Leakage of von Willebrand Factor and Mast Cell Degranulation in the Skin of Patients with Systemic Sclerosis

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Immunohistochemical expression of von Willebrand factor (vWF), the number and intensity of mast cell degranulation, and small blood vessel electron microscopical morphology in the group of 9 patients with an established diagnosis of systemic sclerosis (SSc), aged 29–54 years (mean, 47.5) was studied. The control group consisted of 10 age-matched subjects free of any systemic disease. All skin biopsies were obtained from the forearm.

A coincidence of extensive extravascular vWF leakage, small blood vessel damage, and perivascular mast cell degranulation in the early edematous stage of SSc was found.

In the late fibrotic stage of SSc neither vWF leakage nor mast cell degranulation were prominent, if any. In the mid stages of SSc pathogenesis, intensive mast cell degranulation but no vWF leakage were present.

Key words: von Willebrand factor, blood vessels, mast cells, systemic sclerosis, skin biopsy

INTRODUCTION

Vascular involvement in systemic sclerosis (SSc) has been known from the early descriptions of the disease. The disease is characterized by more or less specific microvascular changes such as "bushy" capillaries, hemorraghes and thrombosis gradually leading to diminished number of capillary blood vessels in affected sites, accompanied by fibrosis in the damaged area. The first target for vascular deterioration in SSc is endothelium (12, 16). SSc patients are in hypercoagulable state with elevated plasma levels of fibrinogen and von Willebrand factor (vWF), defective tissue plasminogen activator release, elevated plasminogen activator inhibitor, enhanced thrombin generation and increased fibrin formation (3). Excessive thrombin acts as a mitogen for fibroblasts, upregulates vWF and plasminogen activator inhibitor, enhances endothelin production by vascular endothelial cells and facilitates fibrin deposition on vessel wall (3, 5, 17). vWF is secreted by endothelial cells constitutively into circulation, but also is deposited in specific storage organelles known as Weibel–Palade bodies. The high molecular weight, polyvalent vWF is released by exocytosis upon vascular stimulation, irritation and/or damage (6, 9, 26, 33, 35).

There are evidences that mast cells are involved in the development of interstitial edema and in very early stages of SSc pathogenesis (2, 11).

The aims of the present study were to asses vascular damage/vWF release into peri- and extravascular space and mast cell degranulation as eventual local pathomechanisms in SSc.

PATIENTS AND METHODS

Patients and biopsies

Nine women patients aged 29–54 years (mean, 47.5) were examined clinically before a biopsy was taken. All SSc patients were hospitalized, gave their informed consent and met the criteria established by the American College of Rheumatology for SSc (15, 32). All patients were classified as suffering from a systemic form of scleroderma. Ten control skin sam-

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ples were taken from patients in Surgical Department. They patients didn't suffer from any systemic disease. All biopsies were from the forearm skin. One half of the material, intended for immunohistochemical analysis, was fixed in 10% neutral formalin and ethanol, embedded in paraffin and then processed for staining with hematoxilyn-eosin and toluidine blue at pH of 2.0 for the mast cell detection and evaluation. Mast cells were identified by their metachromatic granules and unilobed nuclei. Another half of every biopsy was fixed in 2.5% glutaraldehyde followed by 2% osmium tetroxide, and embedded in Epons for further electron microscopic analysis.

Imunohistochemistry

The primary antibodies used were serum protein absorbed rabbit anti-human vWF IgG (1:500, Dakopats A/S, Glostrup, Denmark). Paraffin sections (5 μm) were mounted on DAKO Capillary slides (TechMate™ DAKO, Glostrup, Denmark), deparaffinized in xylene and rehydrated in a graded ethanol series and 10 mM phosphate-buffered, 0.9 M saline, pH 7.4 (PBS). For antigen retrieval the slides were pretreated with 0.4% pepsin in 1N HCl at +37 °C for 30 minutes. Then the slides were washed and stained automatically in a staining robot by the following protocol: 1) the primary antibody, diluted with DAKO ChemMate™ antibody diluent, for 30 minutes; 2) secondary antibody containing biotinylated goat anti-rabbit IgG for 30 minutes; 3) peroxidase block for 30 minutes; 4) peroxidase-conjugated streptavidin 3 times for 3 minutes; 5) HRP Substrate Buffer, and finally 6) substrate working solution containing 3,3-diaminobenzidine tetrahydrochloride (ChemMate™ Detection Kit) for 5 minutes. Between each step, the sections were washed with DAKO ChemMate™ washing buffers three times and dried in absorbent pads. After staining the sections were removed from the robot, counterstained with hematoxylin or left without counterstaining, washed, dehydrated in ethanol series, cleared in xylene and mounted in synthetic mounting medium (Diatex, Beckers Industrifärg AB, Märsta, Sweden) Replacement of the primary antibody with normal rabbit IgG in a corresponding dilution was used as negative staining control. All incubations were performed at +22 °C.

Microscopic examination

Microscopic assessment was done using a low light-charge screen mounted with a 12-bit PC digital image camera (SensiCam, Kelheim, Germany) on a Leitz Diaplan light microscope (Wetzler, Germany). The whole (papillary and reticular) derma section areas were analyzed. Diffusion of vWF from the blood vessels into extracellular/perivascular space and perivascular mast cell degranulation were scored as none (0), mild (+), moderate (++), or strong (+++). The score evaluation was done by two microscopists independently.

Ultrathin sections were prepared with LKB ultratome, stained with saturated uranyl acetate and lead citrate, and examined in electron microscope JEM 100 B.

RESULTS

Histological analysis of SSc biopsies disclosed the earliest pathological changes in the skin. Perivascular edema was an early feature. With progression of the pathogenesis, an inflammatory cell infiltration into the dermis and platelet aggregation within vessels developped. Further clinical progression was associated with loss of adnexae, vascular effacement and increasing dermal fibrosis.

Immunotopochemical detection of vWF showed its expression in the skin blood vessel network which corresponded to the network revealed by the standard reference stainings. The degree of vWF expression in the particular vessels and particular areas could significantly differ. In the early stage of SSc pathogenesis, the atypical forms of papillary layer vessels occasionally were observed (Fig. 1a). In some

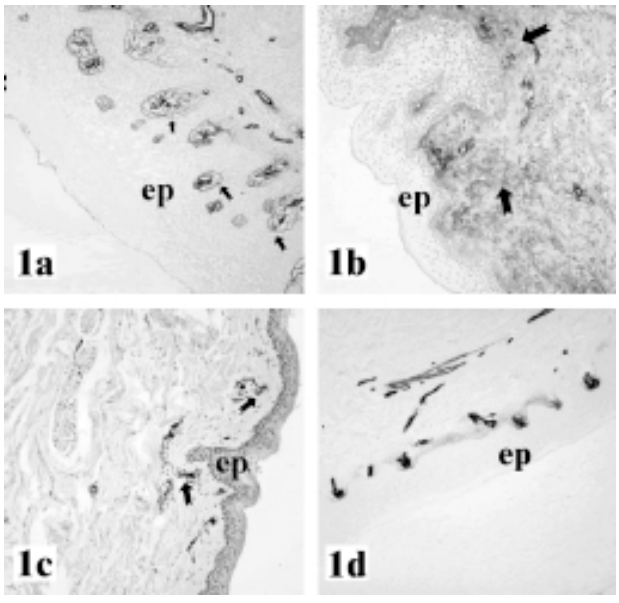

Fig. 1. Expression of vWF in skin in systemic sclerosis: **a** – vWF in "bushy" capillaries (arrows); **b** – extensive extravasation of vWF (arrows). Counterstained with hematoxylin; **c** – reduction of capillary network (arrows). Counterstained by hematoxylin; **d** – vWF in nornal control. Original magnification: ×250. Abbreviation: ep – epidermium

Table. **vWF leakage and mast cell degranulation in SSc patients' skin**

Patient	Age (years)	Duration of disease (years)	vWF extravasation	Perivascular mast cell degranulation	Vascular damages
1	52	2	+	+	Endothelial edema
2	54	1	++	++	Endothelial edema
3	53	4	0	+	Endothelial shrinkage
4	54	15	0	0	Fibrosis
5	29	1	++	+++	Endothelial edema
6	49	21	0	0	Fibrosis
7	45	6	0	++	Necrobiosis
8	43	8	0	+	Fibrosis
9	52	3	++	++	Endothelial edema

Score value: 0 – none, + – mild, ++ – moderate, +++ – strong.

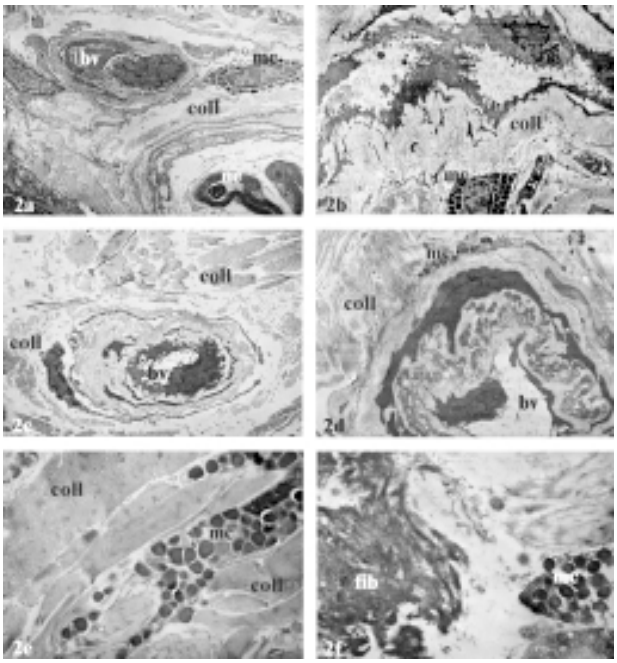

Fig. 2. Ultrastructural changes in SSc skin: **a, b** – mast cells and degenerating nerves close by deteriorating blood vessel. ×3000; **c** – fibrosing blood vessel. ×3000; **d** – edematous blood vessel. ×3000; **e** – mast cell granules among fibrous tissue. ×6000; **f** – fibrin and mast cell granules in the derma. ×8000. Abbreviations: bv – blood vessel, coll – collagen fibers, fib – fibrin, mc – mast cell granules, ne – nerve fibers

areas more or less evident leakage of vWF into extracellular matrix was found (Table, Fig. 1b). In the advanced fibrotic stage of SSc when the blood vessel network was dramatically reduced, no vWF leakage into the perivascular interstitial matrix was detectable (Fig. 1c), and the vWF staining was usually restricted to endothelial cells. No vWF leakage was found in the healthy skin from control subjects (Fig. 1d).

We have shown an increased mast cell number and intensity of their degranulation in SSc patients (Table) with the early indurative phase of the disease. It was especially remarkable in the papillary dermis. In the immediate proximity to adventitia, extensive mast cell degranulation and fibrin deposition was found next to small blood vessels both in papillary and reticular layers of the skin (Fig. 2e–2f). In patients with the late fibrotic phase of SSc, the mast cell number and degranulation decreased as compared to the initial phase of the disease and the controls. At this stage the papillary layer was filled with homogeneous collagen bundles. A change in mast cell number and the intensity of degranulation reflected a change in interstitial collagen bundles in the papillary layer.

The ultrastructural analysis of SSc skin biopsies, even in the early stages of the disease, revealed a serious damage in small blood vessels and perivascular areas (Fig. 2a–2b). It comprised edema, mast cell infiltration and degranulation, nerve fiber dystrophy, and mild interstitial fibrosis. Blood vessel walls underwent necrobiosis and dystrophy (Fig. 2c–2d), the lumina gradually narrowed. The endothelial cells were shrunk. In the late stages of SSc small blood vessel occlusion, atresia and a progressing tissue fibrosis dominated.

DISCUSSION

Angiogenesis and microvascular remodeling are the known features of chronic inflammatory diseases. Angiogenesis is the growth of new blood vessels from existing ones, whereas microvascular remodeling involves structural alterations (usually enlargement) of arterioles, capillaries or venules, without the formation of new vessels (20). In the present study, in patients with the initial stage of SSc we found signs of atypical capillary proliferation (bushy capillaries).

This is in agreement with experimental observations (27) that SSc initially can evoke angiogenesis. In the late stages of SSc we found only blood vessel deterioration and atresia, no longer angiogenesis. In surrounding vessel-free tissues dominated firm fibrosis. Other, typical of SSc blood vessel damages (24, 33) were also found in our study.

In normal vessels, histamine, bradykinin, substance P, and 5-hydroxytryptamine (5-HT) cause plasma leakage through the formation of focal gaps among endothelial cells [18]. Many substances are known to cause plasma leakage by increasing vascular permeability but only few have the opposite effect. B_2-Adrenergic agonists are among them (1, 14). Endothelial fenestrae, transcytotic vesicles, vesico-vacuolar organelles (VVOs), and monolayer defects may contribute to increased plasma extravasation in pathological conditions accompanied by angiogenesis and microvascular remodeling (7, 10, 28).

Newly formed and remodeled blood vessels typically have abnormalities in endothelial barrier function (8, 13). Several factors are likely to participate in the leakiness of remodeled blood vessels. An increase in endothelial permeability resulting from focal separations ~400 nm (the largest particles to pass across being < 2 μm in diameter) between endothelial cells is likely to be involved (4, 19). Also the enlargement of arterioles may lower the upstream resistance and increase the transmural driving force for leakage. Impaired clearance of extravasated proteins via lymphatics could be another factor (our unpublished data on vascular endothelium growth factor receptors, VEGFR, 20).

In our observations, the SSc patients in the early stage of disease were characterized by a severe or moderate release of endothelial vWF into the perivascular and/or interstitial matrix, whereas normal control skin biopsies were characterized by no vWF diffusion or leakage. No vWF expression was found in negative control samples. Many observations indicate an increase of vWF in circulation in SSc patients (3, 5, 17, 29).

Extravasation of vWF may contribute to pathogenesis of chronic inflammation (6, 23, 31, 25). Abnormalities in the vascular remodeling are potentially reversible by therapeutic intervention (20).

Mast cells in scleroderma have been discussed for past decades without any definite conclusion. It was shown (2, 22) that in SSc skin in the edematous stage in both papillary and reticular dermis mast cell density was significantly increased as compared with normal skin. It was found that mast cells were involved in the development of interstitial edema. But in the sclerotic stage characterized by homogenization of collagen bundles, skin mast cell density was significantly decreased. This is not a statement of all authors (30). There is no comprehensive analysis in the literature concerning the role of mast cells in SSc, probably because of their usual association with immediate hypersensitivity phenomena, graft-versus-host reaction, and very broad spectrum of physiological effects. In normal subjects mast cells are unevenly distributed around dermal appendages, blood vessels, and nerves. Cutaneous mast cell density is highly variable among persons and is known to decrease with age. It is conceivable that mast cells have direct effects on fibroblast physiology. In humans, mast cells are located predominantly in the skin, lungs, and gastrointestinal tract, the organs most seriously affected by the sclerotic process of SSc. The mere presence of mast cells alone, however, is not sufficient for development of fibrosis (21). In our SSc biopsy specimens, in the early stages of disease we found a coincidence of vWF leakage and extensive perivascular mast cell degranulation. The specific mechanism of the coincidence remains to be elucidated.

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VON WILLEBRANDO VEIKSNIO PRASISUNKIMAS IR TUKLIŲJŲ LĄSTELIŲ DEGRANULIAVIMASIS SISTEMINE SKLEROZE SERGANČIŲJŲ ODOJE

S a n t r a u k a

Ištyrėme devynių ligonių, sergančių sistemine skleroze, odos bioptatus. Imunohistochemiškai ir elektroniniu mikroskopu išanalizavome von Willebrando veiksnio ekspresiją, smulkiųjų kraujagyslių morfologiją ir tukliųjų ląstelių degranuliavimąsi. Kontrolinę grupę sudarė dešimt atitinkamo amžiaus žmonių, nesergančių sistemine skleroze.

Nustatėme dažną ekstravaskulinį von Willebrando veiksnio prasisunkimo, smulkiųjų kraujagyslių pažeidimo ir tukliųjų ląstelių degranuliavimosi sutapimą ankstyvojoje sisteminės sklerozės stadijoje.

Vėlyvojoje fibrozinėje sisteminės sklerozės stadijoje jau neaptikome nei intensyvaus von Willebrando veiksnio prasisunkimo iš kraujagyslių sienelių ir spindžių į ekstravazalinį tarpą, nei tukliųjų ląstelių degranuliavimosi. Taip pat pastebėta dalies smulkiųjų odos kraujagyslių atrezija.

Analizės rezultatai parodė ankstyvą kraujagyslių pažeidimą sisteminės sklerozės patogenezėje.

Raktažodžiai: sisteminė sklerozė, kraujagyslės, von Willebrando veiksnys, tukliosios ląstelės, oda