the receptor pathway, inducing the anti-apoptotic action of NF-κB (45,63).

Extremely enhanced spontaneous expression of Bcl-2 in peripheral mononuclear cells and its high increase mediated by camptothecin and IL-2 were demonstrated in a female patient with systemic sclerosis and breast cancer. Increased expression of Bcl-2 was likely to be caused by the coexistence of two diseases. The authors suggest that further studies involving a larger population of patients are required to interpret explicitly the pathogenetic and diagnostic role of Bcl-2 (62).

The findings reported by Stummvoll et al., who studied 17 patients with diffuse and 22 patients with limited SSc, did not reveal significant differences in Bcl-2 expression in CD4+ and CD8+ lymphocytes. The authors suggest that expression of Bcl-2, as a marker of apoptosis, may not be dependent on the clinical form of systemic sclerosis (60). Similar results were presented by Cipriani et al. in 17 patients with dSSc and 5 with ISSc, which is likely to indicate that Bcl-2-mediated apoptosis is not dependent on the clinical form of SSc (59). Moreover, there were no significant relations between the expression Bcl-2 in peripheral blood lymphocytes in SSc patients and the duration of disease, its activity, degree and extent of skin lesions, duration of sclerotic microangiopathy, organ changes, antinuclear antibodies or treatment applied (59,60).

The Bcl-2 – Bax ratio is thought to be essential for apoptosis - due to antagonistic effects of these proteins, the ratio decides about cell survival or otherwise (64).

The Bcl-2-associated X protein (Bax) is one of the best-known proteins of the Bcl family. It has an important function in pro-apoptotic regulation of programmed cell death via the mitochondrial pathway. In its inactive form, it is localized in the cytoplasm. Having stimulated the cell to apoptotic death, Bcl-2 translocates to the outer mitochondrial membrane, where it is oligomerised (63). The functional molecule is the 21 kDa protein of the structure similar to Bcl-2. The action of p53 results in increased amounts of Bax and decreased amounts of Bcl-2, which leads to their imbalance and formation of Bax-Bax homodimers. This results in formation of the mitochondrial membranous channel, release of cytochrome c to the cytoplasm, activation of caspases and disintegration of cell structures (40,46). Moreover, Bax accelerates the transition of the cell into the phase of genetic material replication, which suggests its relevant role in proliferative processes, i.e. promoting
neoplasia. This could explain worse prognosis in neoplasms with high Bax expression and better prognosis in cancers with low Bcl-2/Bax ratio (46).

According to the study carried out by Stummvoll et al. in 39 SSc patients and 47 healthy controls, there were no significant differences in Bax expression in CD4+ and CD8+ lymphocytes, which is likely to suggest that Bax does not play any significant role in apoptosis regulation in SSc patients. Moreover, there were no significant differences in Bax expression in relation to the clinical subtype, duration of disease, or immunosuppressive therapy administered (60). Our findings in 40 patients with systemic sclerosis revealed higher expression of Bax in CD8+ lymphocytes in patients with active disease (65). This enhanced Bax expression in CD8+ lymphocytes may suggest the increased loss of these cells through the process of apoptosis. The pathogenesis of SSc is associated with increased proliferation of CD4+ and loss of CD8+ lymphocytes. Apoptosis appears to be one of the possible mechanism for CD8+ loss (14).

5. The role of NF-κB in SSc apoptosis

Another relevant transcription factor responsible for activation and regulation of expression of genes involved in apoptosis is the nuclear factor κB (NF-κB) (66). It plays a crucial role in regulation of the immune response, inflammatory processes, oncogenesis, and virus replication. Moreover, it is necessary for activation of lymphocytes, proliferation and expression of cytokines (61). The NF-κB–activated genes include genes encoding cytokines IL-1, IL-2, IL-6, IL-12, TNF, LTα/β), granulocyte macrophage-colony stimulating factors (GM-CSF), immunoreceptors (with the MHC ligand), cell adhesion molecules (ICAM, VCAM, ELAM), acute phase proteins (SAA – serum amyloid), enzymes (inducible nitric oxide synthase - iNOS, cyclooxygenase-2 – COX-2) and genes encoding oncogenesis-involved factors (cIAP1, cIAP2, fasl, c-myc, p53, cyclin D1) (67). To date, ten various transcription factors belonging to the NF-κB family (Rel) have been identified in mammals. Five of them are transcription regulators: Rel/NF-κB (p50/p105 – NF-κB1, p52/p100 – NF-κB2, c-Rel-Rel, RelA – p65 and RelB); the remaining ones have inhibitory properties (IκBα, IκBβ, IκBγ-p105, IκBδ-p100, Bcl-3) (68). All regulatory factors contain the rel homology domain (RHD), composed of 300 amino acids, which is responsible for formation of dimmers, their permeation to the nucleus and binding to an appropriate DNA fragment (69). The terminal fragment of RHD contains a nuclear location sequence (NLS), which permits binding to the nucleus (67).

The NF-κB proteins may be homo- and heterodimers (except for RelB). The majority of homodimers are not capable of inducing transcription whereas heterodimetric structures contain transactivating domains indispensable for induction of genes involved in the immune response (68,70). The best-known heterodimer is p50/RelA, composed of two subunits, p50, a product of NF-κB1 gene, and p65, a product of RelA gene (67). NF-κB, found bound to IκB in all cells, except for lymphocytes B, is activated in the cytoplasm, following the cell exposure to pro-inflammatory factors, e.g. lipopolysaccharides, (LPS), the tumour necrosis factor (TNF-α, TNF-β), epidermal growth factor (EGF), free radicals, cytokines, viruses, ionizing or ultraviolet radiation. NF-κB, released during IκB degradation, is translocated to the nucleus, where it binds to DNA and activates suitable genes, e.g. mediators of inflammation, carcinogenesis or IκB mediators (68, 70).
Numerous data highlight a significant regulatory role of NF-κB in the process of apoptosis. Being involved in various pathways of programmed cell death, NF-κB exerts anti- and pro-apoptotic effects, which is most likely dependent on the predominance of factors activating or inhibiting the expression of the cascade of apoptotic events. Apoptosis is inhibited due to NF-κB-induced transcription of Bcl anti-apoptotic genes (Bcl-xl, BFl/A1) and inhibitors of apoptosis (cIAP1, cIAP2) (which indirectly reduces the activity of cytochrome c). Moreover, the mechanism of activation of tumour receptor-associated factors (TRAF1, TRAF2) and IAPs (cIAP1, cIAP2, XIAP), resulting in inhibition of the caspase cascade, is involved; caspase 8 is deactivated by TRAF1, TRAF2, cIAP1, cIAP2, whereas caspase 3 mainly by activated proteins cIAP1 and cIAP2 (Fig.3) (61,66,71,72).

An example of NF-κB anti-apoptotic action is its involvement in transcriptional regulation of genes associated with liver regeneration after partial hepatectomy or protection of cortical neurons against apoptotic effects of β-amyloid (exposure of cortical neurons to β-amyloid is connected with an increase in IkB-α mRNA level, which reduces the NF-κB activity) (73,74).

On the other hand, the role of NF-κB in transcriptional regulation of several pro-apoptotic genes is noteworthy. It is highly likely that this process results from rapid activation of NF-κB in response to the apoptotic signal and from effects of NF-κB on expression of some genes associated with programmed cell death, e.g. TNF, c-myc or fasl genes (66). By increasing the expression of FasL, NF-κB enhances the Fas-FasL interactions. Moreover, as demonstrated earlier, the transcription factor RelA (p65) is essential for activation of the promoter fragment FasL (75,76). It should be emphasized, however, that some researches do not confirm possible NF-κB-activated apoptosis mediated by expression of Fasl gene (77,78).

Another indirect example of pro-apoptotic NF-κB action is activation of nitric oxide synthase required for production of nitric oxide. The process results in inhibition of caspase
cascade, which leads to cell apoptosis (61). It is worth noting that apoptosis may be regulated by the antagonistic action of protein p53 towards NK-κB, which compete for binding to the co-activator p300 (33).

The involvement of NF-κB in cell cycle regulation involves facilitation of transition from phase G1 to S through inhibition of activation or function of p53 and increased expression of the cyclin D1. Additionally, NF-κB can activate the transition from phase G2 to M by inhibiting the expression of the growth arrest DNA-damage protein 45 (GADD45), which blocks the cyclin B/CDK2 complex (66).

The ability of transcription factor NF-κB proteins to suppress apoptosis and regulate the cell cycle indicates that NF-κB may play an essential role in oncogenesis. Enhanced expression of NF-κB has been demonstrated in numerous neoplastic diseases, e.g. breast, lung or thyroid cancer, T and B cell leukaemia, malignant melanoma, prostate, gallbladder, head and neck cancer (34,35,79-82).

In the study performed in 27 SSc patients and 28 healthy controls, Kassel et al. observed reduced expression of NF-κB in CD8+ lymphocytes of SSc patients compared to controls; additionally, an inverse correlation was found between the percentage of anti-apoptotic CD8+ T lymphocytes and NF-κB expression. The authors believe that decreased NF-κB expression in CD8+ lymphocytes in peripheral blood is likely to be one of the mechanisms of enhanced apoptosis of CD8+ lymphocytes in SSc patients. This weighs in favour of the anti-apoptotic action of NF-κB in systemic sclerosis and thus confirms an important role of NF-κB in regulation of homeostasis and tolerance of T lymphocytes (61, 83). The exact mechanism leading to decreased NF-κB expression in CD8+ lymphocytes in SSc patients has not been fully explained. The ability of NF-κB to regulate the expression of anti-apoptotic genes, such as cellular inhibitors of apoptosis (c-IAP1, c-IAP2, IXAP), TNF receptor-associated factors (TRAF1 and TRAF2) as well as Bcl-2 proteins, appears to be crucial (83,72). Importantly, NF-κB, as a nuclear transcription factor, and pathways of its anti-apoptotic action can be activated by various factors: cytokines, free radicals, lipopolysaccharides, or directly acting receptors, e.g. TNF receptor (61). The NF-κB involvement in regulation of apoptosis has been confirmed in experimental studies carried out for over ten years. Numerous reports indicate that NF-κB activation is necessary for protection of lymphocytes against apoptosis induced by various factors (83). In 1997, Ivanov et al. suggested a possible relevant role of NF-κB in the regulation of Fas receptor-induced apoptosis of T lymphocytes (84). Two years later, Dudley et al. confirmed protective effects of NF-κB on T lymphocytes against Fas receptor- and TNF-induced apoptosis (85). The recent reports demonstrate that NF-κB activation is indispensible for protection of T lymphocytes against apoptosis induced by mutagens and anti-Fas antibodies (78).

According to Auphan et al. and Lanza et al., steroid preparations are likely to contribute to NF-κB inactivation, hence increasing the percentage of apoptotic cells. Glucocorticosteroids, as one of the most powerful anti-inflammatory and immunosuppressive agents, inhibit the synthesis of cytokines and many cell surface molecules required for induction of immune responses. NF-κB is inactivated due to steroid-induced increased synthesis of IκB. IκB, a nuclear factor inhibitor, retains NF-κB in the cytoplasm in the form of inactive complexes (86,87).

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6. The role of mitochondrial membrane potential in SSc apoptosis

Many researchers stress an important role of mitochondria in programmed cell death (88-90). The majority of human cells undergo apoptosis via the intrinsic pathway (91). It has been shown that the key point in induction of mitochondrial pathway of apoptosis is increased permeability of the outer mitochondrial membrane, usually accompanied by decreased potential of inner mitochondrial membrane ($\Delta\Psi_m$). The differences result from metabolic features of membranes. High values of inner mitochondrial membrane ($\Delta\varphi_m$) have to be maintained for proper mitochondrial energetic processes, which lead to the formation of adenosine triphosphate (ATP). In normal cells, the inner mitochondrial membrane is virtually impermeable; however, it is equipped with transport systems for selected metabolites, whose weight does not exceed 1.5 kDa. Thanks to the presence of voltage-dependent anion channels (VDACs), the outer mitochondrial membrane acts as a molecular sieve, which is permeable to the majority of ions and low-molecular substances dissolved in water of a molecular weight below 5 kDa. VDACs are characterized by reversibility and selectivity, both for anions and cations; at low voltages, they are open for anion metabolites. Thus, under normal conditions they are impermeable to positively charged cytochrome c. The mechanism for opening and closing of VDACs is regulated by Bcl proteins (55,63,92-94).

The results of studies in patients with chronic B-cell leukaemia reveal that decreased mitochondrial potential is a marker of early apoptosis mediated by the mitochondrial permeability transition pores (MPTPs) formed in the inner mitochondrial membrane. The major constituents of MPTPs are adenine nucleotide translocase (ANT) and cyclophilin D, located in the inner mitochondrial membrane as well as VDACs and the peripheral benzodiazepine receptor located in the outer membrane. In normal mitochondria, VDACs and ANTs form a macromolecular complex responsible for transport of adenine nucleotides from the site of ATP production within the mitochondrial matrix to that of ATP consumption in the cytosol. Since apoptosis is relevant for the development of systemic sclerosis, impaired production of ATP should be expected in T lymphocytes of SSc patients. It is known that the mitochondria are essential for apoptosis, which results from the fact that permeability of the mitochondrial membrane and activation of caspases determine irreversibility of the process. Interestingly, permeability of the outer mitochondrial membrane is a constant feature of apoptosis. The opening of several MPTPs, or even one of them, leads to depolarization of mitochondria, impaired oxidative phosphorylation and marked swelling of mitochondria. With progression of programmed cell death, the mitochondrial potential decreases, which results in the release of proteins closed within the intermembrane space (e.g. cytochrome c, apoptosis-inducing factor (AIF), pro-caspase 2, 3, 9, adenylate kinase, the second mitochondrial activator of caspases). The outflow of these molecules is necessary for quick activation of the cascade of programmed cell death events (95,96).

In contrast to the outer membrane, apoptotic permeability of the inner membrane is not a constant feature of apoptosis and does not cause such an intense release of proteins from the matrix. An increase in inner membrane permeability to dissolved molecules of molecular weight of about 1.5 kDa is a characteristic feature, which is associated with dispersion of the proton gradient responsible for mitochondrial transmembrane potential ($\Delta\Psi_m$) (97,98).

The available literature lacks studies assessing the mitochondrial membrane potential in the population of CD4+ and CD8+ lymphocytes. In our study, the percentage of apoptotic cells was analysed using chloromethyl-X-rosamine (CMXRos) (65). The method assesses the
mitochondrial potential, an indicator of the induction of an intrinsic apoptotic pathway. The method was chosen as it enabled the assessment of the very early stages of apoptosis, before the cells undergoing apoptosis are eliminated from the circulation through phagocytosis (89). Our findings show higher percentages of CD4+ and CD8+ lymphocytes with reduced mitochondrial membrane potential ($\Delta\Psi_m$) in patients compared to healthy controls, which is likely to suggest the activation of early CD8+ T lymphocyte apoptosis through the mitochondrial pathway in patients with systemic sclerosis (65). It is noteworthy to mention that a decrease in mitochondrial potential is characterized by specificity, as the process involves only the cells entering apoptosis, and universality, as it regards all cells entering the programmed cell death pathway. Moreover, decreased mitochondrial potential is characterized by irreversibility since the cells of decreased $\Delta\Psi_m$ undergo apoptosis even when the triggering stimulus is removed (99,100). Thus, the measurement of mitochondrial potential seems to be an extremely sensitive and precise method for assessment of apoptotic cell percentages.

7. The role of Fas receptor and Fas ligand in SSc apoptosis

The role of the soluble Fas (sFas) in initiation of programmed cell death is illuminated by the results of studies in patients with systemic sclerosis and other autoimmune diseases (101). The Fas receptor (APO-1, CD95) and Fas ligand (FasL) belong to the family of TNF receptors (102). Soluble Fas (sFas) is a 4 kDa glycosylated type I membrane protein, whereas FasL is synthesized as a 40 kDa type II transmembrane protein. The Fas receptor is expressed on the surface of various types of normal and neoplastic human cell lines, e.g. T and B lymphocytes, macrophages, hepatocytes or thymocytes. FasL is produced by activated CD4+, CD8+ T lymphocytes and NK cells; it is expressed in the eyes and testes and is characterized by high cytotoxic activity towards the Fas receptor-bearing cells. The activity of FasL is stimulated by UV radiation, gamma radiation and some drugs, e.g. bleomycin, anisomycin and doxorubicin, and inhibited by cyclic adenosine monophosphate (cAMP), retinoic acid, nitric oxide and vitamin D$_3$ (39,75,76,103).

In response to an apoptotic signal, FasL binds to the Fas receptor, which results in Fas trimerisation. The interactions between Fas and FasL are relevant for induction of lymphoid line apoptosis and systemic immune response. The pro-apoptotic action is possible thanks to the complex of adaptor proteins, the mediators of the reaction, or to cell contact. This happens because Fas receptor fragments are deprived of catalytic domains. One of the adaptor proteins contains the death domain (DD) - the sequence of specific amino acids, which enables interactions of FADD protein with the cytoplasmic fragment of activated Fas receptor. Consequently, the death-inducing signalling complex (DISC) is formed. In addition to DD, the FADD protein has the death effector domain (DED), to which pro-caspase 8 binds with its DED. This complex is necessary for autocatalytic activation of pro-caspase 8. At this stage, two pathways of further signalling leading to apoptosis are possible. In the first one, active caspase 8 is sufficient to activate pro-caspase 3, which finally leads to condensation of nuclear chromatin and DNA degradation. The cells characterized by this signalling on the extrinsic pathway are called type I cells. In contrast, in type II cells, activation of caspase 8 is insufficient for induction of apoptosis as it is usually weak, and thus does not lead to formation of sufficient amounts of the product. The signal has to be enhanced on the mitochondria-dependent intrinsic pathway. A link between both apoptotic pathways is the Bid protein (Fig.4) (39,76,104,105).
Wetzig et al. demonstrated significantly increased levels of sFas in the group of 30 patients with systemic sclerosis compared to 15 healthy controls. The authors suggested that increased sFas levels might be an important marker of prevention of T lymphocyte apoptosis in systemic sclerosis (103). Similar results were reported by Dziankowska-Bartkowiak et al., who studied the group of 29 SSc patients and 10 healthy controls and found significantly higher levels of sFas in SSc patients, which is likely to implicate an important role of sFas in apoptosis prevention in systemic sclerosis (106). By affecting FasL-Fas coupling sFas may prevent the induction of apoptosis, thus promote the activation of T lymphocytes in systemic sclerosis. The available results suggest that sFas may be essential for inhibition of apoptosis in the pathogenesis of systemic sclerosis. By preventing the initiation of programmed cell death, sFas is likely to increase the proliferative response of lymphocytes to autoantigens, ultimately leading to excessive activation of T lymphocytes (14,101,107). Stummvoll et al. observed statistically significantly higher concentrations of
sFas in serum of SSc patients compared to healthy controls. Additionally, they showed higher Fas expression in CD8+ lymphocytes in SSc patients compared to controls, which is likely to suggest increased apoptosis of these lymphocytes. However, they did not observe any significant differences in Fas expression in CD4+ lymphocytes. Abnormal serum sFas levels in SSc patients are likely to be a marker of T lymphocyte activation during systemic sclerosis (60). Cipriani et al. demonstrated significantly higher serum sFas concentrations in 22 SSc patients in comparison with healthy controls, which also seems to confirm the earlier implicated role of the receptor pathway of apoptosis in the pathogenesis of systemic sclerosis (59). Moreover, elevated sFas levels in SSc patients compared to healthy controls were observed by Nozawa et al., yet the differences were not statistically significant, which may be associated with the smaller population of patients included in their study (only 16 patients) (108).

The literary data indicates that SSc patients are characterized not only by increased numbers of activated T lymphocytes but also by the enhanced expression of Fas receptors in these cells, compared with healthy controls (60). This shows that increased serum levels of sFas in SSc patients may protect autoreactive T lymphocytes against apoptosis induced by the Fas-ligand system and lead to excessive activation of T lymphocytes (14,101,107). Increased concentrations of sFas may be indicative of inhibition of apoptosis induction by the receptor pathway, and thus contributes to the activation of T lymphocytes in this disease.

It is worth mentioning, however, that there are studies in which no significant differences in serum sFas levels in SSc patients were found compared to healthy controls, which may be associated with different study designs, differences in disease activity or therapy administered (101,109,110).

Apoptosis appears to be mediated by the Fas receptor pathway in both forms of disease; nevertheless, considering the clinical picture of both forms, higher levels of sFas should be expected in SSc patients, whose disease develops more rapidly and affects the internal organs, especially in early stages (103,111-113). In the study by Wetzig et al., involving 16 lSSc and 14 dSSc patients, there were no significant differences in sFas levels according to the clinical form of disease (103). Similar results were reported by Stummvoll et al. Additionally, they demonstrated a positive correlation between Fas receptor expression and the age of the patients. Their findings, showing statistically significant differences in Fas expression and sFas concentration, are likely to indicate enhanced activation of T cells resulting from impaired apoptosis of lymphocytes (60). Otherwise, the findings reported by Dziankowska-Bartkowiak et al. revealed statistically significant differences in sFas levels depending on the disease form. Their study involved two size-comparable groups of patients (15 dSSc and 14 lSSc patients). The sFas concentrations in dSSc patients were found higher in comparison with lSSc patients (52,106). According to Ingegnoli et al., expression of Fas receptor in CD4+ and CD8+ lymphocytes was significantly higher in dSSc patients. These findings are likely to confirm impaired lymphocyte homeostasis in systemic sclerosis. The authors suggested that enhanced Fas expression in dSSc patients might lead to the development of autoregulation mechanisms due to abnormal immune response. sFas-induced excessive activation of T lymphocytes is likely to lead finally to the elimination of autoreactive lymphocytes through Fas receptor-activated apoptotic pathways (16).

In the studies carried out by Wetzig et al. in SSc patients, only a slight correlation between sFas concentration and disease activity was found. The activity of disease was assessed...
based on elevated CRP, SR and/or presence of immune complexes, leucocytosis and clinical markers of skin involvement (swelling, redness, or tenderness). The diagnostic criteria of inactive disease included normal SR and CRP, as well as skin sclerosis without swelling or atrophies. Elevated sFas levels were more common in patients with active disease, although in single cases high sFas levels were also observed in patients with inactive SSc (103).

Ates et al. found no significant correlations between sFas concentration and degree or extent of skin involvement in SSc patients. They assessed the severity and extent of sclerosis using the 4-degree (0-3) scoring method of Kahaleh et al. in 15 body areas (101). Different results were reported by Dziankowska-Bartkowiak et al, who also used the Kahaleh scale and observed a positive correlation between sFas concentration and severity of skin lesions and a directly proportional relation between the serum sFas level and osteoarticular involvement. The authors suggest that elevated sFas levels in systemic sclerosis may be a marker of skin and osteoarticular involvement (106).

Ates et al. did not show significant differences in serum sFas concentrations of patients with lung fibrosis and those without HRCT-detected chest lesions. Moreover, they did not observe significant correlations between serum sFas levels and lung diffusion capacity (101). Similar results were presented by Luzin et al. and Wetzig et al. (103,114). However, there are also reports stressing the role of sFas in the development of interstitial lung disease in SSc patients. The evidence can be found in studies devoted to the role of apoptosis in the pathogenesis of systemic sclerosis induced by bleomycin. According to Kuwano et al., anti-FasL antibodies administered in injections may prevent lung fibrosis induced by bleomycin in SSc patients. Anti-FasL antibodies are most likely to lead to inhibition of apoptosis induced via the Fas-FasL pathway (115). This mechanism, however, does not seem sufficiently protective as lung fibrotic processes are induced not only through the ligand-receptor pathway (49).

Taking into account the treatment used, Ates et al. demonstrated significantly higher serum sFas levels in untreated SSc patients in comparison to healthy controls and patients undergoing therapy (101).

The available literature does not provide evidence for significant correlations between Fas protein concentrations and disease duration or presence of oesophageal or cardiac lesions (59,103).

8. The role of cytochrome c in SSc apoptosis

Furthermore, the role of cytochrome c in apoptosis should be highlighted. Cytochrome c is a water-soluble 15 kDa haem protein, consisting of a 104 amino acid-long peptide chain combined with the haem molecule. It plays an essential role in oxygen phosphorylation and apoptosis, being involved in caspase 3 activation and DNA fragmentation (94,116,117). Like the majority of mitochondrial proteins, cytochrome c is encoded by the nuclear gene and synthesized in the cytoplasm as a precursor 12 kDa molecule, called apocytochrome c. It translocates from the cytoplasm, independently of the receptors, along the outer mitochondrial membrane to the perimitochondrial space where functionally active molecules of cytochrome c are formed mediated by the inner mitochondrial membrane enzyme – cytochrome c haem lyase. During programmed cell death, cytochrome c translocates from the mitochondria to the cytosol in response to the apoptotic signal. The molecular mechanism of this translocation is not fully explained. As demonstrated earlier, it
results from decreased membranous mitochondrial potential characteristic of early stages of apoptosis. The release of cytochrome c during apoptosis is regulated by Bcl proteins. Under normal conditions, VDAC, formed in the outer mitochondrial membrane by the mitochondrial channel protein porin, is impermeable to cytochrome c. Mediated by pro-apoptotic proteins (Bax, Bak, tBid), VDAC opens and releases cytochrome c from the intermembrane space, whereas anti-apoptotic proteins, e.g. Bcl-xl, close the channel retaining cytochrome c within the mitochondria. Cytochrome c in the cytoplasm initiates programmed cell death events through the caspase cascade-dependent pathway (44). By catalysing the heptameric complex of caspase 9 and apoptosis protease activating factor 1 (Apaf 1), a proenzyme of caspase 9, cytochrome c acts as a cofactor of the reaction. Cytochrome c binds Apaf 1 without the involvement of deoxyadenosine triphosphate (dATP); subsequently, in the presence of cytochrome c and with dATP involved, pro-caspase 9 can bind to Apaf 1, which results in caspase 9 activation. In cases of cytochrome c deficiency, even if dATP is available, this reaction is infeasible, which points to the relevant role of cytochrome c in initiation of the cascade of caspases - executors of the death signal. The consequence of caspase 9 activation is indirect involvement of cytochrome c in activation of caspase 3, which leads to DNA fragmentation and cell death (Fig.5) (45,117,118).

Fig. 5. The role of cytochrome c in apoptosis (117)

9. The role of caspase 9 in SSc apoptosis

Caspase 9 (ICE-LAP6, Mch6) belongs to the intracellular proteases of cysteine, whose common feature is hydrolysis of protein substrates at the place of asparagine acid carboxyl residue. These enzymes play a key role in apoptosis; capable of destroying the enzymatic and effector proteins, they ultimately lead to complete cell disintegration. Caspase 9 is
synthesized in its pro-enzymatic form as a zymogene. Unlike other caspases, the caspase 9 zymogene shows high chemical activity, which may suggest that pro-caspase 9 proteolysis is not necessary for enzyme activation (119,120). Like other cysteine proteases, pro-caspase 9 has a N-terminal pro-domain consisting of a larger subunit – p20 (20kDa) and a smaller subunit – p10 (about 10 kDa), joined with a short linker. This pro-domain is involved in dimerisation of pro-caspase molecules and their maintenance in inactive forms. According to its structure, caspase 9 is a caspase with a long pro-domain, with the caspase activation and recruitment domain (CARD). The enzyme is activated due to binding of the homological fragment of 85 amino acids of NH$_2$-terminal fragment of Apaf to CARD 1 in the presence of cytochrome c and dATP and due to the effects of caspase 3 and granzyme B on the pro-caspase 9 molecule, which was demonstrated under in vitro conditions (118,121-124). Caspase 9 can also be activated with involvement of active caspase 8 during Bid disintegration, which results in the release of cytochrome c to the cytosol. Moreover, pro-caspase 9 may be proteolysed through the apoptosome-independent pathway using caspase 12 (125,126). The enzymatically active caspase 9 acts as a tetramer formed of two heterodimers consisting of a small and large subunit (p20$^2$ – p10$^2$) (40,127-129). Ultimately, it is located in the cytosol, where to pro-caspase 9 translocates from the perimitochondrial space of various organs in response to the apoptosis-inducing stimulus. High expression of caspase 9 was shown in the heart, ovaries and testes. Its presence was also detected in the liver, kidneys, brain, spleen and lymphoid cell lines and neuroblastoma lines (45,130).

In the process of apoptosis, caspase 9 plays an important role in induction of caspase cascade - the pathway of biochemical events directly responsible for programmed cell death. By activating the effector caspase 3 and 7, it substantially contributes to degradation and fragmentation of cytoplasmic and nuclear proteins. Additionally, since it can be activated by caspase 3, caspase 9, as an active enzyme, is crucially involved in irreversible changes occurring in the cells during apoptosis (128,130,131). The caspase 9 involvement in apoptosis is regulated by specific inhibitors, such as the tumour-up-regulated CARD-containing antagonist of caspase nine (TUCAN) protein, Akt kinase (protein kinase B), anti-apoptotic Bcl proteins and IAPs (132).

The role of cytochrome c and caspase 9 in apoptosis has not been fully elucidated; therefore, further research is required.

10. Key points
1. The Bcl family appears to play a significant role in the regulation of T lymphocyte apoptosis in SSc patients. Enhanced expression of Bax in CD8$^+$ lymphocytes in patients with active disease suggests increased loss of these lymphocytes through intensified apoptosis.
2. Decreased expression of NF-kB in activated CD8$^+$ lymphocytes in peripheral blood is likely to be one of the mechanisms potentiating apoptosis of CD8$^+$ lymphocytes in patients with systemic sclerosis.
4. Fas is likely to play an important role in prevention of T lymphocyte apoptosis during systemic sclerosis.
11. References


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Apoptosis of T Lymphocytes in Systemic Sclerosis

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Systemic sclerosis (SSc), or often referred to as Scleroderma (tight skin), is characterized by an exaggerated formation of collagen fibers in the skin, which leads to fibrosis. Accumulating evidence now points toward three pathological hallmarks that are implicated in Ssc, the order of which has yet to be determined: endothelial dysfunction, autoantibody formation, and activation of fibroblasts. This current book provides up-to-date information on the pathogenesis and clinical features of this severe syndrome. It is our hope that this book will aid both clinicians and researchers in dealing with patients with this clinical syndrome. In addition, we hope to shed more light on this rare and severely disabling syndrome, ultimately leading to better research and successful therapeutic targeting.

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