

# *Acta Medica Okayama*

---

*Volume 50, Issue 6*

1996

*Article 2*

DECEMBER 1996

---

## Increased endothelial and epidermal thrombomodulin expression and plasma thrombomodulin level in progressive systemic sclerosis.

Hitoshi Mizutani\*

Tatsuya Hayashi†

Nobuhiro Nouchi‡

Shin Inachi\*\*

Koji Suzuki††

Masayuki Shimizu‡‡

\*Mie University Faculty of Medicine,

†Mie University Faculty of Medicine,

‡Mie University Faculty of Medicine,

\*\*Mie University Faculty of Medicine,

††Mie University Faculty of Medicine,

‡‡Mie University Faculty of Medicine,

# Increased endothelial and epidermal thrombomodulin expression and plasma thrombomodulin level in progressive systemic sclerosis.\*

Hitoshi Mizutani, Tatsuya Hayashi, Nobuhiro Nouchi, Shin Inachi, Koji Suzuki, and Masayuki Shimizu

## Abstract

To clarify the relation between systemic and cutaneous vascular endothelial injury in progressive systemic sclerosis (PSS), we examined thrombomodulin (TM) expression in PSS skin lesions immuno-histopathologically and compared it with plasma soluble TM levels measured by specific enzyme-linked immunosorbent assay. The plasma soluble TM level in PSS patients was significantly higher than that of normal controls and was as high as the levels of SLE patients. In relation to disease activities, the plasma TM levels of sclerotic phase PSS patients were significantly higher than that of atrophic phase PSS patients. The plasma samples with anti-Scl-70 antibody showed a high TM level than samples with anti-centromere antibody or anti-RNP antibody. Barnett's types or systemic corticosteroid treatment did not affect the TM level. Histopathologically, the dermal endothelial TM expression significantly increased in the sclerotic skin and moderately increased in the non-sclerotic skin of PSS compared with that of normal control skin. In addition, immunoreactive TM expression in the epidermis also increased in PSS. Disease activity-dependent elevation of plasma TM levels and immuno-histopathological expression of TM suggested generalized endothelial and epidermal cell involvement in PSS, and compensation in part by overproduction of TM by endothelial cells.

**KEYWORDS:** thrombomodulin, scleroderma, skin, endothelial cells, keratinocyte

---

\*PMID: 8985465 [PubMed - indexed for MEDLINE]

Copyright (C) OKAYAMA UNIVERSITY MEDICAL SCHOOL

## Increased Endothelial and Epidermal Thrombomodulin Expression and Plasma Thrombomodulin Level in Progressive Systemic Sclerosis

Hitoshi MIZUTANI\*, Tatsuya HAYASHI<sup>a</sup>, Nobuhiro NOUCHI, Shin INACHI, Koji SUZUKI<sup>a</sup> and Masayuki SHIMIZU

Department of Dermatology and <sup>a</sup>Department of Molecular Biology on Genetic Disease, Mie University Faculty of Medicine, Mie 514, Japan

To clarify the relation between systemic and cutaneous vascular endothelial injury in progressive systemic sclerosis (PSS), we examined thrombomodulin (TM) expression in PSS skin lesions immuno-histopathologically and compared it with plasma soluble TM levels measured by specific enzyme-linked immunosorbent assay. The plasma soluble TM level in PSS patients was significantly higher than that of normal controls and was as high as the levels of SLE patients. In relation to disease activities, the plasma TM levels of sclerotic phase PSS patients were significantly higher than that of atrophic phase PSS patients. The plasma samples with anti-Scl-70 antibody showed a higher TM level than samples with anti-centromere antibody or anti-RNP antibody. Barnett's types or systemic corticosteroid treatment did not affect the TM level. Histopathologically, the dermal endothelial TM expression significantly increased in the sclerotic skin and moderately increased in the non-sclerotic skin of PSS compared with that of normal control skin. In addition, immunoreactive TM expression in the epidermis also increased in PSS. Disease activity-dependent elevation of plasma TM levels and immuno-histopathological expression of TM suggested generalized endothelial and epidermal cell involvement in PSS, and compensation in part by overproduction of TM by endothelial cells.

**Key words:** thrombomodulin, scleroderma, skin, endothelial cells, keratinocyte

**T**hrombomodulin (TM) is a membrane protein expressed on the surface of endothelial cells which expresses anticoagulation activity via activation of protein

C after binding to thrombin (1, 2). Recently, a soluble form of TM was recognized in vasculitic disease patients' plasma (pTM) and some relation between the pTM level and disease activity has been reported in systemic lupus erythematosus (SLE) (3-5). However, the function, precise mechanism of elevated pTM and the source of pTM are still unclear. We focused on progressive systemic sclerosis (PSS) as a collagen disease with vascular involvement, including inflammation followed by sclerotic fibrosis, vascular spasm and long lasting vasomotor disorders. We measured the pTM level in PSS patients and compared immuno-histopathological TM expression in the sclerotic skin.

### Patients and Methods

**Patients.** Thirty-seven PSS patients aged 27 to 72 years old (mean 49.9) and 21 SLE patients aged 25 to 65 years old (mean 45.3) who met the American Rheumatism Association criteria. PSS patients were subdivided according to their autoantibodies: anti-Scl-70 antibody (Scl, n = 20), anti-RNP antibody (RNP, n = 6), anti-centromere antibody (ACA, n = 6) and other autoantibody groups (other, n = 5), Barnett's types (6), and groups with or without systemic corticosteroid treatment. PSS patients were also divided into sclerotic phase (n = 22) and atrophic phase groups (n = 15). Normal control values were obtained from 24 age-matched volunteers. All the patients and controls were female.

**Plasma sample preparation.** Blood samples were obtained from brachial vein puncture with 10% volume of 10mM sodium citrate. Plasma was immediately separated by centrifugation and stored at -70°C before preparation.

---

\* To whom correspondence should be addressed.

**Human-TM specific monoclonal antibodies.**

Monoclonal antibody: mouse monoclonal antibody for 5th EGF domain of TM (MFTM-5) was raised and purified as reported previously (7). MFTM-5 recognizes the thrombin binding sites and competes with thrombin for binding to TM. Another mouse monoclonal antibody raised to purified human TM is MFTM-6, which recognizes non-thrombin binding sites of TM without competing with MFTM-5 (7, 8).

**Recombinant human TM.** Recombinant human TM was purified by thrombin affinity column chromatography from culture condition medium of COS-1 cells transfected with the full length h-TM DNA plasmid cloned, as reported previously (9).

**Human TM ELISA.** Concentration of plasma samples were measured by solid phase sandwich enzyme-linked immunosorbent assay (ELISA) system reported previously (3). Serially diluted known concentrations of affinity purified human TM or plasma samples were applied to microtubes containing MFTM-5-coated beads (Sumitomo, Tokyo, Japan). After 2h incubation and washing at room temperature, peroxidase conjugated MFTM-6 was applied as the second antibody and developed with tetramethyl benzidine. The absorbance of each tube at 450nm was measured by spectrophotometer. The TM concentration of the samples was calculated from the standard curve. This ELISA system recognizes 1 to 100 ng/ml of TM.

**Statistical analysis.** Data was analyzed by the Mann-Whitney U-test.

**Immunohistochemical study.** Skin biopsy specimens were obtained from sclerotic forearm skin lesions of PSS patients and normal volunteers. Non-sclerotic skin of PSS samples were obtained from the upper arm. Freshly obtained 6mm punch biopsy skin specimens were fixed with poly-l-lysine paraformaldehyde solution or without fixation and were frozen embedded in OTC compound (Miles, MA, USA). All samples were stored at  $-70^{\circ}\text{C}$  before processing. The tissue samples were processed as reported previously (10), 5- $\mu\text{m}$  sections on poly-l-lysine-coated slides were incubated with 1  $\mu\text{g}/\text{ml}$  of MFTM-5 and/or MFTM-6 as the first antibody after blocking by normal rabbit sera. Rabbit anti-mouse antibody and alkaline phosphatase/anti-alkaline phosphatase antibody complex were used as the second antibody complex. Sections were developed with fast red substrates (APAAP Kit, DAKO-Japan, Kyoto, Japan).

**Results**

**Plasma TM concentration.** The plasma TM level in PSS patients ( $1.71 \pm 0.07 \text{ ng/ml}$ , mean  $\pm$  SE,  $P < 0.01$ ) was significantly higher than that of normal controls ( $1.38 \pm 0.12 \text{ ng/ml}$ ). This PSS TM level was as high as the plasma TM level in SLE patients ( $1.72 \pm 0.12 \text{ ng/ml}$ ,  $P < 0.05$ ) measured as positive control (Fig. 1).

The plasma TM level from sclerotic phase patients ( $n = 22$ ) was  $1.97 \pm 0.09 \text{ ng/ml}$ , which was significantly higher than that of atrophic phase patients ( $n = 15$ ,  $1.49 \pm 0.07 \text{ ng/ml}$ ,  $P < 0.01$ , Fig. 1).

Plasma TM level in the Scl, ACA, RNP and other groups were  $1.79 \pm 0.10$ ,  $1.59 \pm 0.25$ ,  $1.33 \pm 0.07$ ,  $1.80 \pm 0.10 \text{ ng/ml}$ , respectively (Fig. 2). The Scl and other groups TM levels were significantly higher than that of the normal controls ( $P < 0.01$ ).

The TM level of corticosteroid-treated patients ( $1.66 \pm 0.09 \text{ ng/ml}$ ) was slightly lower than that of the non-corticosteroid treated group ( $1.74 \pm 0.14 \text{ ng/ml}$ ). There was no difference in the TM levels among Barnett's three types (type 1:  $1.75 \pm 0.14$ , type 2:  $1.66 \pm 0.13$ , type 3:  $1.66 \pm 0.10 \text{ ng/ml}$ ).

**Immunohistochemical expression of TM in the skin.** Normal human dermal capillaries, venule, lymph vessels and small arteries showed moderately but

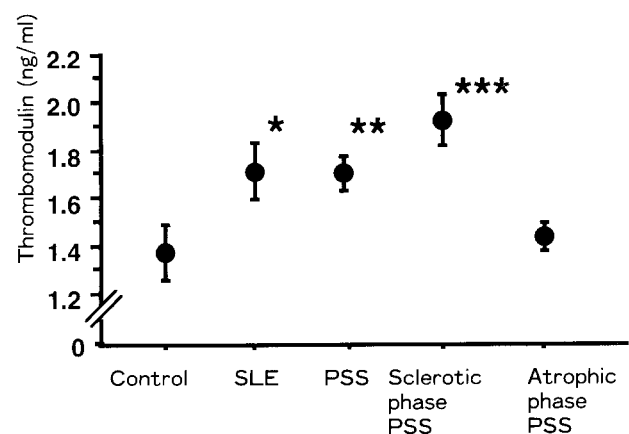
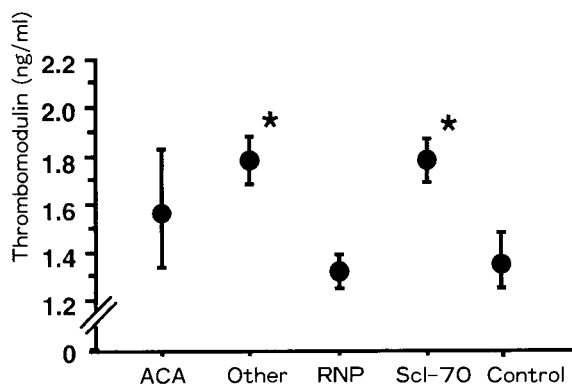


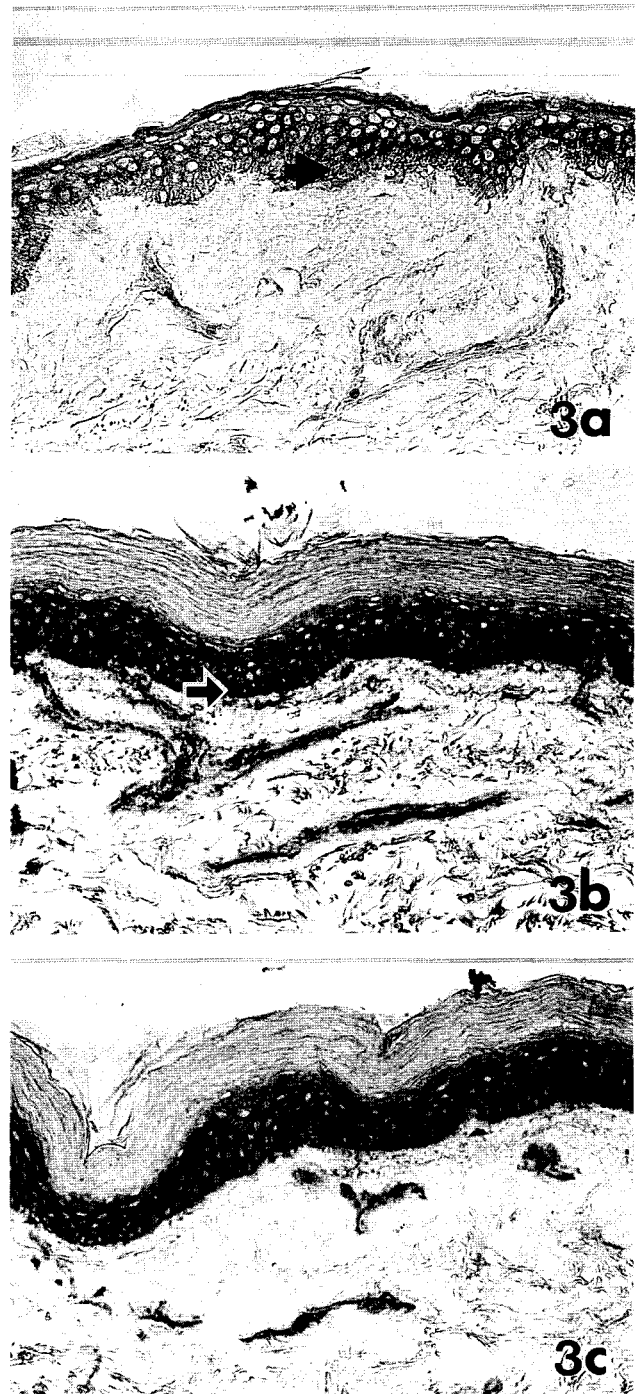
Fig. 1 Plasma thrombomodulin (TM) level in progressive systemic sclerosis (PSS) was significantly higher than age-matched normal controls (\*\* $P < 0.01$ ), which was as high as the pTM level in SLE (\* $P < 0.05$ ). The plasma TM level varied between disease phases. Active sclerotic phase samples showed significantly higher TM level than that of atrophic phase patients (\*\* $P < 0.01$ ).

clearly positive staining (Fig. 3a). Epidermal keratinocytes were also stained positively from suprabasal to subcorneal layer cells. We could not find positive staining at the basal layer cells in ten normal skin samples from various regions including the upper arm, face, trunk and forearm. The immunoreactive TM distributed around the cell membrane and showed a basket-like pattern. No samples showed cytoplasmic staining pattern. These staining patterns did not differ between samples stained by MFTM-5 and MFTM-6. Since the background staining was slightly low in MFTM-5 samples, we used MFTM-5.

The clinically and histopathologically sclerotic forearm skin of PSS patients showed marked greater expression of TM on the dermal capillaries, veins and arterioles compared with normal control skin obtained from the corresponding part of the body (Fig. 3b). The epidermis also showed a highly dense staining pattern. Different from normal skin, we found intense immunoreactive TM distribution through basal layer cells to subcorneal layer cells. A positive basal cell pattern was observed in all of



**Fig. 2** The plasma TM level of Scl, ACA RNP and other antinuclear antibody groups were  $1.79 \pm 0.1$ ,  $1.59 \pm 0.25$ ,  $1.33 \pm 0.07$  and  $1.80 \pm 0.1$ , respectively. In Scl and other groups TM levels were significantly higher than those of normal control ( $*P < 0.01$ ). TM: See Fig. 1.



**Fig. 3** Immunohistochemical expression of thrombomodulin in normal and PSS skin. **a:** The dermal vessels of normal skin showed moderate staining for MFTM-5 monoclonal anti-TM antibody. Epidermis was also stained at Malpighian layer cells, but not at basal cells (arrow) ( $50 \times$ ). **b:** The sclerotic forearm skin in PSS showed significantly stronger staining for TM on the dermal vascular endothelial cells. Epidermis also showed more intensive staining than normal epidermis. Different from normal basal cells, the sclerotic skin basal cells showed intensive immunoreactive TM expression (arrow), as in the upper epidermis ( $50 \times$ ). **c:** Biopsy specimen from non-sclerotic upper arm skin from PSS also shows intense immunoreactive TM expression on the dermal vasculature close to the sclerotic skin lesions. Basal layer cells also expressed TM at a level similar to the sclerotic skin basal cells ( $50 \times$ ). TM: See Fig. 1.

the PSS samples examined. In contrast to the clear membrane distribution of TM in normal skin, the augmented epidermal TM staining of PSS skin showed thick and diffuse staining around the cell membrane. Three samples from normal looking, non-sclerotic upper arm skin were examined. While these samples did not show sclerotic changes histologically, the dermal and epidermal immunoreactivity to anti-TM antibodies were close to the sclerotic skin lesions (Fig. 3c).

## Discussion

In contrast to the well known anti-coagulation function of the endothelial cell surface TM, little is known about the function of the plasma TM. Elevation of pTM has been reported in vasculitic diseases (11), disseminated intravascular coagulation (12) and SLE (5, 13, 14). Since the extracellular domain of endothelial TM is susceptible to enzymatic degradation, including trypsin (15), it has been speculated that TM is shed from endothelial cell membranes by enzymes from infiltrated leukocytes. The smaller molecular weight and much lower cofactor activity of pTM compared with membrane-bound TM also support the idea that pTM is shed endothelial TM. Therefore, pTM has been regarded as a new marker for endothelial damage (11).

Disease activity elevation of plasma and serum TM levels were reported in SLE. Because of the milder clinical course and less significant histopathological vasculitic changes, there are few reports on the pTM level in PSS (16). However, the present results showed a significant increase of pTM in the sclerotic phase of PSS but not in the atrophic phase. In addition, the pTM level was significantly higher in the Scl group, and moderately high in the ACA group but not in the RNP groups. Anti-Scl-70 antibody is a marker for severe PSS (17), and Scl groups show progressive, widespread skin involvement and lung fibrosis. ACA and RNP manifest a milder clinical course. Thus, it is likely that the PSS pTM level reflects the disease activity of PSS as it does in SLE. Vasculitis of PSS is very limited morphologically. However, vasomotor disturbances, Raynaud's phenomenon, thrombosis and ischemic vascular lesions involve PSS systematically. Since ischemia is a potent inducer of pTM (11), elevation of pTM in PSS can be attributed to vascular disturbances.

Inflammatory cytokines such as IL-1 and TNF-alpha down-regulate endothelial cell TM expression *in vitro* (18).

The high-pTM systemic disease is equal to the inflammatory cytokine productive disease (19-22). Scleroderma mononuclear cells produce inflammatory cytokines, causing the plasma cytokine level to become elevated (23, 24). The *in vivo* TM regulation system is still obscure. Endothelial TM may be down-regulated once by inflammatory cytokines. *In vivo*, different from *in vitro*, there must be some feedback system which protects endothelial cells from over coagulation due to TM depletion. It is possible to hypothesize that over-expression of endothelial TM in PSS antagonized autoimmune injury.

Another novel finding was enhanced epidermal TM expression through the basal layer to the Malpighian layer. In the normal condition, epidermal basal cells do not express TM (10, 25). Keratinocyte TM expression can be augmented by differentiation with elevated levels of calcium (10, 26). Epidermal damage in PSS has not been studied as intensively as the epidermal liquefaction degeneration in SLE. However, the sclerotic skin lesion with hyperpigmentation and depigmentation shows keratin-positive amyloid deposition (27), which strongly suggests persistent basal cell degeneration in PSS. The immunological attack against basal keratinocytes may induce cellular damage with calcium flux. Thus, PSS basal cells showed a phenotypic alteration mimicking differentiated keratinocytes with TM expression.

Epidermal TM is a full-sized and functional membrane-bound form (10), which can be cleaved enzymatically into soluble form (data not shown). The contribution of epidermal TM to the elevation of plasma TM in PSS can not be eliminated. However, the epidermis does not come into direct contact with the blood, but endothelial cells do. It is likely that the shed endothelial cell TM is a major source of plasma TM and it has been compensated for overproduction. Histological endothelial TM expression is more extensive in the active sclerotic skin but less significant in non-sclerotic upper arm skin. The plasma TM level was also elevated in the sclerotic phase PSS and progressive anti-Scl-70 antibody-positive cases. Thus, the plasma TM level appears to be related to tissue TM expression.

Plasma TM has been regarded as an indicator of endothelial cell damage resulting from vasculitis. The present data imply an active compensation mechanism of endothelial cells, and its contribution to pTM elevation.

Acknowledgments. This work was supported in part by a grant from the Ministry of Education and the Ministry of Health and Welfare of Japan.

## References

1. Suzuki K, Kusumoto H and Hashimoto S: Isolation and characterization of thrombomodulin from bovine lung. *Biochim Biophys Acta* (1986) **882**, 343-352.
2. Suzuki K, Kusumoto H, Deyashiki Y, Nishioka J, Maruyama I, Zushi M, Kawahara S, Honda G, Yamamoto S and Horiguchi S: Structure and expression of human thrombomodulin, a thrombin receptor on endothelium acting as a cofactor for protein C activation. *EMBO J* (1987) **6**, 1891-1897.
3. Kodama S, Uchijima E, Nagai M, Mikawatani K, Hayashi T and Suzuki K: One-step sandwich enzyme immunoassay for soluble human thrombomodulin using monoclonal antibodies. *Clin Chim Acta* (1990) **192**, 191-199.
4. Ishii H and Majerus PW: Thrombomodulin is present in human plasma and urine. *J Clin Invest* (1985) **76**, 2178-2181.
5. Boehme MW, Nawroth PP, Kling E, Lin J, Amiral J, Riedesel J, Raeth U and Scherbaum WA: Serum thrombomodulin: A novel marker of disease activity in systemic lupus erythematosus. *Arthritis Rheum* (1994) **37**, 572-577.
6. Barnett AJ: *Scleroderma*, Charles C ed, Thomas, Springfield (1974) pp 74-93.
7. Hayashi T and Suzuki K: Monoclonal antibodies for human thrombomodulin which recognize binding site for thrombin and protein C. *J Biochem* (1990) **108**, 874-878.
8. Hayashi T, Zushi M, Yamamoto S and Suzuki K: Further localization of binding sites for thrombin and protein C in human thrombomodulin. *J Biol Chem* (1990) **265**, 20156-20159.
9. Suzuki K: Gene structure of human thrombomodulin, a thrombin receptor on endothelium acting as a cofactor for thrombin-catalyzed activation of protein C. *Nippon Ketsueki Gakkai Zasshi* (1988) **51**, 1655-1664.
10. Mizutani H, Hayashi T, Nouchi N, Ohyanagi S, Hashimoto K, Shimizu M and Suzuki K: Functional and immunoreactive thrombomodulin expressed by keratinocytes. *J Invest Dermatol* (1994) **103**, 825-828.
11. Wada H, Ohiwa M, Kaneko T, Tamaki S, Tanigawa M, Shirakawa S, Koyama M, Hayashi T and Suzuki K: Plasma thrombomodulin as a marker of vascular disorders in thrombotic thrombocytopenic purpura and disseminated intravascular coagulation. *Am J Hematol* (1992) **39**, 20-24.
12. Wada H, Minamikawa K, Wakita Y, Nakase T, Kaneko T, Ohiwa M, Tamaki S, Deguchi K, Shirakawa S, Hayashi T and Suzuki K: Increased vascular endothelial cell markers in patients with disseminated intravascular coagulation. *Am J Hematol* (1993) **44**, 85-88.
13. Karmochkine M, Boffa MC, Piette JC, Cacoub P, Wechsler B, Godeau P, Juhan V and Weiller PJ: Increase in plasma thrombomodulin in lupus erythematosus with antiphospholipid antibodies (letter). *Blood* (1992) **79**, 837-838.
14. Takaya M, Ichikawa Y, Kobayashi N, Kawada T, Shimizu H, Uchiyama M, Moriuchi J, Watanabe K and Arimori S: Serum thrombomodulin and anticardiolipin antibodies in patients with systemic lupus erythematosus. *Clin Exp Rheumatol* (1991) **9**, 495-499.
15. Kurosawa S, Galvin JB, Esmon NL and Esmon CT: Proteolytic formation and properties of functional domains of thrombomodulin. *J Biol Chem* (1987) **262**, 2206-2212.
16. Soma Y, Takehara K, Sato S and Ishibashi Y: Increase in plasma thrombomodulin in patients with systemic sclerosis (letter). *J Rheumatol* (1993) **20**, 1444-1445.
17. Catoggio LJ, Bernstein RM, Black CM, Hughes GR and Maddison PJ: Serological markers in progressive systemic sclerosis: Clinical correlations. *Ann Rheum Dis* (1983) **42**, 23-27.
18. Archipoff G, Beretz A, Freyssinet JM, Klein-Soyer C, Brisson C and Cazenave JP: Heterogeneous regulation of constitutive thrombomodulin or inducible tissue-factor activities on the surface of human saphenous vein endothelial cells in culture following stimulation by interleukin-1, tumor necrosis factor, thrombin or phorbol ester. *Biochem J* (1991) **273**, 679-684.
19. Hemmer CJ, Bierhaus A, von Riedesel J, Gabat S, Liliensiek B, Pitronik P, Lin J, Grauer A, Amiral J, Ziegler R: Elevated thrombomodulin plasma levels as a result of endothelial involvement in plasmodium falciparum malaria. *Thromb Haemostasis* (1994) **72**, 457-464.
20. Takakuwa T, Endo S, Nakae H, Kikichi M, Suzuki T, Inada K and Yoshida M: Plasma levels of TNF-alpha, endothelin-1 and thrombomodulin in patients with sepsis. *Res Commun Chem Pathol Pharmacol* (1994) **84**, 261-269.
21. Takakuwa T, Endo S, Nakae H, Yamada Y, Inada K, Yoshida M, Ogawa M and Uchida K: Relationship between plasma levels of type II phospholipase A2, PAF acetylhydrolase, endothelin-1, and thrombomodulin in patients with infected burns. *Res Commun Mol Pathol Pharmacol* (1994) **86**, 335-340.
22. Neumann FJ, Ott I, Gawaz M, Richardt G, Holzapfel H, Jochum M and Schomig A: Cardiac release of cytokines and inflammatory responses in acute myocardial infarction. *Circulation* (1995) **92**, 748-755.
23. Mizutani H, Mizutani T, Yamakami A and Shimizu M: Disease activity dependent IL-6 production by peripheral blood mononuclear cell from PSS in *Current Topics in Scleroderma*, Seino Print Co., Gifu (1992) pp 193-197.
24. Needleman BW, Wigley FM and Stair RW: Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor alpha, and interferon-gamma levels in sera from patients with scleroderma (see comments). *Arth Rheum* (1992) **35**, 67-72.
25. Mizutani H, Ohyanagi S, Hayashi T, Groves RW, Suzuki K and Shimizu M: Functional thrombomodulin expression on epithelial skin tumors as a differentiation marker for suprabasal keratinocytes. *Br J Dermatol* (1996) **135**, 187-193.
26. Raife TJ, Lager D, Madison KC, Piette WW, Howard EJ, Sturm MT, Chen Y and Lentz ST: Thrombomodulin expression by keratinocytes: Induction of cofactor activity during epidermal differentiation. *J Clin Invest* (1994) **93**, 1846-1851.
27. Ogiyama Y, Hayashi Y, Kou C, Matsumoto Y and Ohashi M: Cutaneous amyloidosis in patients with progressive systemic sclerosis. *Cutis* (1996) **57**, 28-32.

---

Received May 13, 1996; accepted August 6, 1996.