Multiplex Reverse Transcription Polymerase Chain Reaction Assessment of Sialyltransferase Expression in Peripheral Blood Mononuclear Cells in Systemic Sclerosis

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ABSTRACT. ***Objective.*** To assess sialyltransferase expression in peripheral blood mononuclear cells (PBMC) of patients with systemic sclerosis (SSc) and to correlate this expression with the clinical features of the disease.

Methods. Using a multiplex reverse transcription polymerase chain reaction (RT-PCR) method, we simultaneously measured the expression of 5 sialyltransferases (ST3Gal IV, ST3Gal III, ST3Gal I, ST3Gal II, and ST6Gal I) and of one reference housekeeping gene, Tata box binding protein (TBP), in PBMC of 28 patients with SSc and 18 healthy controls. Expression of each sialyltransferase was defined by the ratio sialyltransferase amplification product intensity/TBP amplification product intensity, and was evaluated according to the skin sclerosis extension and the presence of lung fibrosis and/or of pulmonary hypertension.

Results. ST3Gal I and ST6Gal I expressions were lower in patients with SSc than in healthy controls (median 0.48 vs 1.30, $p < 0.0001$, and 0.71 vs 1.96, $p < 0.01$, respectively). No difference was observed for ST3Gal III and ST3Gal IV expression. ST3Gal IV/ST6Gal I ratio was higher in SSc patients than in controls (0.37 vs 0.28, $p = 0.03$). ST3Gal II was either weakly or more often not expressed in both groups. Ten patients had isolated pulmonary hypertension. They had higher ST3Gal IV expression than patients without pulmonary hypertension (0.73 vs 0.29, $p = 0.04$) and a higher ST3Gal IV/ST6Gal I ratio than patients without pulmonary hypertension (1.03 vs 0.27, $p = 0.03$) and controls (1.03 vs 0.28, $p = 0.02$). No difference was found in the sialyltransferase expression and soluble E-selectin concentration according to the cutaneous sclerosis extension or the presence of lung fibrosis.

Conclusion. Sialyltransferase expression is modified in PBMC of patients with SSc compared to healthy subjects. Low ST6Gal I expression associated with normal ST3Gal IV expression, resulting in a higher ST3Gal IV/ST6Gal I ratio, suggests an enhanced expression of Sialyl-Lewis^X at the surface of PBMC in SSc, and therefore an active interaction between activated endothelial cells and PBMC through the binding between E-selectin and Sialyl-Lewis^X. This suggested that higher expression of Sialyl-Lewis^X concerned patients with isolated pulmonary hypertension more specifically. Binding between PBMC surface Sialyl-Lewis^X and activated endothelial cell E-selectin might therefore play a role in the pathogenesis of SSc-related isolated pulmonary hypertension. (J Rheumatol 2004;31:88–95)

Key Indexing Terms:
SYSTEMIC SCLEROSIS SIALYLTRANSFERASE ENDOTHELIAL CELLS PULMONARY HYPERTENSION

Systemic sclerosis (SSc) is a connective tissue disease characterized by collagen accumulation and vascular injury in the skin and internal organs[1]. The pathogenesis of SSc remains unclear, but some clinical vascular manifestations such as Raynaud's phenomenon, pulmonary hypertension[2], and vascular histological abnormalities[3] suggest a central role of endothelial cells[4]. Moreover, there is endothelial cell activation in SSc, as shown by increased expression of E-selectin in the skin[5] and in minor labial salivary glands[6,7], and by increased serum circulating soluble E-selectin (sE-selectin) concentrations[6,8]. E-selectin is an adhesion molecule whose expression is restricted to activated endothelial cells[9]. E-selectin is involved in adhesion and rolling of

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leukocytes to blood endothelial cells and in their extravasation into perivascular areas, playing an important role during the early inflammatory response[10]. Interaction between endothelial cells and leukocytes is allowed by the very specific binding between E-selectin and its ligand [E-selectin ligand-1 (ESL-1)], found on the surface of the leukocytes[11]. This occurs when the amino-terminal lectin-like domain of E-selectin recognizes the sialylated and fucosylated tetrasaccharides Sialyl-Lewis^X (SLe^X) and Sialyl-Lewis^a (SLe^a), found on the surface of ESL-1[12]. Biosynthesis of SLe^a and SLe^X involves sialyltransferases ST3Gal III (CMP-NeuAc: Gal1-3GlcNAc 2,3-sialyltransferase) and ST3Gal IV (CMP-NeuAc: Gal1-4GlcNAc 2,3-sialyltransferase), respectively[13,14]. An important point is that cell surface SLe^X and SLe^a expression is mainly regulated by sialyltransferase activity that is correlated with sialyltransferase gene expression[15-17]. For example, ST3Gal IV has been shown to increase the level of cell surface SLe^X expression in transfected cells[16]. Therefore, studying sialyltransferase gene expression in leukocytes is a way to assess interaction between these cells and activated endothelial cells through the binding between ESL-1 SLe^X or SLe^a and E-selectin[17].

A perivascular mononuclear cell infiltrate is present in SSc lesions and probably plays an important role in the pathogenesis of SSc[18,19]. However, the setting up of this infiltrate is not fully understood[19]. Rudnicka, *et al* showed that peripheral blood mononuclear cells (PBMC) from patients with SSc adhere abnormally to endothelial cells, with an overall reduction in binding but with enhanced binding of a subfraction of activated cells[20]. Sollberg, *et al* showed that lymphocytes in close proximity to small blood vessels in the skin of patients with SSc were positive for beta-1 and beta-2 integrins and that endothelial cells highly expressed E-selectin[21]. However, to our knowledge, the expression of SLe^X and SLe^a in PBMC has not been previously studied.

We assessed this interaction through study of the expression of 5 sialyltransferases, ST3Gal I, ST3Gal II, ST3Gal III, ST3Gal IV, and ST6Gal I, in SSc patients' PBMC using a multiplex reverse transcription-polymerase chain reaction (RT-PCR) technique.

MATERIALS AND METHODS

Patients. This study was performed on 28 consecutive patients with SSc fulfilling the American College of Rheumatology (ACR) criteria[22]. All patients had recently diagnosed disease, and none of them had received immunomodulatory or antifibrotic therapy. There were 26 women and 2 men, with a mean age of 56.9 ± 10.5 years (range 36–75). Disease duration was measured from the onset of the first symptom (Raynaud's phenomenon in all cases), and was 5.3 ± 4.8 years (range 0–21). Cutaneous extension of sclerosis was graded according to LeRoy's classification system[23]: that is, limited (hands, forearms, face, or feet; n = 20) or diffuse (truncal and acral; n = 8). The presence of lung fibrosis was determined in each patient by chest computed tomodensitometry and pulmonary function tests. Lung fibrosis was defined by the presence of parenchymal micronodules, honeycombing, or linear opacities on chest computed tomodensitometry and abnormal lung function (lung volumes < 80% of predicted values and/or diffusing capacity < 75% of that predicted)[24,25]. According to these criteria, 14 patients had lung fibrosis. Systolic pulmonary arterial pressure was estimated in each patient by Doppler echocardiography[26]. Pulmonary hypertension (PH) was diagnosed when systolic pulmonary arterial pressure was > 40 mm Hg (n = 10)[2]. C-reactive protein (CRP) concentration was measured in 24/28 patients at the time of blood sample.

Eighteen healthy subjects were also included in this study as controls, 17 women and one man, with a mean age of 55.3 ± 9.3 years (range 32–69).

Isolation of PBMC. PBMC were isolated using mononuclear cell preparation tubes (Vacutainer CPT, Becton-Dickinson, Franklin Lakes, NJ, USA) by density gradient centrifugation.

Isolation of total RNA. Total RNA of PBMC was isolated using the RNeasy Minikit (Qiagen, Courtaboeuf, France). The amount of the RNA was quantified by measuring the absorbance at 260 nm.

Multiplex RT-PCR. We have described the method[27]. Briefly, total cellular RNA (1 µg in 8 µl MilliQ water) was heated at 65°C for 10 min and placed on ice for 2 min. Reverse transcription into cDNA was achieved using the First-Strand cDNA Synthesis kit (Pharmacia Biotech, Piscataway, NJ, USA), according to the manufacturer's protocol using oligo-d(T) as initiation primer in a final reaction volume of 15 µl. One microliter of the retrotranscription reaction was subjected to PCR amplification using sialyltransferase-specific derived primers and Tata box binding protein (TBP)-specific derived primers (Table 1). TBP is a housekeeping gene used to reflect the cell transcription level[28]. The 24 µl quantity of PCR mixture for the multiplex PCR of the 5 sialyltransferase- and TBP-specific fragments consisted of 3 units of Pyra exo(–)DNA polymerase (QBiogen, Evry, France), 10 mM Tris-HCl, pH = 8.8, 3 mM $MgSO_4$, 0.1% Triton X-100, 0.2 nM dNTP, 0.4 µM of each TBP primer, and 0.3 µM of each sialyltransferase primer. Reactions were run in the GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) using the following conditions: 2 min at 94°C then 30 cycles including 1 min at 94°C, 1 min at 63°C, and 2 min at 72°C, and finally, after the 30 cycles, 8 min at 72°C. In all experiments, negative control reactions were performed by replacing cDNA template with sterile water.

Quantification of amplification products. The sense primer of TBP was 5' fluorescent labeled with 6-carboxyfluorescein and the sense primers of the sialyltransferases were 5' fluorescent labeled with 6-carboxy-2',4',7',4,7 hexachlorofluorescein. After completion of the PCR, 1/50 of the PCR reaction mixture was coelectrophoresed through a 6% polyacrylamide-8 M urea gel on a 373 DNA sequencing system from Applied Biosystems (Perkin-Elmer) with molecular mass markers fluorescently labeled with 6-carboxy-X-rhodamine (Applied Biosystems). The results were analyzed with Genescan 672 Software (Applied Biosystems). Levels of each sialyltransferase expression were presented as the ratio enzyme-related area/TBP-related area. Similarly to other studies concerning sialyltransferase expression[29], we also calculated the ratio ST3Gal IV expression/ST6Gal I expression. This ratio was used to best assess cell surface SLe^X expression, which is positively regulated by ST3Gal IV and negatively regulated by ST6Gal I expression[15,16].

Concentrations of serum circulating soluble E-selectin. Serum samples were obtained during the centrifugation performed for isolation of PBMC. Concentrations of circulating sE-selectin were measured in duplicate using a commercial ELISA kit (R&D Systems, Abington, UK) as described[30]. To avoid a possible rise in sE-selectin concentration associated with impaired liver or kidney functions, we verified that all patients had normal liver (serum bilirubin ≤ 30 µmol/l, aspartate transaminase ≤ 25 IU/ml) and renal (serum creatinine ≤ 130 µmol/l) function.

Statistical analyses. Results are presented as median (range). Comparisons between the SSc group and controls were performed using the Wilcoxon nonparametric test and the chi-square test with Yates' correction if necessary. The same tests were used to compare sialyltransferase expression according to the clinical and biological features of SSc. A value of $p < 0.05$

Table 1. Sequences of the 6 primer pairs used for PCR amplification.

Target cDNA	Primer Set	Tm, ˚C	PCR Product Size, bp
TBP	5'-CACGAACCACGGCACTGATT-3' 3'-CAGGTCTGACCGTCGTTCTTTT-5'	62	88
ST3Gal III	5'-CGGATGGCTTCTGGAAATCTGT-3' 3'-AGTTTCTCAGGACCTGCGTGTT-5'	57	300
ST6Gal I	5'-TATCGTAAGCTGCACCCCAATC-3' 3'-GAAGGCCTGGTAAGTGACGATT-5'	56	371
ST3Gal IV	5'-CCCAAGAACATCCAGAGCCTCA-3' 3'-CTAATTCGTCTTCGGGTGGTGC-5'	58	458
ST3Gal I	5'-TCAGAGTGGTGCCTGGGAATGT-3' 3'-CGTTTCCCTTGACCGTGGTGAT-5'	58	537
ST3Gal II	5'-TGGTTTGACAGCCACTTTGACG-3' 3'-CCCTACGACCACGAAAAGAAAC-5'	57	609

TBP: Tata box binding protein.

was considered statistically significant. Graphic representations were made using the box-plot method[31].

RESULTS

Sialyltransferase expression (Figure 1). Expressions of ST6Gal I and ST3Gal I were lower in the SSc group than in controls [0.48 (range 0.02–2.24) vs 1.30 (0.03–2.81), $p < 0.0001$, and 0.71 (range 0.06–2.99) vs 1.96 (0.60–3.35), $p < 0.01$, respectively)]. There was no significant difference between the SSc group and controls for ST3Gal III [0.72 (0.04–2.03) vs 0.83 (0.34–1.54), $p = 0.14$] and ST3Gal IV [0.38 (0.01–2.95) vs 0.52 (0.04–1.38), $p = 0.31$] expression. The ST3Gal IV/ST6Gal I ratio was higher in SSc patients than in controls [0.37 (0.09–17.06) vs 0.28 (0.05–0.46), $p = 0.03$]. ST3Gal II was either weakly or more often not expressed in both groups. An example of the sialyltransferase expression profile in a patient and a healthy control is shown in Figures 2a and 2b, respectively. Circulating sE-selectin concentrations were higher in the SSc group than in controls [45.5 (14.4–136.9) vs 37.7 (20.1–55.1) ng/ml, $p = 0.03$]. There was no significant systemic inflammation by CRP level in any patient.

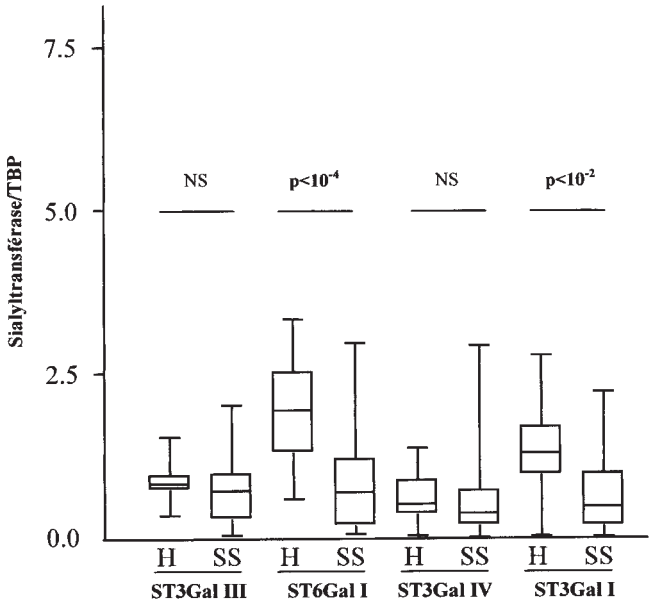

Figure 1. Sialyltransferase expression in patients with systemic sclerosis (SS) compared to healthy controls (H). NS: nonsignificant.

Correlation with clinical features. Ten patients had pulmonary hypertension. All had the limited form of SSc, with no pulmonary fibrosis. No remaining patient had any evidence of pulmonary hypertension on Doppler echocardiography, and systolic pulmonary arterial pressure was < 30 mm Hg in each case. Patients with pulmonary hypertension had significantly higher ST3Gal IV expression than patients without [0.73 (0.12–1.18) vs 0.29 (0.01–2.95), $p = 0.04$]. Conversely, no difference was found for ST3Gal III, ST6Gal I, and ST3Gal I expression or for circulating sE-selectin concentrations according to the presence of pulmonary hypertension. An example of the sialyltransferase expression profile in a patient with pulmonary hypertension is shown Figure 2c.

The ratio ST3Gal IV/ST6Gal I was higher in patients with pulmonary hypertension than in patients without [1.03 (0.33–6.89) vs 0.27 (0.09–17.06), $p = 0.03$] as well as in controls [1.03 (0.33–6.89) vs 0.28 (0.05–0.46), $p = 0.02$] (Figure 3). Conversely, no significant difference was observed between patients without pulmonary hypertension and controls [0.27 (0.09–17.06) vs 0.28 (0.05–0.46), $p = 0.11$]. The median of the ratio ST3Gal IV/ST6Gal I was 0.37 in the SSc group. All 10 (100%) patients with pulmonary hypertension had a ratio ST3Gal IV/ST6Gal I higher than 0.37 compared to 5 of 18 (27.7%) patients without pulmonary hypertension ($p < 0.01$) and 4 of 18 (22.2%) controls ($p < 0.001$).

No difference was found in the sialyltransferase expression and sE-selectin concentrations according to the extent of cutaneous sclerosis or the presence of lung fibrosis.

DISCUSSION

We used a multiplex RT-PCR assay to compare the expression of 5 sialyltransferases in PBMC of patients with SSc and healthy subjects. Although RT-PCR assesses sialyltransferase activity in an indirect way, this may be better than direct measurement[27,32] for several reasons: First, functional activity assessment does not allow identification of the activity of each sialyltransferase, as one activity can be supported by more than one enzyme[32]. In contrast, multiplex RT-PCR is accepted as a very sensitive and highly reproducible method to assess each sialyltransferase expression at the mRNA level and to determine a pattern of expres-

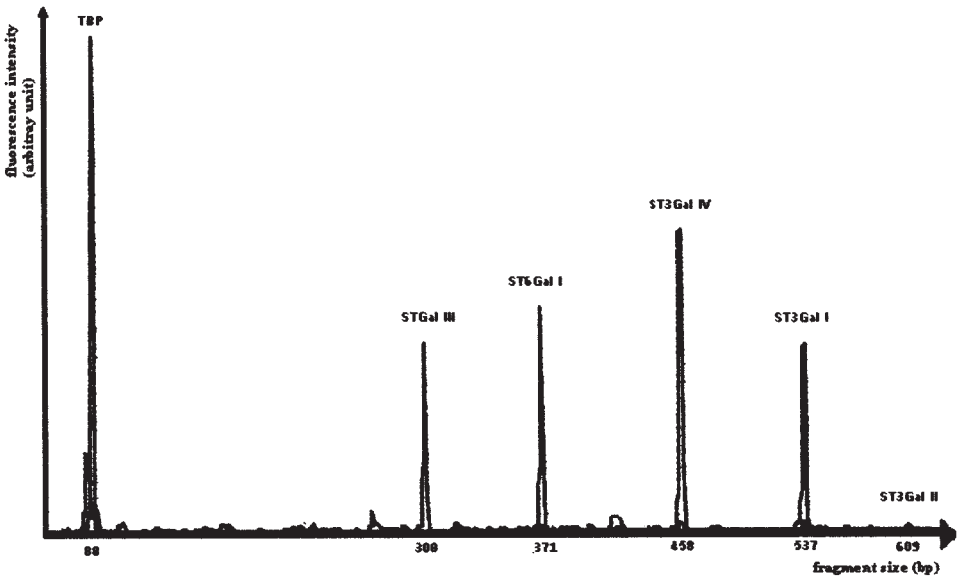

Figure 2a. Fluorescent peaks corresponding to the 5 sialyltransferases and Tata box binding protein expression in a patient with SSc. In SSc patients, ST6Gal I and ST3Gal I expression was lower than in healthy controls, whereas ST3Gal IV and ST3Gal III expression was not different.

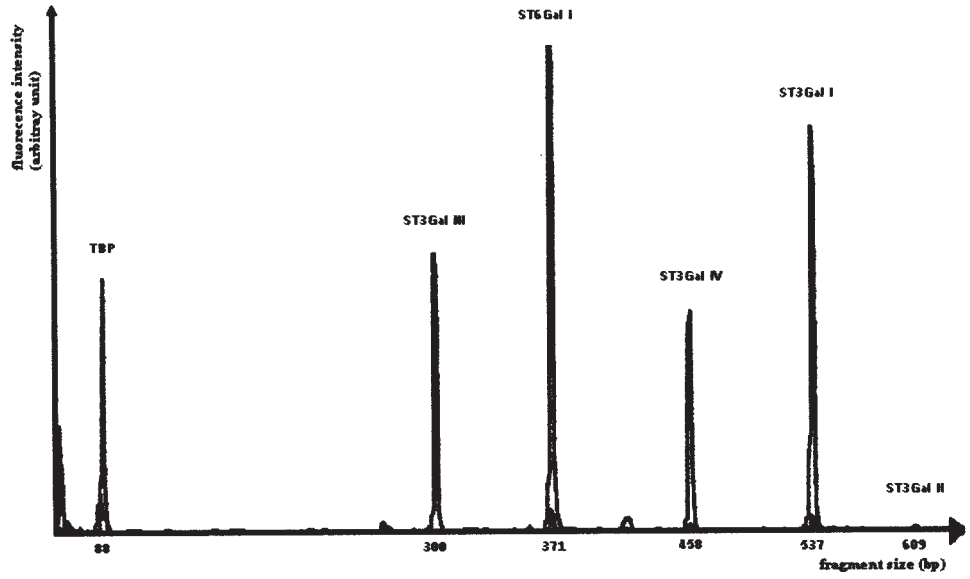

Figure 2b. Fluorescent peaks of the 5 sialyltransferases and Tata box binding protein in a healthy control.

sion[27,32]. Second, sialyltransferase activity is mainly regulated at the transcriptional level, giving a high value to the study of sialyltransferase mRNA[13]. Third, there is a good correlation between sialyltransferase mRNA expression and sialyltransferase activity at the cell surface. For example, many investigators have found correlation between ST6Gal I mRNA level and cell surface α2,6-sialic acids[15,33]. Moreover, a high expression of ST3Gal III and ST3Gal IV mRNA is associated with a higher level of cell surface SLe^a and SLe^X expression, respectively, either in tumor cells[16] or in PBMC[17]. Indeed, sialyltransferase assessment at the mRNA level by RT-PCR is an accepted way to assess sialyltransferase activity[14,27,29,32].

To our knowledge, only one study has focused on sialyltransferase activity in SSc[34]. Using an enzymatic spectrofluorometric assay, Gabrielyan, *et al* found that global sialyltransferase blood plasma activity was decreased in patients with SSc compared to healthy subjects[34]. Multiplex RT-PCR assay allows analysis of the individual expression of sialyltransferases[27]. We found that sialyltransferase gene

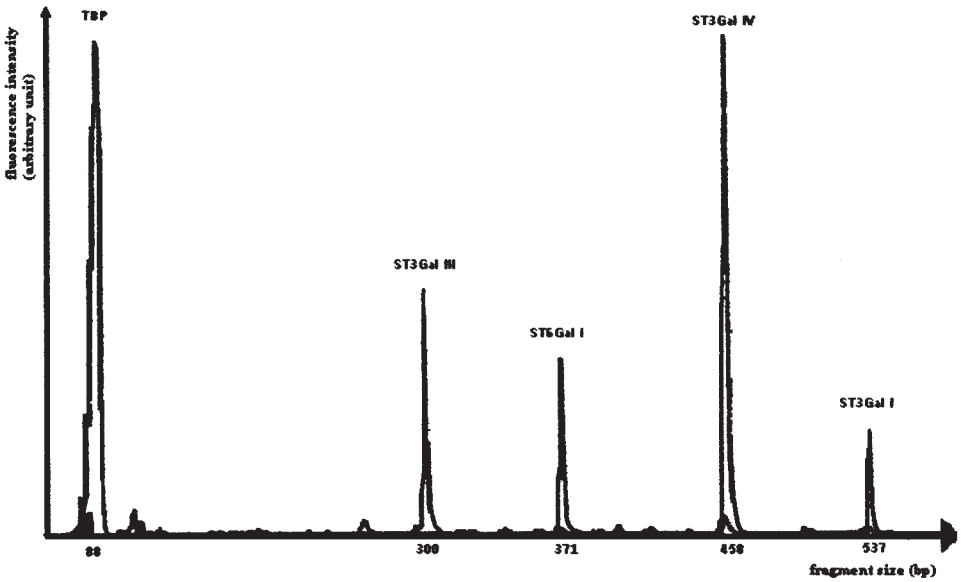

Figure 2c. Fluorescent peaks of the 5 sialyltransferases and Tata box binding protein in a patient with SSc with pulmonary hypertension. Note the high expression of ST3Gal IV and low expression of ST6Gal I.

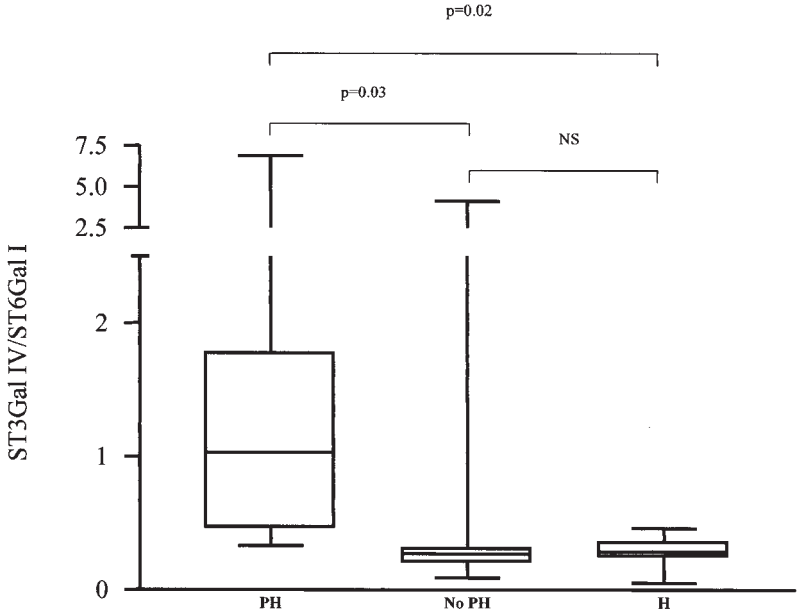

Figure 3. Ratio of ST3Gal IV/ST6Gal I in patients without and with pulmonary hypertension (PH) and healthy controls (H). NS: nonsignificant.

expression is modified in PBMC of patients with SSc compared to healthy subjects. ST3Gal I and ST6Gal I expression was lower in SSc patients than in healthy subjects, whereas ST3Gal III and ST3Gal IV expression was similar in both groups. ST3Gal II was weakly (or not) expressed in PBMC from SSc patients and healthy controls.

ST3Gal II shares the same substrate activity with ST3Gal I. Both enzymes are able to transfer a sialic residue onto the 3-OH group of the Gal residue of the type III disaccharide structure, and could be involved in sialylation of T-antigen (Galß1-3GalNAcα-O-Ser/Thr)[13]. In PBMC, α2,3 sialylation of T-antigen might thus be essentially catalyzed by ST3Gal I rather than ST3Gal II. Moreover, this sialylation is decreased in SSc patients' PBMC compared to controls, as shown by the lower expression of ST3Gal I in SSc patients, and this might be explained by the reduction of T cell activity observed in SSc[35-37].

ST3Gal III is involved in SLe^a synthesis. The expression level of SLe^a on the cell surface is positively correlated with the expression level of ST3Gal III[38]. ST3Gal III expression is not significantly different in SSc patients and healthy subjects.

Thus, our results suggest that PBMC SLe^a surface expression might be not significantly different between the 2 groups.

Cell surface SLe^X expression is known to be positively regulated by ST3Gal IV and negatively regulated by ST6Gal I expression[15-17]. This explains why the ratio ST3Gal IV expression/ST6Gal I expression might best indicate cell surface SLe^X expression. ST3Gal IV and ST6Gal I share the same substrate specificity (Galß1-4GlcNAcß-). Low ST6Gal I expression may therefore increase the number of substrate acceptors for ST3Gal IV, and has been shown to result in an increased cell surface SLe^X expression[15]. Moreover, Okajima, *et al* have recently suggested that decreased ST6Gal I expression could, in the same way, increase the number of substrate acceptors for ST6Gal VI, which is a recently cloned sialyltransferase involved in synthesis of SLe^X precursor[39]. In our study, we found that ST3Gal IV expression was not significantly different in SSc patient and control PBMC. In contrast, ST6Gal I expression was significantly decreased in SSc patient PBMC, resulting in a higher ST3Gal IV/ST6 Gal I ratio than in healthy subjects. Together, these results suggest that SLe^X expression is increased on the PBMC surface in SSc. However, we did not measure PBMC surface SLe^X expression directly. This expression is therefore only suggested, as in other studies[27-29] and deduced from the literature[15-17]. This suggested higher SLe^X expression associated with the high circulating serum sE-selectin concentrations is evocative of an interaction in SSc between PBMC and activated endothelial cells through the binding between SLe^X on the ESL-1 surface and E-selectin.

In this study, 10 patients had pulmonary hypertension. This small number of patients does not permit firm conclusions, but the results are interesting. Pulmonary hypertension is a severe complication of SSc and probably one of the leading causes of morbidity and mortality in this condition[2,40,41]. Doppler echocardiography was performed in each patient to evaluate systolic pulmonary artery pressure. Although some authors suggest that systolic pulmonary artery pressure can be underestimated by the Doppler technique as a result of technically inadequate signals[42], several studies report very close correlations between direct measurements of systolic pulmonary artery pressure by right-heart catheterization and noninvasive estimates based on continuous-wave Doppler measurements[43]. We found that patients with pulmonary hypertension had a significantly higher ST3Gal IV/ST6Gal I ratio than patients without hypertension and healthy subjects. Conversely, we found no difference between patients without pulmonary hypertension and healthy subjects. Moreover, a higher ST3Gal IV/ST6Gal I ratio was found in each of the patients with pulmonary hypertension and significantly more often than in patients without hypertension and healthy subjects. This suggests that higher expression of SLe^X at the surface of PBMC is more specifically found in sclerodermic patients, whose disease is complicated by pulmonary hypertension. In our study, all patients with hypertension had the limited form of SSc without pulmonary fibrosis, and therefore made up a homogeneous group. As we found no statistically significant difference in sialyltransferase expression or in circulating serum E-selectin concentration according to the extent of cutaneous SSc or the presence of lung fibrosis, there is little risk that the difference observed in pulmonary hypertension was due to confounding factors. The absence of lung fibrosis strongly suggests an isolated pulmonary hypertension due directly to pulmonary artery lesions[2,44]. The presence of a mononuclear cell infiltrate (including macrophages and T and B lymphocytes) around these lesions is well established in SSc[19,45]. Our results suggest that the binding between SLe^X on PBMC and E-selectin on endothelial cells might play a role in setting up this perivascular inflammatory mononuclear infiltrate. A central role of inflammation in SSc-related isolated pulmonary hypertension has been suggested[19,45]. Voelkel and Tuder report that the interactions between perivascular inflammatory cells and pulmonary endothelial cells may induce a release of platelet-derived growth factor, vascular endothelial growth factor, and transforming growth factor-ß leading to endothelial cell proliferation, intima and media thickening, and *in situ* thrombosis, therefore playing a major role in the pathogenesis of pulmonary hypertension in SSc[45].

No difference was found in sialyltransferase expression profiles and sE-selectin concentrations according to the extent of cutaneous sclerosis or presence of lung fibrosis. One could suggest that mechanisms other than interaction between PBMC and activated endothelial cells may be involved in the inflammatory infiltrate in these organs. For example, some authors suggest that fibroblasts in skin or lungs could promote mononuclear leukocytes across endothelial cells via a monocyte chemoattractant protein-1-dependent mechanism[46,47]. Other investigators report that high E-selectin concentrations were associated with disease severity, i.e., lung and renal involvement[48].

We found no studies focusing on sialyltransferase expression in other systemic diseases where endothelial cells are activated, such as vasculitis or lupus. Basset, *et al* described enhanced sialyltransferase activity in B lymphocytes from patients with primary Sjögren's syndrome. However, these results did not concern interaction between activated endothelial cells and PBMC[49]. In contrast, there are a lot of data for cancers, mainly breast and colon cancers. In breast cancers, high concentrations of circulating soluble E-selectin are associated with a poor prognosis[30]. Recchi, *et al*[27] report that a high breast tumor cell expression of ST3Gal III, which is involved in SLe^a synthesis, is associated with a poor prognosis. These results suggest endothelial cells expressing E-selectin may be more receptive to tumor cells highly expressing some sialyltransferase, with a

higher risk of metastasis and a poorer prognosis. The same results were found in colon cancers[50].

Our results show that sialyltransferase gene expression is modified in PBMC of patients with SSc. The sialyltransferase expression pattern is suggestive of a high level of PBMC SLe^X expression, although we did not measure it directly. In SSc, PBMC could therefore interact with activated endothelial cells through the binding between SLe^X and E-selectin. Moreover, we found that patients with pulmonary hypertension had a sialyltransferase gene expression profile evocative of a higher expression of SLe^X than patients without pulmonary hypertension and healthy subjects. Therefore, the interaction between PBMC and activated endothelial cells through the binding between SLe^X and E-selectin might play a role in the pathogenesis of SSc-related pulmonary hypertension. Studies of longitudinal assessment of changes in sialyltransferase expression over time in a cohort of patients with SSc could yield useful information.

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