ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. LXII, N 1, 54 SECTIO D 2007

Chair and Department of Dermatology, Venereology and Pediatric Dermatology
Medical University of Lublin

GRAŻYNA CHODOROWSKA, BARTŁOMIEJ WAWRZYCKI,
MAŁGORZATA DĄBROWSKA-CZŁONKA, JOANNA BARTOSIŃSKA,
IWONA JAZIENICKA

Immunological abnormalities in the localized scleroderma

Scleroderma is a chronic disease of unknown aetiology characterized by skin fibrosis and is divided into two clinical entities: localized scleroderma and systemic sclerosis (SSc). The term scleroderma refers to syndromes that are confined to the skin and subcutaneous tissues (localized scleroderma, morphea) and to those in which multiple visceral organs can be involved (systemic sclerosis). Although examination of involved skin may be similar or identical in localized and systemic syndromes, the clinical presentation and pattern of cutaneous involvement however, is different from that of SSc. Morphea can be differentiated from SSc by the distribution of lesions – no sclerodactyly or perioral involvement, absence of Raynaud's phenomenon and rarity of severe systemic findings. The life prognosis of patients with localized scleroderma is good.

In localized scleroderma the lesions are usually limited to the skin. Localized scleroderma is characterized by round, oval, linear or irregular plaques. The plaques are initially red, smooth and indurated. Then plaque or plaques progress to ivory white atrophy sometimes with surrounding violaceous halo. Sclerosis can sometimes also involve subcutaneous tissue, muscles and bones, resulting in functional disabilities and cosmetic problems. In some of most severe cases atrophy of the extremities, deformities, contractures and limb length discrepancies develop. In the *en coup* the sabre type, which affects the face, involvement of the underlying structures may cause hemiatrophy of the face and facial deformity. Localized scleroderma is classified morphologically into five variants: plaque (morphea), linear, bullous, deep and generalized morphea (13).

Expression of cytokines with fibrogenic activity is thought to play an important role in the development of fibrosis in scleroderma patients. Fibrosis develops as a result of increased proliferation rate and excessive production of extracellular matrix components, such as collagen by fibroblasts.

Transforming growth factor (TGF)-β is a multifunctional cytokine with impact on cell growth, differentiation and biosynthesis of connective tissue. TGF-β is a potent stimulator of many extracellular matrix genes, including collagen types I, II, III, IV, V and VII, elastin and fibronectin.

So far, three isoforms, TGF-β1, TGF-β2, and TGF-β3, have been identified in mammals. TGF-β is a mediator released not only by inflammatory cells such as lymphocytes and macrophages, which are the major component of cell infiltrates in fibrotic lesions, but also by endothelial cells and fibroblasts. Thus, the inflammatory cells present in the skin of patients with fibrotic skin diseases could release TGF-β, resulting in increased production of collagen and leading to clinical manifestations of dermal fibrosis. Fibrosis induced by TGF-β may be also facilitated by other cytokines such as connective

tissue growth factor (CTGF), IL-4 and IL-13. Some literature data indicate that TGF-β signaling is enhanced in sclerotic skin, resulting in increased fibroblast growth, collagen synthesis and its own synthesis by autoinduction. Expression of the TGF-β receptors seems to be of great interest. Levels of TGF-β receptor types I and II (TGF-βRI and II) have been reported to be elevated in scleroderma fibroblasts (3).

Querefeld et al. demonstrated the presence of m RNA for all three isoforms of TGF-β in inflammatory skin areas of systemic and localized scleroderma, but not in sclerotic or healthy skin. Immunohistochemical analysis also confirmed expression of β1 and β2 proteins in skin biopsies taken from patients with the inflammatory stage of the disease (11). Other authors also reported enhanced expression of both type I collagen and TGF-β1 genes in cell populations within keloids, as well as in progressive systemic sclerosis and generalized morphea, measured with immunohistochemical and *in situ* hybridization techniques.

Connective tissue growth factor (CTGF) is a cysteine-rich peptide, that exhibits platelet-derived growth factor activities and is produced by skin fibroblasts after activation with transforming growth factor-β. Coordinate TGF-β expression followed by CTGF during wound repair suggest that this cascade process may be responsible for the control of tissue regeneration and repair. It is known that CTGF mRNA is strongly expressed in the fibroblasts located in sclerotic lesions from patients with systemic sclerosis. In localized scleroderma expression of CTGF mRNA was found to be scattered throughout the sclerotic lesions whereas the adjacent nonaffected dermis was negative for CTGF mRNA. In the same study CTGF was also found to be present in keloids and scars (3, 11).

The microvasculature (endothelial cells platelets, capillaries) is thought to be one of first affected systems in scleroderma. In systemic sclerosis it may sometimes precede the outbreak of the disease even by years, e.g. Raynaud's phenomenon. There is also some evidence that perivascular cellular infiltration and endothelial cell injury occur in early stages of LS. The vascular abnormality may be caused by toxic factors like proteases, lipoperoxides, IgG antiendothelial autoantibodies, and free radicals.

Endothelial cell apoptosis may be a primary event in scleroderma. Data from Sgonc et al. showed that endothelial cells are the first to undergo apoptosis in the skin of UCD-200/206 chickens, which are animal models of hereditary scleroderma (12). All UCD-200/206 samples were taken already in an early stage of disease, before clinical manifestations. There were no other alterations seen in microscopically compared to control. Later with disease progression perivascular infiltrations of CD4+, CD8+ lymphocytes, and TCR1+ cells were observed. Detection of apoptotic cells indicated their presence in the deeper dermis of all patients with acute SSc and loSc samples, compared to chronic fibrotic skin biopsies, healthy controls and keloid sections where no apoptotic cells were found. This apoptotic process is probably caused by antiendothelial cell antibodies (AECA) (12).

Another evidence for the vascular changes in limited scleroderma was provided by Kobayasi and Serup (8). They found three patterns of vascular alteration in LS. Biopsies of both uninvolved and involved skin showed stimulated endothelial cells and a thickened vascular wall infiltrated with macrophages and mast cells. Two additional vascular patterns were present in inflammatory and sclerotic areas. The second pattern was characterized by thick basal lamina of pericytes and the third by activated pericytes with infliltrating lymphocytes and plasma cells. Authors concluded that activated pericytes are the most essential changes in vessels of morphea. There is another theory claiming that damage to vascular endothelium might be caused by autologus complement. Venneker et al. showed either undetectable or low levels of membrane cofactor protein (MCP) and decay accelerating factor (DAF) in the endothelium of skin affected by morphea. DAF and MCP are complement regulatory molecules found in endothelial cells. MCP and DAF inhibit formation of C3/C5 convertases of the

classical and alternative pathways, thereby protecting cells from complement mediated damage. Decreased expression of MCP and DAF provides evidence for vascular susceptibility to damage by complement (15). Kowalewski et al., in their study presented alterations in vascular network in skin lesions of morphea. Three-dimensional reconstruction, showed increased angiogenesis only in the early inflammatory stage of morphea, whereas in inactive morphea and lichen sclerosus various numbers of enlarged vessels were visible (6).

Some authors hypothesized that the generation of free radicals could be a common mechanism through which different etiological agents can provoke scleroderma in genetically predisposed individuals. Oxygen free radicals in high concentrations can inhibit collagen formation and even cause its degradation. Conversely, the repeated release of free radicals in low concentrations stimulates fibroblast proliferation with narrowing of the vessel wall, ischemia, and the release of more free radicals. It was suggested that free radicals can cause sclerosis either through direct tissue damage leading to vascular endothelial necrosis or through the chronic stimulation of collagen formation. Some authors even suggest that the release of oxygen free radicals and the associated damage to the vascular endothelium could be the primary event, which is followed by the stimulation of the immune system (3)

Superoxide dismutase (SOD) is a family of enzymes that catalyse the dismutation of $O_2^{\cdot}$ to H_2O_2 and O_2, protecting aerobic cells from damage by oxygen-free radicals. Human tissues have three major SOD isozymes: copper-zinc SOD, manganese SOD (Mn SOD), and extracellular SOD. The two first isozymes have intracellular distribution, and elevation of their serum levels is being considered to indicate a high production of oxygen-free radicals. Furthermore, it is believed that SOD in the serum is released during tissue breakdown (5). Thus, the elevation of the serum levels of SOD seems to reflect both the increase of oxygen-free radicals and severity of tissue injury. Increased free-radical production by peripheral neutrophils or monocytes is supposed to be involved in the pathogenesis of SSc. In localized scleroderma elevated plasma, lesional and nonlesional skin SOD activity have been reported. Also serum, lesional and nonlesional skin lipid peroxide levels were higher compared with normal controls. Moreover, the degree of skin induration could be correlated with changes in lesional SOD activity and lipid peroxide levels, respectively, but there was no correlation between SOD or lipid peroxide and antinuclear antibody titer. In more recent paper from Jinnin et al., authors report significantly higher serum levels of Mn SOD in patients with generalized morphea than those in healthy individuals. Patients with elevated serum Mn SOD had also a significantly larger number of sclerotic lesions and significantly higher serum levels of sIL-2. Concluding, the authors suggest that this enzyme might be a serological marker for disease activity and extent of skin involvement (5).

Vascular endothelial cell injury represents an early event followed by the up-regulation of the expression of adhesion molecules, and cytokines. Inflammatory cells, including CD4+ T cells, monocytes, macrophages, and eosinophils, are subsequently recruited and infiltrate through the endothelium to the reticular dermis of the skin. Cytokines and growth factors released by endothelial and inflammatory cells result in fibroblast proliferation and increased deposition of extracellular matrix (3).

It is well known that IL-2 is produced by activated T-cells, and the IL-2 receptor is shed from them, proportionally to the state of activation. Uziel et al. demonstrated significantly elevated levels of sIL-2R in patients with active localized scleroderma as compared with patients with inactive disease (14). Moreover, other authors noted that higher levels of sIL-2 in localized scleroderma were correlated with the number of sclerotic lesions, the number of involved areas, the levels of anti-ssDNA, and the levels of antihistone antibody immunoglobulin. These data suggest that lymphocyte activation is one of the early processes in the development of scleroderma. It appears that collagen

promotes the IL-2 production, and that laminin induces IL-2 receptor expression on lymphocytes. Endothelial cell membranes increase c-fos expression in T-cells, which themselves induce IL-2 expression. This may explain the presence of activated T-cells in the perivascular infiltrate of scleroderma skin.

It has been estimated that Il-4 promotes T-cell adhesion to endothelial cells (EC), differentiation of lymphocytes and stimulates fibroblast proliferation and extracellular matrix synthesis. Human fibroblasts synthesize elevated levels of collagen and fibronectin in response to IL-4 and fibroblasts from scleroderma patients are hyperresponsive to this cytokine. SSc fibroblasts express also higher levels of IL-4Rα. An increased number of cells synthesizing IL-4 was identified in biopsies from systemic scleroderma patients, and elevated IL-4 levels were noticed in the serum of scleroderma patients.

IL-6 is typical pleiotropic cytokine, produced by various cell types including T cells, fibroblasts and endotheial cells. One of the major roles of these cytokines is stimmulation of immunoglobulin production in B cells. IL-6 induces in concentration dependent manner production of collagen and glycosaminoglycans from human dermal fibroblasts *in vitro*. Il-6 exerts its biological function through a cell surface receptor IL6R, and a transducing 130 kDa glycoprotein (gp130). It has been proved that soluble Il-6 (sIL-6R) receptor binds to Il-6. The Il-6/sIl-6R complex is as efficient as the Il-6/membrane Il-6R complex in the binding and subsequent activation of signal transduction. Soluble gp130 binds to Il-6/sIl6r complex and prevents its interaction with membrane bound gp130 on target cells, resulting in inhibiting the Il-6 activities.

It regulates the high affinity Il-2 R, in lymphocyte cultures, most likely regulating the effect of IL-2 on immune activation in scleroderma 39. Ihn et associates investigated serum levels IL-2, IL-4 and IL-6 in a cohort of 48 patients suffering from localized scleroderma. As a result they found that serum levels of IL-4 were elevated in 17% of patients, serum IL-2 levels in 27% and serum IL-6 levels in 47% of patients suffering from localized scleroderma, while none of the healthy controls had elevated levels of these cytokines. Interestingly, all three cytokines were detected more frequently in patients with generalized morphea (4). Kreuter et al. studied levels of these pro-inflammatory cytokines in tissue biopsies of lesional skin from patients with localized scleroderma. They found that IL-2 and IL-4 were weakly expressed in lesional skin, whereas IL-6 and IL-8 were detectable at high levels and significantly decreased after UVA1 phototherapy. In addition, the authors also investigated mRNA levels of β-defensins – cysteine-rich peptides with antimicrobial activity, and also implicated in tissue injury, scarring and wound healing. Human beta defensin-1, -2 and -3 levels were higher in lesional skin compared with healthy controls (7).

Serum levels of soluble Il-6 receptor and sgp130 were found to be significantly higher in patients with localized scleroderma compared with healthy controls. In patients with localized scleroderma elevated sIL-6R levels significantly correlated with levels of antihistone antibodies, the presence of rheumatoid factor, the number of linear lesions, and the number of body areas involved. Elevated sgp 130 levels were significantly associated with levels of antihistone antibodies, the number of plaque lesions, the total number of lesions, and the number of body areas involved (4).

TNF is produced mainly by activated monocytes and macrophages. It possesses multiple inflammatory and immunoregulatory properties, including the ability to influence fibroblasts growth or collagen synthesis (3). TNF plays also an important role in leukocyte movement within inflamed tissues by activating chemokines or endothelial cells, which on the other hand, may indicate its engagement in an early phase of morphea. It is believed that TNF may act *in vivo* by regulating other fibrogenic mediators like IL-6, rather than stimulating collagen directly.

Il-13 is secreted by activated T cells, mast cells, and natural killer cells. Il-13 promotes B cell proliferation, induces cell surface expression of integrin, MHC class II and CD23. It has also ability to suppress proinflammatory cytokine production in monocytes and macrophages. Il-13 and Il-4 share a common cellular receptor, which accounts for many of the similarities between these two cytokines. Il-13 is like Il-4 a potent stimulator of fibroblast proliferation. Serum levels of both IL-13 and TNF were reported to be significantly higher in patients with localized scleroderma. Moreover, the presence of IgM antihistone antibodies, anti-single-stranded DNA antibodies and elevated serum Il-6 levels was correlated with elevated TNF levels. Clinically, elevated serum levels of TNF were correlated with the number of linear lesions and muscle involvement. Elevated Il-13 levels were significantly associated with the number of plaque lesions and the total number of lesions.

In scleroderma EC express increased numbers of adhesion molecules and therefore may facilitate the interaction with lymphocytes. In this way the transcapillary migration of inflammatory cells is mediated, leading to prominent T-cell infiltrates around blood vessels in early lesions.

Vascular cell adhesion molecule 1 (VACAM-1) is a member of the immunoglobulin gene superfamily and is induced on the surface of endothelial cells within hours of stimulation by the inflammatory cytokines, such as IL-1 or tumor necrosis factor (TNF)α. VACAM-1 is expressed by activated endothelial cells and several extravascular cell types. E-selectin is a member of the selectin family and is induced on vascular endothelial cells by cytokines such as IL-1, TNF-γ, interferon gamma (INFα), and IL-4. Unlike other endothelial adhesion molecules such as VCAM-1, E-selectin has been reported to be expressed only on endothelial cells. Both of these proteins have their soluble forms soluble VCAM-1 (sVCAM-1) and soluble E-selectin (sE-selectin). sVACAM-1 and sE-selectin share most of the structure and exhibit most of the function of the extracellular portion of cell-bound adhesion molecules. It appears that the sources of sVCAM-1 and sE-selectin *in vivo* may be mainly cell-bound adhesion molecules expressed on activated endothelial cells, which means that high levels of sVACAM-1 and E-selectin may be correlated with endothelial activation. In localized scleroderma serum levels of sVCAM and sE-selectin were found to be higher than those in healthy controls. Patients with GM, the most severe form of limited scleroderma, had the highest levels of sVACAM-1 and E-selectin. In addition, the serum levels of these soluble adhesion molecules were significantly correlated with both the number of sclerotic lesions and the number of involved areas (13).

Laboratory evidence of autoantibody is often seen in patients with limited scleroderma. Whether autoantibodies are pathogenic or merely ephipenomena is unclear. Certainly, many patients with LS do not have detectable circulating antibodies. When laboratory abnormalities are present however, the most commonly detected are antinuclear antibody (ANA), rheumatoid factor RF, hypergammaglobulinemia, and eosinophilia.

The frequency of ANA positivity in LS varies from 23% to 73%. The various nuclear immunofluorescence patterns include homogeneous (75%), nuclear speckled (17%), and nucleolar ones (8%). However, the antigens that are recognized by the ANA in localized scleroderma remain to be determined. Falanga et al. studied 31 patients with linear scleroderma and found that the presence of ANA was more common in patients with more severe and extensive disease. In his further study he did not however, note such a correlation between the presence or absence of ANA and disease activity in patients with morphea and generalized morphea (1).

The same author reported the high frequency (59%) of anti-single-stranded DNA antibodies in morphea and generalized morphea, with the highest levels of ssDNA binding observed in patients with generalized morphea. The frequency of antibodies to ssDNA was higher in patients with clinical evidence of active lesions. In linear scleroderma the presence of anti-ssDNA antibodies was more common in patients with joint contractures and disease duration of over 2 years (2). Rufftatti

et al. also confirmed the high frequency of anti-ssDNA in localized scleroderma, and found that generalized morphea and linear scleroderma had higher antibody prevalence and higher levels than morphea (10).

The prevalence of rheumatoid factor in localized scleroderma is approximately 30%. Falanga et al. showed the presence of RF in 9 of 34 patients wih linear scleroderma and showed that a higher titer was associated with more severe cutaneous and articular disease (1). More recently other researchers reported that all three isotypes of RF from generalized morphea were significantly higher than those from healthy subjects (13). Moreover, they found that serum levels of IgM RF were correlated with the number of sclerotic lesions.

Antihistone and antinucleosome antibodies are often identified in patients with limited scleroderma. Sato et al. demonstrated the presence of AHA in 47% of patients with localized scleroderma and in 87% of patients with generalized morphea. Further investigations revealed that the predominant antigens were histones H1 and H3. In his recent study he also found high prevalence of antinucleosome antibodies which were detected in localized scleoderma even more frequently than antihistone antibodies (82% vs. 53%). Author concluded that antinucleosome antibody might be a major autoantibody in this disease (13).

Anti-Fcγ receptor autoantibodies were found in 33% of patients with localized scleroderma, with no correlation to clinical data. Anti-agalactosyl immunoglobulin G antibodies have been reported to be detected in 19% of patients with localized scleroderma, with significant correlation between anti-AG IgG levels and the number of involved areas and sclerotic lesions (13).

Antibodies to U1 RNP and Th/To ribonucleoprotein were found in 3% and 4% of patients with LS respectively. It appeared that the frequency of anti-Th/To was similar to that in patients with SSc, whereas the frequency of anti-U1 RNP as significantly lower in patients with LS compared to SSc 3% vs. 13% (13).

As described above, the presence of autoantibodies and elevated cytokine levels, suggests immune activation, and shows that localized scleroderma is one of the organ-specific autoimmune disorders targeting skin. Weather the presence of various autoantibodies and cytokines is related to the pathogenesis of cutaneous lesions remains to be determined.

REFERENCES

1. Falanga V. et al.: Antinuclear and anti-single-stranded DNA antibodies in morphea and generalized morphea. Arch. Dermatol., 123, 350, 1987.
2. Falanga V. et al.: Linear scleroderma. Ann. Intern. Med., 104, 849, 1986.
3. Haustein U. F., Anderegg U.: Pathophysiology of scleroderma: an update. J. Eur. Acad. Dermatol. Venereol., 11, 1, 1998.
4. Ihn H.: Demonstration of interleukin-2, interleukin-4, and interleukin-6 in sera from patients with localized scleroderma. Arch. Dermatol., 287, 193, 1995.
5. Jinnin M. et al.: Serum levels of manganese superoxide dismutase in patients with localized scleroderma. Exp. Dermatol., 13, 357, 2004.
6. Kowalewski C. et al.: Alterations of basement membrane zone and cutaneous microvasculature in morphea and extragenital lichen sclerosus. Am. J. Dermatopath., 6, 489, 2005.
7. Kreuter A. et al.: Ultraviolet A1-induced downregulation of human β-defensins and interleukin-6 and interleukin-8 correlates with clinical improvement in localized scleroderma. Br. J. Dermatol., 155, 600, 2006.

8. Kobayasi T., Serup J.: Vascular changes in morphea. Acta Derm. Venereol. (Stockh), 65, 116, 1985.
9. McGaha T. L., Bona C. A.: Role of profibrogenic cytokines secreted by T cells in fibrotic process in scleroderma. Autoimm. Rev., 1, 174, 2002.
10. Ruffatti A. et al.: Prevalence and characteristics of anti-single-stranded DNA antibodies in localized scleroderma. Arch. Dermatol., 127, 1180, 1991.
11. Querfeld C. H. et al.: Expression of TGF- β1, TGF- β2, and – β3 in localized and systemic scleroderma. J. Dermatol. Sci., 21, 13, 1999.
12. Sgonc R. et al.: Endothelial cell apoptosis is a primary pathogenic event underlying skin lesions in avian and human scleroderma. J. Clin. Invest., 98, 785, 1996.
13. Takehara K., Sato S.: Localized scleroderma is an autoimmune disorder. Rheumatol., 44, 127, 2005.
14. Uziel J. et al.: Serum levels of soluble interleukin-2 receptor a marker of disease activity in localized scleroderma. Arthritis Rheum., 6, 898, 1994.
15. Venneker G. T et al.: Morphea lesions are associated with aberrant expression of membrane cofactor protein and decay accelerating factor in vascular endothelium. Br. J. Dermatol., 131 (2), 237, 1994.

SUMMARY

Localized sclerodema (LS) is a connective issue disorder characterized by immunogical dysregulation, vascular alteration and fibrosis of the affected tissue as the most prominent features. Localized scleroderma, especially generalized morphea which is the most severe form of his disorder, has been reported to be accompanied by variety of abnormal immune reactions. Impaired T-cell function mediated through the release of various cytokines is considered to contribute to the development of LS. In this context, interleukin (IL)-2, IL-4 and IL-6 together with other cytokines, have been demonstrated at increased levels in the sera of patients with LS correlating with the severity of the disease. Localized scleroderma may be also accompanied by the presence of antinuclear antibody (ANA), anti-single-stranded NA antibody (anti-ss-DNA), antihistone antibody (AHA), rheumatoid factor (RF) and other antibodies.

Zaburzenia immunologiczne w twardzinie ograniczonej

Twardzina ograniczona jest schorzeniem tkanki łącznej, u którego podłoża leżą zaburzenia immunologiczne, zmiany naczyniowe oraz włóknienie w obrębie zajętej skóry. Twardzinie ograniczonej, a w szczególności jej najcięższej postaci – odmianie rozsianej, towarzyszyć mogą zaburzenia immunologiczne. Uważa się, iż nieprawidłowości w funkcjonowaniu limfocytów T, polegające m. in. na zmianie profilu wydzielanych przez te komórki cytokin, mogą brać udział w patogenezie twardziny ograniczonej. W surowicy pacjentów z twardziną ograniczoną obserwowano m. in. podwyższone poziomy interleukiny 2 (IL)-2, IL- 4, IL- 6 oraz innych cytokin, korelujące z nasileniem zmian chorobowych. Innymi zaburzeniami immunologicznymi towarzyszącymi omawianej jednostce są: obecność przeciwciał przeciwjądrowych (ANA), przeciwciał przeciwko jednoniciowemu DNA (anty-ss-DNA), przeciwciał przeciwko histonom (AHA), czynnika reumatoidalnego (RF) oraz szeregu innych odchyleń w zakresie odpowiedzi humoralnej.