Systemic sclerosis (SSc) is characterised by ischemic damage, impaired angiogenesis and skin fibrosis. Tissue kallikrein (t-kallikrein) is involved through kinins in inflammation, vasorelaxation and angiogenesis. T-kallikrein is synthetised by endothelial, smooth muscle, and inflammatory cells and, in skin, also by dark cells of the sweat glands, where it is involved in sweat formation.

Our aim was to analyse, by immunohistochemistry and RT-PCR, the expression of t-kallikrein in the skin of patients with different SSc subsets, limited (lSSc) and diffuse (dSSc), and phases, early and advanced.

Skin biopsies were taken from 18 SSc patients and 10 controls. Immunohistochemistry was performed on paraffin sections with an antibody against human urinary t-kallikrein.

For RT-PCR, cDNA from skin biopsies was amplified using primers specific for human t-kallikrein. In the control skin, dark cells of the secretory units of sweat glands showed immunopositivity for t-kallikrein as well as blood vessels. In the lSSc skin, immunoreactivity was observed only in some glands, with weak staining in the advanced phase. In early lSSc skin, immunoreactivity was observed in microvessel walls and in the inflammatory infiltrate. In dSSc skin, dark cells of the glandular fundus units, and the few remaining vessels showed scarcity (early phase) or lack (advanced phase) of immunoreactivity for t-kallikrein. RT-PCR confirmed a decrease of t-kallikrein mRNA levels from early to advanced phase in SSc subsets, reaching its lowest level in advanced dSSc.

In conclusion, immunohistochemical and biomolecular results indicate that t-kallikrein is decreased in the skin of SSc patients and decreases progressively from the early to advanced phase of lSSc and dSSc. The decreased expression of t-kallikrein may be involved in the impairment of the sweating process, vessel functionality and angiogenesis.

Key words: Tissue Kallikrein, Skin, Systemic Sclerosis, Sweat glands

Introduction

T-kallikrein (hK1 or true tissue kallikrein) is a serine protease that cleaves low molecular weight kininogen to produce kinins, which interact with B₁ and B₂ receptors. B₂ receptors are constitutively expressed and mediate most biological effects of bradykinin and Lys-bradykinin, while B₁ receptors are induced by tissue damage and inflammation (Xiong et al., 1992; Emanuelli et al., 2001). T-kallikrein mRNA is widely synthetized in a wide range of tissues including kidney, exocrin glands such as eccrine sweat, salivary and pancreas, endocrine or endocrine-related tissues such as testis, prostate, breast and endometrium and in the central nervous system (Marceau et al., 1998; Mahabeer and Bhoola, 2000; Yousef and Diamandis, 2001; Komatsu et al., 2003). Moreover, it is expressed by endothelial and smooth muscle cells in the aorta as well as in medium and small vessels (Wolf et al., 1999), and neutrophils (Wu et al., 1993). T-kallikrein synthetized at vessel level acts through kinins which modulate a broad spectrum of vascular functions (Emanuelli et al., 2000, 2001), playing an important role in the regulation of vascular homeostasis (Wolf et al., 1999; Mahabeer and Bhoola, 2000) and in angiogenesis (Mahabeer and Bhoola, 2000; Plendt et al., 2000).

It is now clear that human tissue kallikrein gene family contains at least fifteen genes (Yousef and Diamandis, 2001) but this does not necessarily imply that any of these family members has kininogenase activity. In fact, hK1, the classical t-kallikrein, is highly effective, whereas hK3, for example (also called prostate-specific antigen) is inactive and hK2 is of intermediate potency (Deperthes et al., 1997).
In human skin, kinins are involved, e.g., as comitogens in cellular proliferation or in processes propagating pain and inflammation (Schremmer-Danninger et al., 1999). A role for t-kallikrein and kinins in sweat glands function was already proposed since the presence of kallikrein-like enzymes in human sweat was demonstrated (Fox and Hilton, 1958; Frewin et al., 1973; Mayfield et al., 1989; Hibino et al., 1994). The presence of t-kallikrein in the skin was studied by immunohistochemistry, and immunoreactivity was observed in the dark cells in the fundus of the sweat glands (Poblete et al., 1991).

RT-PCR studies showed that t-kallikrein mRNA is constitutively expressed in human skin, as well as both receptors, whereas kinogen is not detectable (Schremmer-Danninger et al., 1999; Komatsu et al., 2003).

The role of kallikrein-kinin system (KKS) is widely studied in many pathologic conditions including inflammation, hypertension, renal disease, pancreatitis and cancer (Yousef and Diamandis, 2001). In skin diseases or conditions involving the skin, t-kallikrein has been scantily investigated (Poblete et al., 1991; Zaccolo et al., 1992; Hibino et al., 1994).

Systemic sclerosis (SSc) is a connective tissue disease of unknown etiology leading to fibrosis of the skin and internal organs (Clements and Furst, 2003). The pathogenesis of SSc includes microvascular alterations with perivascular inflammatory cell accumulation, abnormal collagen deposition and immune dysfunction. In SSc, impaired angiogenesis, due to microvascular involvement, results in reduced blood flow and tissue ischemia. In the progression of the disease, decrease of thickness of epidermis, flattening of epidermal-dermal papillae and fibrosis of connective tissue represent the typical cutaneous alterations of long-lasting SSc. Furthermore, the skin appendages disappear, except for sweat glands, which, surrounded by abnormal collagen, are pushed up to the mid-dermis (Hashimoto and Niizuma, 1983). In SSc patients, the thermoregulation was studied (Hahn et al., 1998), but the functionality of sweat glands was not assessed.

Since the skin represents one of the main target organs in SSc, we performed an immunohistochemical and biomolecular analysis on skin biopsies of SSc patients according to different subsets and phases, in order to investigate the presence of t-kallikrein in sweat glands and vessels. Particularly, we suppose that an altered expression for t-kallikrein can occur in the different phases of the disease, which, in turn, could contribute to the impairment of skin vascularization, due to its potential role in angiogenesis, and sweat glands functionality.

**Materials and methods**

**Patients and controls**

Eighteen SSc patients (13 females and 5 males, mean age: 51±15.9 years) affected by SSc, attending the outpatient clinic of Department of Medicine, Section of Rheumatology of the University of Florence and University of L’Aquila, were enrolled in the study.

In order to have two comparable groups according to disease subsets, patients affected by diffuse (dSSc, nr: 9) and by limited (lSSc, nr: 9) SSc (LeRoy et al., 1988), undergoing skin biopsies to assess the progression of the disease, were chosen. Patients (lSSc and dSSc) were further classified, according to disease duration, in early (nr: 6 lSSc, 3 dSSc) and advanced phase (nr: 3 lSSc, 6 dSSc) (Medsgier et al., 1996). SSc patients were assessed according to the recent guidelines (VVAA, 2003).

Ten, age- and sex-matched subjects (8 females and 2 males; mean age: 54±6.5 years), not affected by dermatological inflammatory or autoimmune diseases, undergoing surgery for traumatic lesions, were used as controls.

Enrolled subjects gave their written informed consent. Italian Law and the ethical guidelines of the Italian National Medical Council were followed throughout clinical and laboratory procedures.

Before biopsy, all SSc patients underwent 15 days of drug wash-out. During this period, only proton pump inhibitors and clebopride were allowed.

**Biopsies**

Full thickness biopsies of clinically involved skin, approximately 1x0.5 cm, were taken from the middle third of the forearm of SSc patients. We consider clinically involved skin for values, in this area, of skin thickness ≥ 2, according to Rodnan modified skin thickness score (Clements and Furst, 2003). In SSc, skin score evaluates the thickness of the skin as assessed by palpation of 17 body areas on 0 to 3 scale (normal, mild, moderate, severe) and from the summation of the scores from all body areas (Clements et al., 1995). Skin samples taken from the same anatomical area were obtained from healthy controls. Each biopsy was divided in two specimens and processed for immunohistochemistry and for biomolecular analysis, respectively. For RT-PCR analysis, the specimens were immediately immersed in liquid nitrogen and then stored at -80 °C until use.

**Immunohistochemistry**

Biopsies were fixed in paraformaldehyde-lysine-periodate (PLP) fixative (Pieri et al., 2002) at room temperature for 4 hours. The tissue specimens were dehydrated in alcohol graded series and embedded in paraffin wax. Sections (5 μm) were mounted on glass slides coated with polylysine. Immunohistochemistry was performed with a polyclonal antibody raised against human urinary tissue kallikrein (Calbiochem, San Diego, CA, USA) (1:1000). The immunoreactivity was detected using the avidin-biotin complex followed by binding horseradish peroxidase (Vectastain Elite ABC kit, Vector.
Laboratories, Burlingame, CA, USA). The sections were then counterstained with hematoxilin. Negative controls were obtained by omitting the primary antibody.

All the sections processed for immunohistochemistry were observed under a light microscope (Nikon Eclipse E400), and photographed by digital camera (Cool pix 2500 Nikon).

**Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Skin samples were homogenised and total RNA was extracted in 1 ml of TRI-Reagent (Sigma-Aldrich, St. Louis, MO), according to the protocol provided by the manufacturer. Single stranded cDNA was obtained from 1 µg of total RNA, 1.6 µg of oligo-p(dT)15 primer and 20 U of AMV Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany) in a total reaction volume of 20 µl. Semiquantitative determination of t-kallikrein (hK1) mRNA levels was done by an internal standard-based PCR assay with serial dilution of cDNA and using GAPDH as a reference gene. A 203-bp segment of the t-kallikrein cDNA sequence (GenBank Accession number NM_002257) was targeted with upstream primer 5'-GCCAAGCAGACGAGGACTAC-3', bases 371-391, and downstream primer 5'-TTTGAGGTCCACACACTGGA-3', bases 573-593. Amplification was performed with Accuprime Super Mix II (Invitrogen, Carlsbad, CA, USA) in a total reaction volume of 50 µl. The PCR profile was 30 sec at 94 °C, 30 sec at 55 °C and 1 min at 68 °C for 35 cycles. Densitometric analysis was perfomed by NIH Image analysis software.

**Statistics**

Descriptive statistics for clinical variables were expressed as mean ± standard deviation (SD) for continuous variables, and as a ratio for categorical variables. To compare for continuous variables and categorical variables, the two-tailed t-test and the Fisher’s exact test were used, respectively. P-values less than 0.05 were considered statistically significant.

**Results**

Demographic and clinical characteristics of SSc patients are shown in Table 1. The mean disease duration was 5.6 ±4.0. Age and disease duration were not different between patients affected by dSSc and ISSc.

**Immunohistochemistry**

In human skin, the secretory fundus of the eccrine sweat glands is localised in the papillary derma and is formed by dark cells surrounding the lumen, with glycoproteic secretory function, and clear cells laying on basal membrane, with electrolytic pump function. In the control skin, the dark cells of the sweat glands showed a cytoplasmic granular pattern of immunostaining for t-kallikrein at the apical side (Fig.1A,B). T-kallikrein immunopositivity was also observed in luminal ductal cells. No immunostained products were observed in the clear cells and myoepithelial cells in the sweat glands. Dermal vessels showed immunopositivity for t-kallikrein in the endothelial cells, smooth muscle cells and pericytes (Fig. 417).

**Table 1.** Demographic and clinical characteristics of SSc patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>SSc</th>
<th>LIMITED SSc</th>
<th>DIFFUSE SSc</th>
<th>P VALUE (ISSc vs dSSc)</th>
<th>HEALTHY CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51±15.9</td>
<td>57±13.1</td>
<td>52±15.1</td>
<td>ns</td>
<td>54 ± 6.5</td>
</tr>
<tr>
<td>Disease subset</td>
<td>9/18</td>
<td>9/18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>5/18</td>
<td>1/9</td>
<td>4/9</td>
<td>2/10</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>13/18</td>
<td>8/9</td>
<td>5/9</td>
<td></td>
<td>8/10</td>
</tr>
<tr>
<td>Disease duration</td>
<td>5.6±4.0</td>
<td>4.2±1.6</td>
<td>7.0±5.2</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Skin score</td>
<td>16.44±11.6</td>
<td>7.2±3.3</td>
<td>25.7±9.1</td>
<td>p&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Calcinosis</td>
<td>3/18</td>
<td>2/9</td>
<td>1/9</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Fingertip ulcers</td>
<td>5/18</td>
<td>2/9</td>
<td>3/9</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Other skin ulcers</td>
<td>6/18</td>
<td>3/9</td>
<td>3/9</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Autoantibodies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANA +</td>
<td>18/18</td>
<td>9/9</td>
<td>9/9</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Scl-70 +</td>
<td>7/18</td>
<td>2/9</td>
<td>5/9</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>ACA +</td>
<td>8/18</td>
<td>6/9</td>
<td>2/9</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>RF +</td>
<td>2/18</td>
<td>1/9</td>
<td>1/9</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>DLCO (%)</td>
<td>72.2±21.2</td>
<td>85.9±12.2</td>
<td>58.6±19.6</td>
<td>p&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

In ISSc skin, both in the early and advanced phase, immunopositivity for t-kallikrein in sweat glands had a patchy distribution: the sections showed immunopositivity only in some sweat glands, while other were negative (Fig. 2A). In the positive glands the staining was not homogeneously distributed in all dark cells (Fig. 2B). In the advanced phase, the few stained cells of the positive glands showed weaker immunoreactivity than in the early phase. In vessels of ISSc skin samples, distinct cytoplasmic reactivity for t-kallikrein was detected in vascular smooth muscle cells and endothelial cells, as well as in pericytes (Fig. 2C). The endothelial cells appeared hypertrophic and partially occluding the lumen of small vessels. Moreover, in early ISSc, inflammatory infiltrate, consisting mainly in neutrophils, monocytes and some lymphocytes, observed around microvessels at the upper papillary derma level, was positive for t-
kallikrein (Fig. 2C). In advanced lSSc, immunoreactivity of vessels was still present.

In early dSSc, immunopositivity of sweat glands for t-kallikrein was present in all dark cells of glandular fundus and in ductal cells, but the staining intensity was weaker than in control and early lSSc samples. In advanced dSSc, scarce or lack of immunoreactivity was detected in most of the dark cells of the fundus units (Fig. 3A,B) and in the ductal cells. Interestingly, in advanced dSSc the glandular cells appeared reduced in size, as well as the whole gland. Exuberant collagen substituted fat tissue, normally present around the glands, and wrapped the glandular fundus. Other appendages, as well as the vessels, mostly disappeared with disease progression and exacerbation of fibrosis.

In early dSSc, vessels were reduced and weakly stained for t-kallikrein. The inflammatory features were less relevant, and the infiltrate mainly consisted in different cells, such as lymphocytes and plasmacells, which were not positive for t-kallikrein. In advanced dSSc, few remaining vessels in papillary and reticular dermis did not show any immunoreactivity (Fig. 3C). Only some microvessels, close to the secretory units of sweat (Fig. 3A) and sebaceous glands and to the nerves, still showed immunoreactivity in endothelial cells.

Reactivity was absent when anti-t-kallikrein antibody was omitted.

**Evaluation of t-kallikrein mRNA expression levels**

Figure 4 shows the representative t-kallikrein bands from SSc and control samples. T-kallikrein expression levels in the skin of early lSSc (lane lSSc e) was comparable to the controls and significantly higher than that obtained from the other SSc samples. In advanced lSSc (lane lSSc a), t-kallikrein mRNA levels resulted similar to that observed for early dSSc (lane dSSc e), where advanced dSSc (lane dSSc a) shows a significant decrease respect to the other samples.

The results of immunohistochemistry and RT-PCR on t-kallikrein are summarised in Table 2.

Table 2. Comparison between t-kallikrein mRNA and immunohistochemistry evaluation.

<table>
<thead>
<tr>
<th>SSc phases</th>
<th>LIMITED SSc</th>
<th>DIFFUSE SSc</th>
<th>HEALTHY CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>early</td>
<td>advanced</td>
<td>early</td>
</tr>
<tr>
<td>mRNA levels</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Immunopositivity</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Fig. 3. dSSc skin, advanced phase. **A.** Cross section. Eccrine sweat gland shows scarce immunoreactivity. The vessels close to the secretory units are still immunopositive (arrow). **Insert:** the dark cells of a fundus unit show scarce or no immunopositivity. **B.** Longitudinal section. Secretory fundus dark cells are weakly stained. **C.** Cross section. Immunonegative vessel is shown. t-kallikrein immunohistochemistry with DAB developing. A, x 20; insert, x 40; B, x 60; C, x 40
Discussion

This is the first report on the expression and localisation of t-kallikrein in the skin of SSc patients.

The presence of t-kallikrein was studied by biomolecular (RT-PCR) and immunohistological analysis in normal human skin and in some diseases involving the skin (Poblete et al., 1991; Zucollo et al., 1992; Ekholm and Egelrud, 1999; Schremmer-Danninger et al., 1999; Komatsu et al., 2003).

In agreement with previous reports, we detected t-kallikrein, by immunohistochemistry, in the dark cells of the secretory fundus units of eccrine sweat glands in healthy human skin (Poblete et al., 1991), and we also found immunopositivity in the ductal luminal cells and in the dermal blood vessel wall (Hibino et al., 1994).

In SSc skin, the distribution and expression of t-kallikrein were different from controls and between the two subsets. In ISSc, immunoreactivity for t-kallikrein in sweat glands had a patchy distribution, similar to the other pathological cutaneous alterations observed in this subset, where areas with pathological features were close, with abrupt transition, to healthy looking areas not different from control biopsies (L. Ibba-Manneschi, unpublished data). Differently from sweat glands, all vessels present in dermis were immunopositive.

In the early phase of ISSc, also the perivascular inflammatory infiltrate was immunopositive. T-kallikrein or other components of KKS amplify the production of some inflammatory (Tiffany and Burch, 1989; Hayashi et al., 1998; Sardi et al., 1998; Knox et al., 2001) and fibrotic factors (Ricupero et al., 2000), which are activated in the early phase of SSc. This may indicate a role of t-kallikrein in perivascular inflammation (Figueroa et al., 1989; Poblete et al., 1991; Wu et al., 1993), relevant in the early phase of the disease.

Fig. 4. Upper panel. Representative RT-PCR analysis for t-kallikrein and GAPDH mRNAs from control patients (C) and SSc patients. Lower panel. Graphic representation of t-kallikrein mRNA levels evaluated by an internal standard-based (GAPDH) semiquantitative RT-PCR technique (see “Materials and methods” for details). Amplification products were run on an agarose gel and the ethidium bromide-stained bands were quantified by densitometric analysis (NIH analysis software). Each histogram represents the mean ± S.E. of three values of t-kallikrein amplification products normalized to the starting cDNA volumes and referred to the corresponding GAPDH values. P<0.05 early ISSc vs each SSc group; advanced dSSc vs each SSc group and control; control vs advanced ISSc and early dSSc.
Nevertheless, in early dSSc, the inflammatory process is less relevant and differs in the involved cell types in respect to early lSSc. This may account, together with the reduced synthesis from the sweat glands and vessels, for the decreased levels of m-RNA t-kallikrein in early dSSc in respect to early lSSc.

In the advanced phase of lSSc, in the positive glands, few cells showed immunoreactivity, suggesting a loss of ability by the dark cells to produce t-kallikrein. PCR results confirmed a reduction of mRNA levels for the enzyme in the advanced phase of lSSc, in comparison both with control and early lSSc samples.

dSSc skin specimens showed scarce or no immunoreactivity for t-kallikrein in the secretory fundus units and luminal ductal cells. The reduction of t-kallikrein was homogeneously widespread in the skin of dSSc. The expression of t-kallikrein mRNA, in advanced dSSc, was significantly decreased when compared to lSSc and control samples and also to early dSSc.

Moreover, in advanced dSSc the cutaneous vasculature is deeply reduced and, since an important source of t-kallikrein is represented by endothelial cells, the decrease of its mRNA level observed in dSSc skin may also be due to the reduced number of vessels and to a functional exhaustion of endothelial cells to produce the protein. In dSSc, the reduction in size, both of the whole glands and of the single secretory units, might suggest a gland hypofunctionality. While in lSSc both immunohistochemical and biomolecular results could suggest that the dark cells of the secretory fundus still secrete t-kallikrein, in dSSc they are unable to express this activity.

T-kallikrein seems to carry out several functions in the sweating process, through kinins generations, including stimulation of sodium transport, as observed in cultured sweat ductal cells (Brayden and Cuthbert, 1990), mitotic activity in the secretory epithelium after recovery from sweating (Rixon and Whitfield, 1973; Poblete et al., 1991), and increase of periglandular blood flow (Hibino et al., 1994). We can assume that the altered or reduced expression of t-kallikrein in SSc patients favours the impairment of the sweating process. Autonomic Nervous System (ANS) plays a relevant role in sweating regulation both directly, by stimulating t-kallikrein secretion from sweat glands (Zucollo et al., 1992) and indirectly, by controlling periglandular blood flow. In SSc skin, a sympathetic overactivity and an impaired parasympathetic activity were shown (Matucci-Cerinic et al., 1996; Bertinotti et al., 2004). In fact, sympathetic skin response, evoked during microneurography by stimulating sympathetic skin activity, was impaired in SSc patients (Bertinotti et al., 2004; Casale et al., 2004).

ANS plays a role in the development of many clinical manifestations in SSc (Matucci-Cerinic et al., 1996; Bertinotti et al., 2004) both in early and advanced phases, and also in the control of blood flow and vascular tone. The impairment of ANS innervation (Casale et al., 2004) and the decreased synthesis of t-kallikrein could contribute to an altered thermoregulatory eccrine sweating in SSc patients.

In summary, by immunohistochemical and biomolecular analysis, we provided evidence that the expression of t-kallikrein is decreased in SSc skin. The t-kallikrein reduction is greater in dSSc than in lSSc and in the advanced than in the early phase, reaching the lowest level in advanced dSSc. This reduction, found both in sweat glands and in vessels, may be involved in the impairment of the sweating process, vessel functionality and angiogenesis. However, further studies are needed in order to clarify whether kallikrein-kinin system alterations have a primary role in the pathogenesis of SSc or are consequences of SSc development and/or course.
Tissue kallikrein expression in the skin of systemic sclerosis

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