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REGULAR ARTICLE

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Detection of human soluble Thy-1 in serum by ELISA

Fibroblasts and activated endothelial cells are a possible source of soluble Thy-1 in serum

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Abstract The functions of Thy-1, a 35-kDa cell-surface glycoprotein, and its natural ligand are still unknown. Anchoring to the membrane via linkage to phosphatidylinositol (PI) raises the possibility of cleavage off the membrane by PI-specific phospholipases. Soluble Thy-1 (sThy-1) could interfere with the binding of the unknown natural ligand followed by regulation of different cell functions. In this study we established an enzyme-linked immunosorbent assay (ELISA) to measure and quantify sThy-1 in serum and wound fluid. Recombinant human Thy-1 (rhThy-1) was expressed in Drosophila S2 cells, purified from culture supernatant and used as standard for quantitation of sThy-1 by the ELISA technique. There were no differences in sThy-1 levels in serum of healthy donors and patients with systemic sclerosis, leg ulcers, or rheumatoid arthritis, respectively, detected by ELISA. In contrast, at the local site of inflammation, in wound fluid of venous leg ulcers and in synovial fluid from joint puncture, we found strongly elevated levels of sThy-1 compared with sThy-1 in the serum of the same patient. Thy-1 is expressed in humans on brain cells, fibroblasts, a subpopulation of CD34+ blood stem cells, and possibly activated human dermal microvascular endothelial cells. In this study, we never found Thy-1 mRNA or protein expression in resting endothelial cells as shown by reverse transcriptase polymerase chain reaction (RT-PCR) and flow-cytometry. Thy-1 expression could be induced on endothelial cells by phorbol myristate acetate and to a lesser extent by tumor necrosis factor-α (TNF-α). In situ, monoclonal antibodies to Thy-1 did not stain endothelial cells in normal skin, whereas endothelial cells in the synovial membrane of rheumatoid arthritis patients and endothelial cells surrounding melanoma express Thy-1. In summary, our data indicate that Thy-1 is present in soluble form in serum. Furthermore, Thy-1 seems to be a marker for endothelial cell activation. Therefore, activated endothelial cells as well as fibroblasts might be a possible source of sThy-1.

Key words Endothelial cell activation · Thy-1 (CD90) · Soluble Thy-1 · Human

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Introduction

Previously, we described a monoclonal antibody against human fibroblasts (Saalbach et al. 1996). This monoclonal antibody recognizes predominantly fibroblasts in human skin. The corresponding antigen was purified, biochemically characterized, and was found to be the human Thy-1 (CD90) antigen (Saalbach et al. 1998). The remarkable diversity of distribution and expression of Thy-1 between species and between tissues of the same species suggest that it might have distinct functions in different tissues and species (Dalchau et al. 1989). In mouse, it has been shown to be expressed on the surface of various cells including thymocytes, T-cells, neurons, fibroblasts, myoblasts, epidermal cells, and bone marrow stem cells.

Conversely, in humans the Thy-1 expression is restricted to neuronal cells, fibroblasts, and a subset of CD34+ blood stem cells. There are conflicting results concerning the expression of Thy-1 on endothelial cells (EC). Mason et al. (1996) have demonstrated Thy-1 on EC in the skin and on microvascular as well as macrovascular EC in vitro. In contrast, Romero et al. (1997) have not detected Thy-1 on resting microvascular EC. Ishizu et al. (1997a) have found Thy-1 on EC in vitro, although the EC did not express Thy-1 in situ.

The functions of Thy-1 and the natural ligand are still unknown. Thy-1 is a cell-surface glycoprotein with a molecular weight of about 35 kDa. The anchoring of the Thy-1 molecule to the membrane via linkage to phospha-

tidylinositol (PI) raises the possibility of cleavage off the membrane by PI-specific phospholipases. In vitro the spontaneous shedding of Thy-1 from the cell surface of murine T-lymphocytes and lymphoblastoid cell lines has been shown (Freimuth et al. 1978; Vitetta et al. 1974). Various other cell surface proteins such as intercellular adhesion molecule-1 (ICAM-1), E-selectin, vascular cell adhesion molecule (VCAM), and receptors for cytokines (interleukin receptors IL-1R, IL-2R) are shed from the cell surface (Liu et al. 1999). Soluble cell-free receptors are able to regulate or block important immunological or biological interactions. Thy-1 belongs to the Ig superfamily, suggesting a role as cell adhesion molecule (Dalchau et al. 1989). Soluble Thy-1 (sThy-1) could interfere with the binding of the unknown natural ligand, followed by regulation of different cell functions. Only few data are available on the in vivo release of Thy-1 in humans (Almqvist and Carlsson 1988).

The first aim of this study was to establish an enzyme-linked immunosorbent assay (ELISA) to measure sThy-1 in order to prove whether sThy-1 is detectable in serum or biological fluids and to obtain primary data on pathological situations showing increased levels of sThy-1. From published data, four cell types might be a source of sThy-1 in humans: brain cells, the subpopulation of CD34+ blood stem cells, fibroblasts, and possibly activated human dermal microvascular endothelial cells (HDMEC; Mason et al. 1996; Romero et al. 1997; Saalbach et al. 1998). In this study, we examined the expression of Thy-1 on activated endothelial cells on protein and mRNA levels in vitro and in situ.

Materials and methods

Materials

Skin biopsies, wound fluid, and serum were obtained from patients at the department of dermatology at the Leipzig University. Samples were obtained from patients with systemic sclerosis (SSc; *n*=12) who fulfilled American College of Rheumatology preliminary criteria (Subcommittee for Scleroderma Criteria 1980), venous leg ulcers (*n*=8), and from healthy donors matched for sex and age (*n*=12). Patients with venous leg ulcers were treated with compression and occlusive hydrocolloid dressings (Varihesive). Serum and synovial fluid from knee joint puncture from patients with rheumatoid arthritis (RA) were kindly provided by Dr. U. Wagner (University of Leipzig).

Expression and purification of recombinant human Thy-1

A 332-bp cDNA fragment (bp 58–387) encoding for human Thy-1 from amino acid 20 to 129 was cloned into the pMT/BiP/V5-His A vector using *Bgl* II/*Xho* I sites (Invitrogen; Caerlsbad, Calif.). This construct yields the mature Thy-1 protein without the signal peptide and glycosylphosphatidylinositol (GPI) anchor. *Drosophila* S2 cells were cotransfected with the construct and the pCoHYGRO harboring the gene for resistance to hygromycin according to the manufacturer's protocol (Invitrogen). Thy-1-expressing cells were cultured in DES expression medium containing 10% FCS and 300 µg/ml hygromycin up to 1.5×10^7 cells/ml. For protein expression, cells were cultured in DES serum-free medium. Thy-1 expression was induced by adding copper sulfate to a final concentration of 500 µM. Culture supernatant was concentrated by ultrafiltration (YM-10; Millipore-Amicon, Eschborn, Germany). Protein purification was performed using affinity chromatography to the His tag of the construct according to the manufacturer's protocol with Ni-based Probond resin (Invitrogen). The eluted recombinant human Thy-1 (rhThy-1) was concentrated by ultrafiltration (YM-10; Amicon) and finally purified by gel filtration (Sephadex-200; Biorad, München, Germany). The Thy-1-containing fraction was concentrated and the protein content was determined according to Lowry (Lowry et al. 1951). The purity of the rhThy-1 was proved by SDS gel electrophoresis and Coomassie staining.

Immunoblotting

Culture supernatant of Thy-1-transfected S2 cells was collected 5 days after induction and separated electrophoretically under nonreducing conditions on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described (Saalbach et al. 1996). The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), blocked for 2 h with 5% nonfat milk powder in PBS, and treated overnight at 4°C with an anti-Thy-1 antibody (mAb AS02; 0.2 µg/ml; Dianova, Hamburg, Germany). After washing, bound antibody was detected using a goat anti-mouse antibody conjugated with alkaline phosphatase (Sigma, Deisenhofen, Germany) followed by a color reaction with nitroblue tetrazolium (NBT) and 5-bromo-1-chloro-3-indolyl phosphate (BCIP; Promega, Mannheim, Germany).

The ELISA

In the first experiments, we investigated whether the mAb AS02 and anti-Thy-1 antibody (Pharmingen, Hamburg, Germany) recognize different epitopes of Thy-1. Fibroblasts were incubated with the anti-Thy-1 antibody for 15 min at 4°C followed by the fluorescein isothiocyanate (FITC)-labeled mAb AS02 (Dianova). Using flow-cytometry we showed that the anti-Thy-1 antibody (Pharmingen) did not block the binding of the labeled mAb AS02 to fibroblasts (data not shown).

Microtiter plates were coated with anti-Thy-1 antibody (0.5 µg/well in PBS; Pharmingen, Hamburg, Germany) overnight at 4°C. Plates were washed three times with PBS and blocked with PBS/10% FCS for 30 min at room temperature. The samples and standard (rhThy-1) were diluted in PBS to achieve a measurement in the linear range of the ELISA (70–300 ng/ml). After further washes, the samples and standard (range for calibration curve, 575–36 ng/ml) were applied in duplicate for 4 h at 37°C. The plates were washed three times with PBS. The biotinylated mAb AS02 (Saalbach et al. 1996) was added for 90 min at 37°C. After three washes with PBS, extravidin-conjugated alkaline phosphatase (Sigma) 1:10 000 diluted in PBS/5% BSA was added for 1 h at room temperature. Plates were rinsed three times with PBS. Subsequently, paranitrophenyl phosphate (pNPP, 1 mg/ml) was used to generate color reaction that was measured at 405 nm. The Thy-1 concentration in the samples was calculated from the standard curve. Total protein concentration in serum and wound fluid was determined according to Lowry (Lowry et al. 1951). The statistical significance was evaluated with Mann-Whitney *U*-Wilcoxon rank sum *W*-test. A *P*-value of less than 0.01 was deemed statistically significant.

Cultures and stimulation of HDMEC

HDMEC from adult skin were purchased from Clonetics (Endo-Pack-MV; distributed by Cellsystems, St. Katharinen, Germany). The EGM-MV Bulletkit was used for culture media according to the original protocol.

Subconfluent monolayers of HDMEC were used for stimulation studies with phorbol 12-myristate 13-acetate (PMA; Sigma), lipopolysaccharide (LPS; Sigma), recombinant human tumor necrosis factor-α (rhTNF-α), basic fibroblast growth factor (bFGF), IL-1β (Strahtmann, Hannover, Germany). After the medium was

removed, cell monolayers were washed twice with HEPES/NaCl (30 mM, 150 mM, pH 7.0) and incubated with fresh medium containing 5% FCS and the appropriate stimulus. Cell stimulation in serum-free media did not result in different data.

Reverse transcriptase polymerase chain reaction

RNA for reverse transcriptase polymerase chain reaction (RT-PCR) analysis was prepared with the "Micro spin mRNA purification kit" (Amersham-Pharmacia Biotech, Freiburg, Germany) according to the original protocol.

An aliquot of the mRNA preparation was used for first-strand synthesis with MLV reverse transcriptase (LifeTechnologies, Eggenstein, Germany) according to the standard protocol. PCR-Supermix (LifeTechnologies) was used for the PCR. The PCR products were analyzed in a 2% agarose gel containing ethidium bromide. PCR primers were synthesized at LifeTechnologies Custom Primer Services. The following primer pairs were used:

GAP-DH	5′	CTTCACCACCATGGAGAAGG	3′
	5′	CCAGTGAGCTTCCCGTTCAG	3′
Thy-1	5′	ATGAACCTGGCCATCAGCATCGC	3′
	5′	CGAGGTGTTCTGAGCCAGCAGGC	3′

The PCR conditions were as follows:

1. GAP-DH: 94°C for 5 min; 35 cycles of 15 s at 94°C, 30 s at 55°C, 45 s at 72°C; 5 min at 72°C
2. Thy-1: 94°C for 5 min; 35 cycles of 15 s at 94°C, 30 s at 61°C, 45 s at 72°C; 5 min at 72°C

FACS analysis

Cells were trypsinized with 0.025% trypsin, 0.01% EDTA after incubation with PMA or cytokines and washed twice with PBS. Cells were labeled with 20 µl of the anti-Thy-1, anti-CD31, and anti-ICAM-1 antibody (Stock, 200 µg/ml), respectively, for 45 min at room temperature. After two washes with PBS, the cells were incubated with the secondary fluorescein isothiocyanate (FITC)-labeled antibody for 45 min. Flow-cytometrical analysis was performed after two final washes using an EPICS-Profile II (Coulter, Krefeld, Germany). Earlier tests proved that trypsin treatment does not alter the levels of cell-bound Thy-1, ICAM-1, and CD31.

Immunohistochemistry

Frozen tissue sections from skin, synovial membrane of RA patients, melanoma, and lymph node metastases of melanoma were fixed with acetone for 10 min and dried. Sections were incubated for 1 h with mAb AS02 (0.4 µg/ml; Dianova) followed by the incubation with goat-anti-mouse antibody and alkaline phosphatase-antialkaline phosphatase (APAAP) complex (Dako, Hamburg, Germany). Washing was performed with 0.9% NaCl/0.05 M TRIS-HCl, pH 7.4. Bound antibodies were detected by a color reaction with the New Fuchsin substrate system (Dako). Counterstaining was performed with hematoxylin.

Results

Detection of sThy-1 in serum

In this study we established an ELISA to investigate whether there is sThy-1 in human serum and biological fluids. Therefore, rhThy-1 was expressed in *Drosophila* S2 cells and purified from culture supernatant. The anti-Thy-1 monoclonal antibody AS02 (Dianova) as well as the anti-Thy-1 antibody (Pharmingen; data not shown)

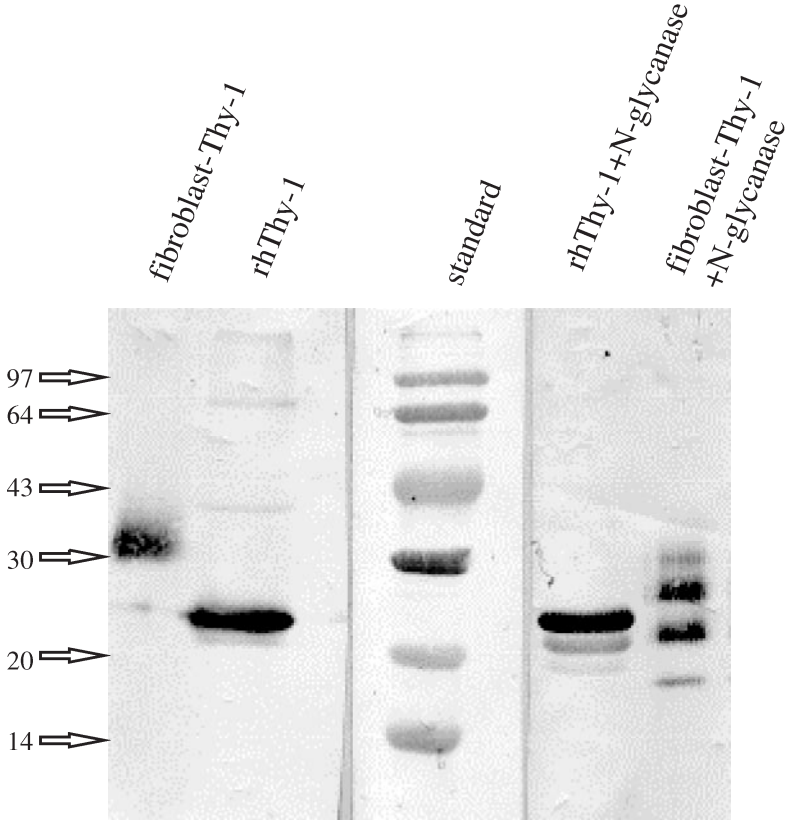
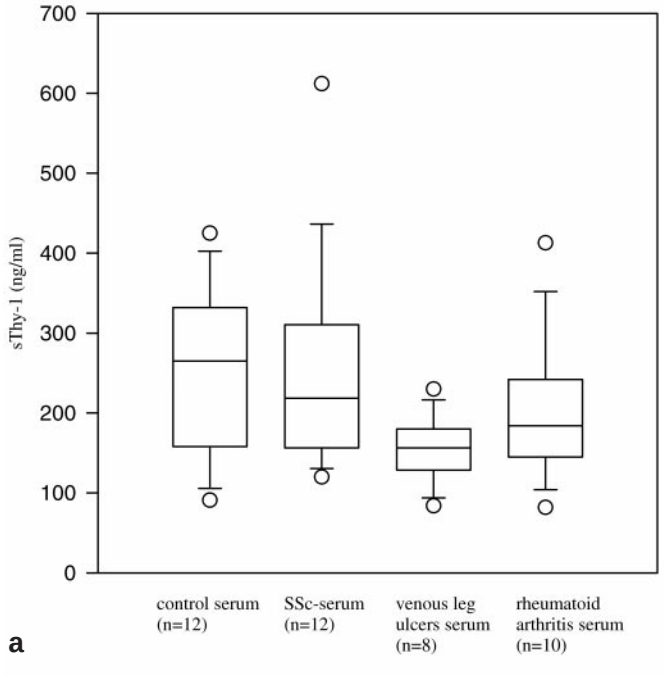
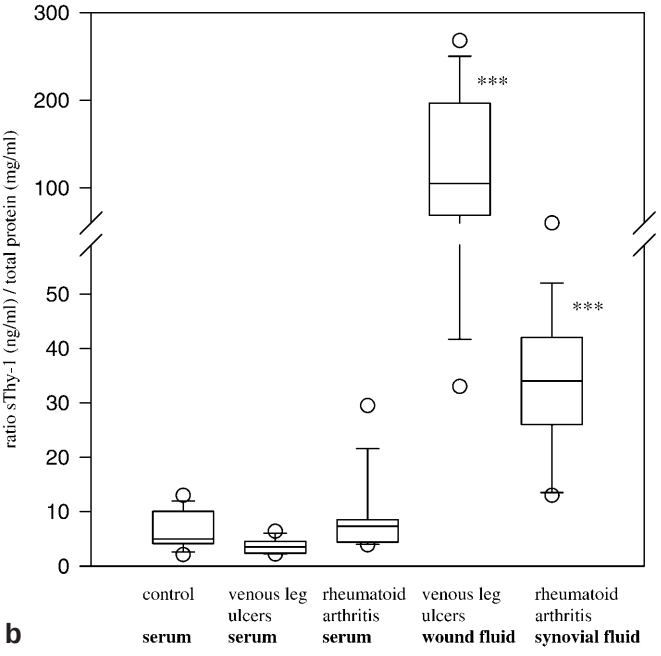


Fig. 1 Comparison of recombinant human Thy-1 (*rhThy-1*) and Thy-1 from human dermal fibroblasts. Human cDNA for Thy-1 was expressed in *Drosophila* S2 cells. SDS-PAGE under nonreducing conditions and immunoblotting revealed a protein band at 25 kDa. In comparison, Thy-1 detected in fibroblast crude extracts has a molecular weight of 30–35 kDa (*left*). After treatment with *N*-glycanase (2 U/ml), deglycosylation products of 19 kDa and 21 kDa (rhThy-1) and 17 kDa, 22 kDa, and 27 kDa (*fibroblast-Thy-1*) are detectable with mAb AS02 (*right*). The several protein bands result from different degrees of cleavage of the carbohydrate chains by the *N*-glycanase. *Arrows* indicate the position of standard proteins





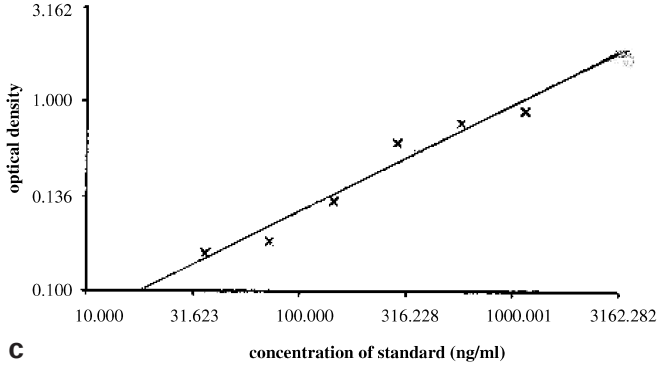


Fig. 2a–c Detection of soluble Thy-1 (*sThy-1*) levels by ELISA in serum. Microtiter plates were coated with an anti-Thy-1 antibody. The sThy-1-standard (range 36–575 ng/ml) and samples (serum from healthy donors, patients suffering from systemic sclerosis, *SSc*, venous leg ulcers, and wound fluid from venous leg ulcers) were applied in duplicate. Bound material was detected by a biotinylated anti-Thy-1 antibody (mAb AS02) and an avidin-alkaline phosphatase conjugate followed by substrate reaction with paranitrophenyl phosphate (pNPP). The extinction was measured at 405 nm. The sThy-1 concentration in the samples was calculated from the standard curve using rhThy-1. **a** Concentration of sThy-1 (nanograms sThy-1 per milliliter) in serum of healthy controls, patients suffering from scleroderma, venous leg ulcers, and rheumatoid arthritis (RA). **b** Ratio of sThy-1 concentration (nanograms sThy-1 per milliliter) to total protein concentration (milligrams total protein per milliliter) in serum of healthy donors, patients with venous leg ulcers and RA as well as in wound fluid from venous leg ulcers and in synovial fluid from knee joint puncture from RA patients. ***Significantly higher values than in control subjects, $P<0.01$ by Mann-Whitney *U*-Wilcoxon rank-sum test **c** The calibration curve (log-log plot) of the ELISA indicates the linear range of measurement

recognize the rhThy-1 expressed by *Drosophila* S2 cells with a molecular mass of about 25 kDa (Fig. 1). The recombinant protein is smaller and has a different glycosylation pattern, as shown by *N*-glycanase treatment, compared with the native Thy-1 isolated from fibroblast membranes (Fig. 1).

The purified rhThy-1 was used for the generation of a standard curve to quantify sThy-1 in different biological fluids. An anti-Thy-1 antibody (Pharmingen) and a biotinylated anti-Thy-1 antibody (mAb AS02; Dianova) were used in a sandwich ELISA system. Blocking experiments revealed that both antibodies recognize different epitopes of Thy-1 (data not shown).

Firstly, we investigated whether sThy-1 is present in serum. Using the ELISA, we measured 251±105 ng sThy-1/ml serum from healthy donors (n=12; Fig. 2a). Furthermore, we studied sThy-1 levels in serum of patients suffering from scleroderma, a disease characterized by fibrosis, vascular damage, and partial involvement of inner organs. In the serum of 12 patients with localized disease as well as with systemic participation, we measured 255±131 ng sThy-1/ml (Fig. 2a). Interestingly enough, in the serum of one patient in whom the disease exhibited strong progression and involvement of the lung, esophagus, kidney, and heart, an elevated level of sThy-1 (612 ng sThy-1/ml) was detectable.

Venous leg ulcers and RA are characterized by inflammation and activation of various cell types present at the local site. In serum from patients with venous leg ulcers and RA, we detected 156±41 ng sThy-1/ml and 207 ng sThy-1/ml , respectively (Fig. 2a). In contrast, in both cases in the local environment, the wound fluid from chronic ulcers, and synovial fluid from knee joint puncture, we found high levels of sThy-1 (2570±1546 ng sThy-1/ml wound fluid; 794 ng sThy-1/ml synovial flu-

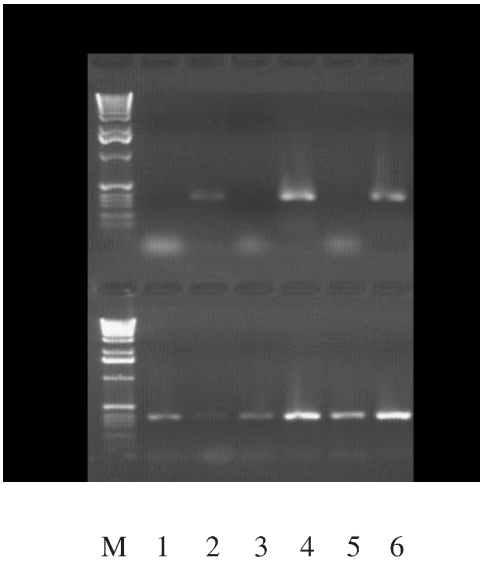


Fig. 3 Induction of Thy-1 mRNA expression in human dermal microvascular endothelial cells (HDMEC) by phorbol 12-myristate 13-acetate (PMA) at various passages. HDMEC were stimulated with PMA (20 ng/ml). mRNA was prepared 24 h after treatment. Aliquots of cDNA were used for reverse transcriptase polymerase chain reaction (RT-PCR). Note that control cells (*lane 1*, 3rd passage; *lane 3*, 4th passage; *lane 5*, 5th passage) never expressed Thy-1 mRNA, whereas the PMA-treated cells (*lanes 2, 4, 6*, respectively) did

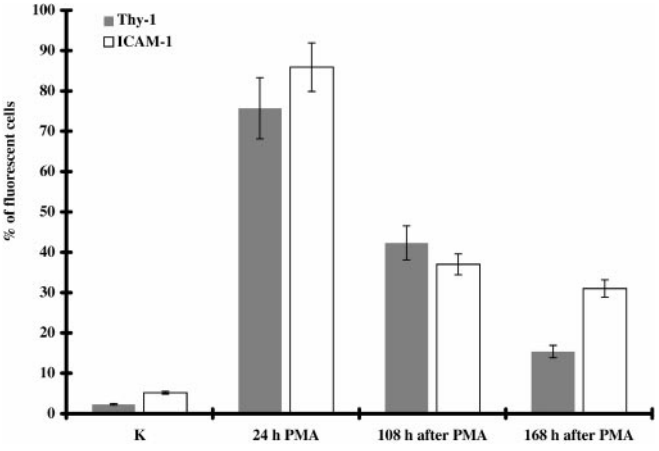


Fig. 4 *Thy-1* and intercellular cell adhesion molecule -1 (*ICAM-1*) reached maximum expression 24 h after PMA treatment and decreased after this time. HDMEC were treated with PMA (20 ng/ml), and Thy-1 and ICAM-1 expression on the cell surface was measured by flow-cytometry using anti-Thy-1 (mAb AS02) and anti-ICAM-1 antibodies (*K* control without PMA)

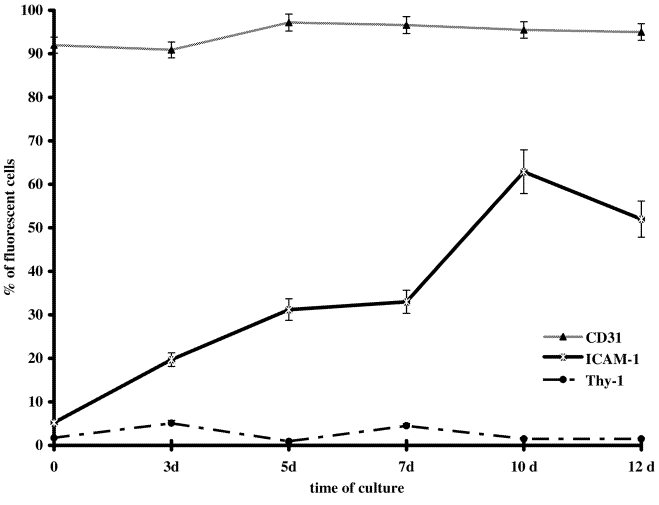


Fig. 5 In contrast to ICAM-1, Thy-1 is not induced in confluent cultures of HDMEC. Six parallel cultures of HDMEC were analyzed at various time points from 90% confluence (day 0) to 12 days of culture without splitting for the expression of *ICAM-1*, *Thy-1*, and *CD31* by flow-cytometry. The medium was changed every 2nd day. About 98% living cells were detected before all measurements were performed

id). To compare sThy-1 levels in serum and wound or synovial fluid, we determined the ratio of sThy-1 concentration (nanograms sThy-1 per milliliter) to milligrams total protein per milliliter. As shown in Fig. 2b, in wound fluid the sThy-1 level per milligram of total protein is significantly enhanced (about 5- to 70-fold) compared with sThy-1 per milligram of total protein in the serum (P=0.0008). Similarly, in synovial fluid the sThy-1 per milligram of total protein is about two- to tenfold increased compared with sThy-1 per milligram of total protein in the serum (P=0.0006; Fig. 2b).

Detection of Thy-1 on HDMEC in vitro

In culture, we found monolayers of cobblestone-like cells that express CD31 antigen, von Willebrand Factor VIII, and small amounts of ICAM-1, but do not express collagens I and III (data not shown).

In vitro, HDMEC do not express Thy-1 protein as well as mRNA. This was proved with different batches of cells. Furthermore, we investigated whether there is expression of low constitutive mRNA levels in resting cells by RT-PCR and whether there are changes in the expression and inducibility of the Thy-1 mRNA over several passages. In contrast to previously published results (Mason et al. 1996), we never found that untreated endothelial cells expressed any detectable amount of Thy-1 mRNA in vitro (Fig. 3).

Using PMA (20 ng/ml) to perform cell activation in vitro, we observed a significant switch of morphology to spindle-shaped cells. In FACS analysis we could show that PMA induces the expression of Thy-1 as well as ICAM-1, a known activation antigen in HDMEC (Fig. 4). Figure 4 shows that Thy-1 protein is induced at 24 h after addition of PMA and the expression declines after this time. Thy-1 is not induced in confluent monolayer cultures of HDMEC, whereas ICAM-1 is induced over the time of cell culture without adding any stimulus, (Fig. 5). The proinflammatory cytokine TNF-α is a less potent inducer than PMA and induces Thy-1 protein expression in

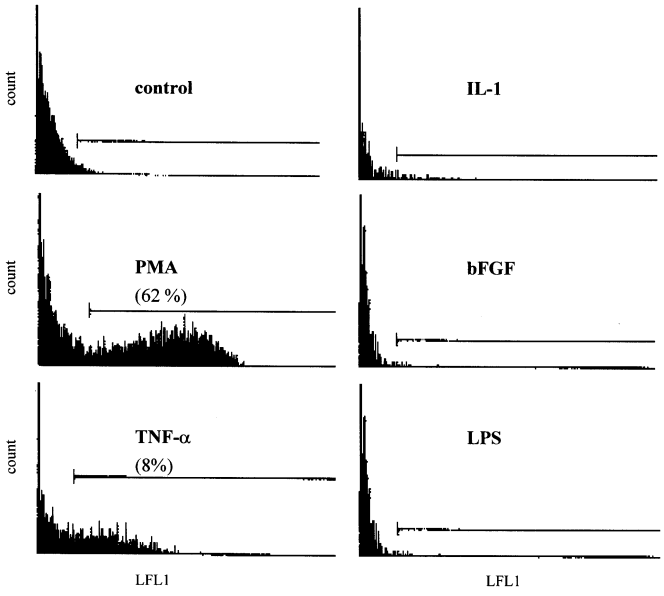


Fig. 6 Induction of Thy-1 protein expression on HDMEC by *PMA* and tumor necrosis factor-α (*TNF-α*). HDMEC were incubated with medium (*control*), with PMA (20 ng/ml), TNF-α (10 ng/ml), interleukin-1β (*Il-1β*; 10 ng/ml), basic fibroblast growth factor (*bFGF*; 200 ng/ml), and lipopolysaccharide (*LPS*; 40 μg/ml). After 24 h, HDMEC were detached and Thy-1 expression was measured by flow-cytometry using the mAb AS02. Only PMA and TNF-α induce the Thy-1 expression on a subpopulation of 62% and 8% of HDMEC, respectively (representing one individual experiment). The induction efficiencies varied depending on the batch of HDMEC used (*LFL1* log fluorescence 1)

Fig. 7 Induction of Thy-1 mRNA expression by PMA and TNF-α in HDMEC detected by RT-PCR. HDMEC were incubated with medium (*control*), with PMA (20 ng/ml), TNF-α (10 ng/ml), Il-1β (10 ng/ml), bFGF (200 ng/ml), and LPS (40 μg/ml). After 24 h, mRNA was prepared and first-strand synthesis was performed. An aliquot of cDNA was used for RT-PCR detecting Thy-1 and GAP-DH (*1* control cells, *2* PMA, *3* Il-1β, *4* TNFα, *5* bFGF, *6* LPS, *W* control without cDNA)

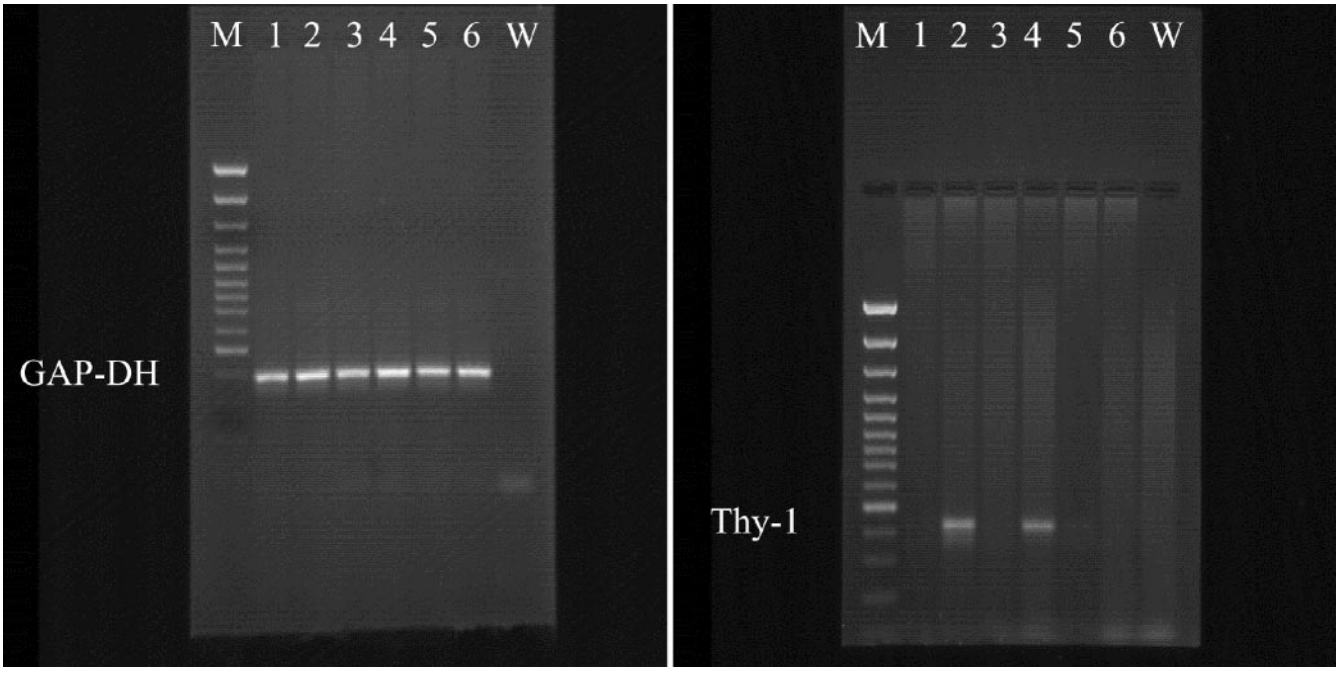
only a small number of the cells (Fig. 6). IL-1β did not enhance the effect of TNF-α. IL-1β alone, bFGF, and LPS did not induce Thy-1 protein expression (Fig. 6). Using RT-PCR we proved that only PMA and TNF-α are able to induce Thy-1 mRNA expression (Fig. 7).

Thy-1 expression on HDMEC in vivo

To obtain data on Thy-1 expression and cell activation in situ, we stained cryostat sections of normal skin (control), synovial membrane (RA), and melanoma with anti-Thy-1 antibody (mAb AS02, Dianova) by immunohistochemistry. In cryostat sections of healthy skin, fibroblasts were stained by mAb AS02 (red color), whereas endothelial cells were not (Fig. 8a). In synovial membranes of RA patients, Thy-1 is expressed on endothelial cells (Fig. 8b). In melanoma (Fig. 8c) and lymph-node metastases (Fig. 8d), stromal fibroblasts as well as endothelial cells closely related to the tumor and endothelial cells of lymph vessels expressed Thy-1.

Discussion

Many proteins expressed on the cell surface can be released from the cell membrane and are found as soluble proteins regulating immunological and biological cell functions (Liu et al. 1999). Thy-1 is a GPI-anchored protein that is constitutively expressed on fibroblasts and can be cleaved off the cell membrane by phospholipase C treatment (Saalbach et al. 1998). In mouse, spontaneous shedding of Thy-1 from the cell surface of T-lymphocytes and lymphoblastoid cell lines was observed (Freimuth et al. 1978; Vitetta et al. 1974). In previous experiments we detected Thy-1 in the culture superna-



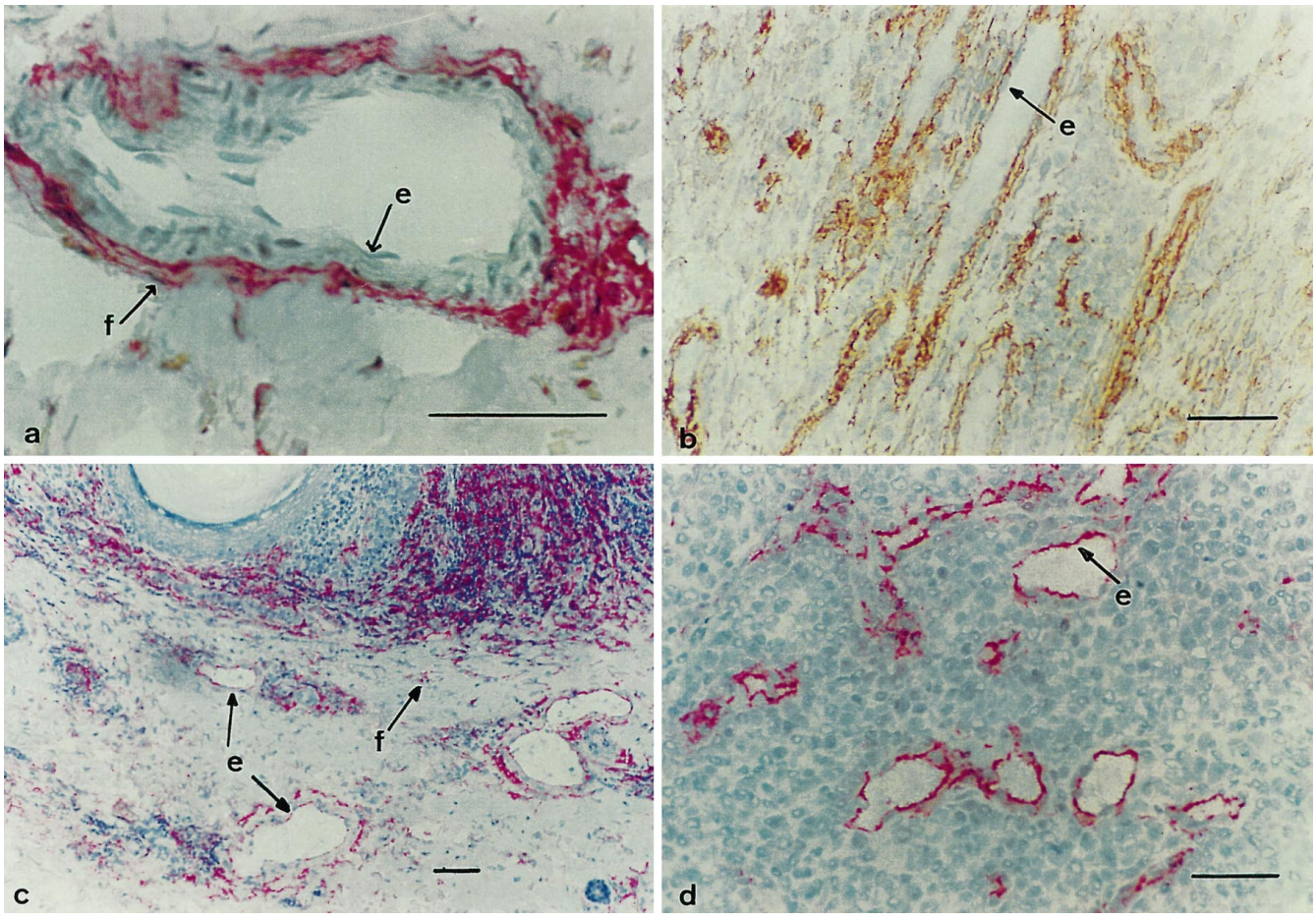


Fig. 8a–d Immunohistochemical analysis of Thy-1 expression in normal skin (**a**), synovial membrane of RA patients (**b**), melanoma (**c**), and melanoma metastases (**d**). In normal skin, endothelial cells were not stained by anti-Thy-1 antibody. In contrast, in synovial membrane of RA patients, melanoma and melanoma metastases Thy-1 is expressed on fibroblasts as well as endothelial cells (*red color*; *f* fibroblast, *e* endothelial cell). *Bar* 25 μm

tant of untreated human fibroblasts by Western blot (Saalbach et al. 1998). Almqvist and Carlsson (1988) detected a hydrophilic form of Thy-1 in human cerebrospinal fluid. From these data arose the question of whether sThy-1 can be found in serum or biological fluids and whether there are situations with increased levels of sThy-1. In our laboratory we established an expression system to produce rhThy-1. The rhThy-1 consists of the complete amino acid sequence of the mature protein except for the amino acids necessary for binding to PI, representing a structural analogue to the shed sThy-1. The smaller molecular weight of rhThy-1 expressed in *Drosophila* S2 is caused by an incomplete glycosylation, because the core proteins show a similar molecular weight. The small difference in the molecular weight of the core protein of both proteins is due to the addition of a V5-His-Tag epitope to the rhThy-1. In spite of the incomplete glycosylation and failing of the GPI anchor, the anti-Thy-1 antibodies used recognize the rhThy-1 in immunoblotting. This expression system producing rhThy-1 provided unique conditions to establish and to validate an ELISA for quantitative determination of sThy-1.

In this paper we demonstrate for the first time that sThy-1 is present in human serum, using the ELISA system. We found similar concentrations of sThy-1 in serum as described for sICAM-1, another cell-surface protein of the immunoglobulin superfamily that is present in soluble form in serum (Okamato et al. 1999). Next, we were interested in whether sThy-1 levels are increased under pathological conditions characterized by cell activation or inflammation. Predominantly, in early inflammatory stages of scleroderma, a strong activation of endothelial cells and fibroblasts occurs, represented by an increased expression of ICAM-1, MHC II antigen, collagen I, presence of autoantibodies, vessel damage, and fibrosis (Haustein and Anderegg 1998). However, we did not measure enhanced levels of sThy-1 in sera from SSc patients (n=12). Interestingly enough, in the serum of one patient in which the SSc exhibited a strong progression and involvement of inner organs, we detected a high sThy-1 level (approx. a threefold increase). Further studies will have to confirm the preliminary results to obtain statistically relevant data. Further, elevated serum levels should be expected in patients with recent onset of SSc or other systemic diseases (e.g., lupus erythematosus).

Next, we determined sThy-1 levels in serum of patients with venous leg ulcers and RA as well as the sThy-1 levels at the local site of inflammation and cell activation (wound fluid, synovial fluid). In both processes, inflammatory processes, tissue destruction, and cell activation occur with an involvement of, e.g., immune cells, fibroblasts or synovial lining cells, and endothelial cells. In serum there were no differences between detectable sThy-1 levels and control sera. Taking into consideration that diseases are characterized by local limited processes without strong systemic participation, the sThy-1 levels in serum should not be affected by these processes. In contrast, at the local site of leg ulcers as well as in affected joints of RA patients, in wound and synovial fluid we found high concentrations of sThy-1. According to the total protein content, we showed significantly elevated sThy-1 levels in the wound and synovial fluid compared with serum, indicating an active production or release of sThy-1 in the local environment of venous leg ulcers and joints of RA patients. The wound and synovial fluid contains cytokines, matrix degradation products, enzymes, and other factors representing a cocktail of factors released by activated cells and tissue destruction. The expression of Thy-1 may be induced or enhanced under these conditions and the membrane-bound Thy-1 is shed or cleaved off the cell surface. Almqvist and Carlsson (1988) detected cell-free Thy-1 in human cerebrospinal fluid and suggested an in vivo release of Thy-1 from brain cells by the action of phospholipase D. Raymond et al. (Raymond et al. 1994) report that phosphoinositol-specific phospholipase D (PL-D) has features characteristic for an acute-phase reactant. Further studies demonstrate an endothelial cell PL-D activation by chemotactic migration of neutrophils through the endothelial monolayer, suggesting an amplifying of target and effector cell reactivity during inflammatory response by this process (Cui et al. 1997). Concerning these data, it is conceivable that endogenous phospholipases or phospholipases present in serum or wound fluid are responsible for the cleavage of membrane-bound Thy-1.

Our data reveal that sThy-1 is present in serum and wound and synovial fluids. From published data, four cell types express Thy-1: fibroblasts, nerve cells, the subpopulation of CD34+ blood stem cells, and possibly endothelial cells (Almqvist and Carlsson 1988; Dalchau et al. 1989; Mason et al. 1996; Romero et al. 1997; Saalbach et al. 1998). In previous studies we demonstrated a high constitutive expression of Thy-1 on fibroblasts (Saalbach et al. 1996). Previously, we detected Thy-1 in culture supernatant of fibroblasts, indicating that human fibroblasts are able to shed or to release sThy-1 and could be a source of sThy-1 found in serum and wound fluid (Saalbach et al. 1998).

The microvascular endothelial cells are involved in thromboresistance, inflammatory processes, extravasation of blood cells into the surrounding tissue, tumor metastasis, and many other physiological and pathophysiological processes. During these processes an activation of endothelial cells occur, including expression of cell-surface molecules (selectins, ICAM-1), shedding of cell-surface proteins (sICAM-1, sE-selectin), secretion of soluble mediators (IL-8, RANTES), and activation of enzymes (PL-D; Cui et al. 1997; Favaloro 1993; Griffith et al. 1991).

Conflicting results on the expression of Thy-1 on endothelial cells have been published. To prove whether endothelial cells could be a possible source of sThy-1 in serum and wound fluid, we studied the expression of Thy-1 on resting and activated endothelial cells at both protein and mRNA level. A few authors (Fivenson et al. 1992; Ishizu et al. 1997b; Lee et al. 1998; Mason et al. 1996; Romero et al. 1997) describe the expression of Thy-1 on resting endothelial cells. However, we never found Thy-1 on resting endothelial cells in vitro. Additionally, using RT-PCR for detection of trace amounts of Thy-1 mRNA, we could clearly demonstrate that resting endothelial cells used in the experiments do not express Thy-1 mRNA. The results are emphasized by the failure to stain any endothelial cells with anti-Thy-1 antibodies in normal skin in situ (Saalbach et al. 1996).

Different results have been published concerning the inducibility of Thy-1 on endothelial cells by phorbol ester and cytokines in vitro (Ishizu et al. 1997a; Mason et al. 1996; Romero et al. 1997). Our data of HDMEC stimulation by PMA show a wide range of Thy-1-expressing cells in the populations, depending on the batch of HDMEC used. Therefore, we suppose individual differences to be activated either in the potency or in the composition of the possibly existing subpopulations of HDMEC purified from the individual biopsy. The strong induction of Thy-1 by PMA indicates a regulation of Thy-1 expression via the protein kinase C pathway activated by PMA. Among the range of cytokines tested, only TNF-α could induce low Thy-1 expression at both protein and mRNA level. Ishizu (Ishizu et al. 1997a) describes the induction of Thy-1 expression on endothelial cells by endogenously produced IL-1 in vitro. In our study, IL-1β neither induced Thy-1 nor strengthened the TNF-α-induced Thy-1 expression on endothelial cells.

In the synovial membranes of RA patients and in the proximity of melanomas, endothelial cell activation as well as neoangiogenesis occurs widely. The induction of Thy-1 on HDMEC in vitro and the staining of endothelial cells in the synovial membrane of joints from patients suffering from active RA, in melanoma, and melanoma metastases in lymph nodes in situ suggests that Thy-1 may be an activation antigen or a marker of neoangiogenesis. These findings are in agreement with data from Lee et al. (Saalbach et al. 1996). Furthermore, the results emphasize the idea that activated endothelial cells are a possible source of sThy-1 detected in wound and synovial fluids. However, the natural mediator of complete and strong Thy-1 induction, the biological function of membrane-bound as well as soluble Thy-1 still remains unknown.

References

Almqvist P, Carlsson SR (1988) Characterization of a hydrophilic form of Thy-1 purified from human cerebrospinal fluid. J Biol Chem 263(25):12709–12715

Cui Y, English DK, Siddiqui RA, Heranyiova M, Garcia JG (1997) Activation of endothelial cell phospholipase D migrating neutrophils. J Invest Med 45(6):388–393

Dalchau R, Daar AS, Fabre JW (1989) The human Thy-1 molecule, in cell surface antigen Thy-1. In: Reif A, Schlesinger M (eds) Immunology series 45. Dekker, New York, pp 189–196

Favaloro EJ (1993) Differential expression of surface antigens on activated endothelium. Immunol Cell Biol 71:571–581

Fivenson DP, Douglass MC, Nickoloff BJ (1992) Cutaneous expression of Thy-1 in mycosis fungoides. Am J Pathol 141: 1373–1380

Freimuth W, Esselmann WJ, Miller HC (1978) Release of Thy-1.2 and Thy-1.1 from lymphoblastoid cells: partial characterization and antigenicity of shed material. J Immunol 120:1651–1658

Griffith CEM, Barker JNWN, Kunkel S, Nickoloff BJ (1991) Modulation of leucocyte adhesion molecules, a T-cell chemotaxin (IL-8) and a regulatory cytokine (TNFalpha) in allergic contact dermatitis. Br J Dermatol 124:519–526

Haustein UF, Anderegg U (1998) Pathophysiology of scleroderma: an update. J Eur Acad Dermatol Venereol 11:1–8

Ishizu A, Ishikura H, Nakamaru Y, Kikuchi K, Koike T, Yoshiki T (1997a) Interleukin-1α regulates Thy-1 expression on rat vascular enothelial cells. Microvasc Res 53:73–78

Ishizu A, Ishikura H, Nakamaru Y, Takeuchi E, Kimura C, Koike T, Yoshiki T (1997b) Thy-1 induced on rat endothelium regulates vascular permeability at sites of inflammation. Int Immunol 7:1339–1347

Lee WS, Jain MK, Arkonac BM, Zhang D, Shaw SY, Kashiki S, Maemura K, Lee SL, Hollenberg NK, Lee ME, Haber E (1998) Thy-1, a novel marker for angiogenesis upregulated by inflammatory cytokines. Circ Res 82:845–851

Liu CM, Sheen TS, Ko JY, Shun CT (1999) Circulating intercellular adhesion molecule 1 (ICAM-1), E-selectin and vascular cell adhesion molecule 1 (VCAM-1) in head and neck cancer. Br J Cancer 79(2):360–362

Lowry O, Rosebrough NJ, Farr AL, Randall RJ (1951) Kolorimetrische Proteinbestimmung mit dem Folin'schen Phenolreagenz. J Biol Chem 19:3265–275

Mason JC, Yarwood H, Tarnok A, Sugars K, Harrison AA, Robinson PJ, Haskard DO (1996) Human Thy-1 is cytokine-inducible on vascular endothelial cells and is a signaling molecule regulated by protein kinase C. J Immunol 157:874–883

Okamato Y, Tsurunaga T, Ueki M (1999) Serum soluble ICAM-1 levels in the patients with cervical cancer. Acta Obstet Gynecol Scand 78(1):60–65

Raymond FD, Fortunato G, Moss DW, Castaldo G, Salvatore F, Impallomeni M (1994) Inositol-specific phospholipase D activity in health and disease. Clin Sci (Colch) 86(4):447–451

Romero LI, Zhang DN, Herron GS, Karasek MA (1997) Interleukin-1 induces major phenotypic changes in human skin microvascular endothelial cells. J Cell Physiol 173:84–92

Saalbach A, Anderegg U, Bruns M, Schnabel E, Herrmann K, Haustein UF (1996) Novel fibroblast-specific monoclonal antibodies: properties and specificities. J Invest Dermatol 106: 1314–1319

Saalbach A, Kraft R, Herrmann K, Haustein UF, Anderegg U (1998) The monoclonal antiboy AS02 recognizes a protein on human fibroblasts being highly homologous to Thy-1. Arch Dermatol Res 290:360–366

Subcommittee for scleroderma criteria of the ARA diagnostic and therapeutic criteria committee (1980) Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 23:581–590

Vitetta E, Uhr JW, Boyse EA (1974) Metabolism of H-2 and Thy-1 alloantigens in mouse thymocytes. Eur J Immunol 4: 276–282