Reduced type I collagen gene expression by skin fibroblasts of patients with systemic sclerosis after one treatment course with rituximab


ABSTRACT

Objective. There is evidence that B lymphocytes play a role in the pathogenesis of systemic sclerosis (scleroderma). Stimulatory autoantibodies targeting and activating normal human fibroblasts in vitro have been demonstrated in sera from scleroderma patients. Rituximab is a monoclonal antibody which selectively targets and depletes CD20⁺ B lymphocytes. We investigated the biological effects of rituximab in six patients affected by scleroderma with severe skin involvement.

Methods. Six patients with severe skin fibrosis, unresponsive to immunosuppressive treatment, were treated with 375 mg/m² per week of intravenous rituximab for a total of four doses. Serum stimulatory autoantibodies to the PDGF receptor were detected. Fibroblast activation was evaluated in fibroblasts grown from skin biopsies performed at baseline and at months 3 and 6 post-treatment. The modified Rodnan's skin score, health assessment questionnaire (HAQ) and visual analogic scale (VAS) for global wellness and B lymphocyte count were performed monthly.

Results. A significant reduction of anti-PDGF receptor autoantibodies was observed in the serum of all patients 3 months after treatment. Fibroblasts showed a significant downregulation of type I collagen gene expression and of the intracellular signalling triggered by anti-PDGF receptor autoantibodies. A decrease of the skin score and an improvement of disability indexes matched with the in vitro results. A single course of rituximab reduced scleroderma fibroblast activation in vitro and the serum levels of anti-PDGF receptor stimulatory autoantibodies.

Conclusion. These data provide further evidence of B-cell involvement in the pathogenesis of scleroderma. Targeting B cells may be a promising treatment for scleroderma patients, and controlled clinical trials are warranted.

Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterised by increased production of extracellular matrix by fibroblasts, both in the skin and in visceral organs. The aetiology is unknown, and key pathogenetic events include immune system activation, endothelial cell dysfunction and fibroblast activation (1-3).

Although current therapies can control end organ damage (4), no disease-modifying agent able to induce complete remission of the disease is available yet, probably due to the poor knowledge of the mechanisms implicated in the pathophysiology of the disease (5).

It has been shown that SSc patients are characterised by activation of humoral immunity. Whitfield et al. using DNA microarrays have revealed up-regulation of genes related to B cells in SSc skin lesions (6). Furthermore, memory B cells have been found to be activated in scleroderma patients and showed enhanced ability to produce IgG in vitro under well defined experimental conditions (7). Increased serum levels of BAFF or B cell-activating factor, a potent B cell survival factor, were detected in an unselected SSc population and found to correlate positively with the extent of skin fibrosis (8). The activation of B cells may lead to increased production of IL-6 and TGF-β which induce fibrosis (9) and IL-10 and IL-6 which drive a Th2-dominant immune response (10, 11) involved in the mechanisms of autoantibody production (12). The description of several autoantibodies directed against non-nu-
clear antigens, that may actually play a pathogenic role in the vascular damage and tissue fibrosis (13-16) has corroborated the hypothesis that activation of humoral immunity is involved in SSc pathogenesis.

Waiting for the definitive demonstration of autoantibody pathogenic role in vivo, it may be postulated that selectively targeting B cells may be a rewarding therapeutic strategy in SSc (17).

Rituximab (RTX) is a chimeric monoclonal antibody that depletes CD20+ B cells via cell-mediated and complement-dependent cytotoxic effects. B-cell depletion affects not only antibody production, but also cytokine and growth factor release, and T cell activation (18, 19). Several pre-clinical (20) and clinical (21-26) reports support the use of this drug in SSc.

On the basis of this evidence, this work was aimed at testing the ability of rituximab to revert the phenotype of fibroblasts cultured from skin biopsies of SSc patients, and to improve the skin involvement of the same patients.

**Patients and methods**

*Patients*

Six consecutive patients with a definite diagnosis of SSc (27) were recruited from the outpatient clinics of two Italian University hospitals (Table I). Patients were considered eligible for treatment with rituximab in the presence of: 1. evidence of worsening of skin fibrosis, defined as an increase of the modified Rodnan skin score (MRSS) of 4 points or more in the last 6 months; 2. documented failure of cyclophosphamide therapy, conducted for 9 months or more (total dose ≥9g), or contraindication to cyclophosphamide or patient’s refusal of cyclophosphamide therapy; 3. stable visceral involvement with no evidence of worsening of cardiac, pulmonary or renal functions in the previous 12 months; 4. positive serum anti-PDGF receptor (PDGFR) autoantibodies at baseline.

Enrolled patients had not received immunosuppressive drugs for at least two months prior to the study. Two patients were on low dose steroids (prednisone, less than 10 mg/day), and anti-platelet drugs. One patient was on bosentan for pulmonary arterial hypertension, two patients continued their monthly infusion of iloprost (0.5 ng/kg/min for 48h) for digital ulcers.

**Study medication and administration**

The protocol was approved by the local Ethics Committee. A written informed consent was obtained from all patients. Enrolled patients had not received immunosuppressive drugs for at least two months prior to the study. Two patients were on low dose steroids (prednisone, less than 10 mg/day), and anti-platelet drugs. One patient was on bosentan for pulmonary arterial hypertension, two patients continued their monthly infusion of iloprost (0.5 ng/kg/min for 48h) for digital ulcers.

**Table I. Main clinical and laboratory characteristics of the enrolled patients.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/gender</th>
<th>Disease duration (years)</th>
<th>SSc subset and autoantibody pattern</th>
<th>Rodnan Skin score baseline</th>
<th>Visceral organ involvement</th>
<th>Previous therapies</th>
<th>Therapy associated to RTX treatment</th>
<th>Rodnan Skin score at month 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>66/ M</td>
<td>2</td>
<td>Diffuse/ANA negative</td>
<td>32</td>
<td>Esophagus Lung</td>
<td>Cyclophosphamide</td>
<td>Steroids, Bosentan Iloprost</td>
<td>22</td>
</tr>
<tr>
<td>B</td>
<td>45/ M</td>
<td>7</td>
<td>Diffuse /Topo I positive</td>
<td>19</td>
<td>Esophagus Lung</td>
<td>Colchicine, Pentosifillin Iloprost</td>
<td>Steroids, Iloprost</td>
<td>16</td>
</tr>
<tr>
<td>C</td>
<td>58/ F</td>
<td>3</td>
<td>Diffuse/ anti-centromere positive</td>
<td>11</td>
<td>Esophagus Lung</td>
<td>Cyclophosphamide</td>
<td>None</td>
<td>7</td>
</tr>
<tr>
<td>D</td>
<td>47/ M</td>
<td>3</td>
<td>Limited /Topo I positive</td>
<td>38</td>
<td>Lung</td>
<td>Iloprost, Steroids Colchicine</td>
<td>Colchicine</td>
<td>21</td>
</tr>
<tr>
<td>E</td>
<td>58/ F</td>
<td>1</td>
<td>Diffuse/Topo I positive</td>
<td>28</td>
<td>none</td>
<td>Penicillamine, Hydroxicoloquine Steroids</td>
<td>None</td>
<td>17</td>
</tr>
<tr>
<td>F</td>
<td>23/ F</td>
<td>2</td>
<td>Diffuse/Topo I positive</td>
<td>18</td>
<td>none</td>
<td>Iloprost</td>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>

SSc: Systemic sclerosis; Topo I: anti-topoisomerase I autoantibody; RTX: rituximab.

**Skin biopsies**

Eight millimeter skin punch biopsy fresh samples were obtained from all patients at baseline, and 3 and 6 months later. All biopsy samples were cut perpendicularly to the skin surface into three specimens: one was routinely embedded in paraffin, the second frozen in OCT medium using liquid nitrogen and the third frozen without OCT medium to obtain fibroblast cultures. Sections from paraffin embedded and OCT frozen samples were stained with haematoxilin and eosin. Immunohistochemistry was performed on sections from frozen tissue using the following antisera: CD3, CD4, CD8, CD20, CD79a, CD45RA.

The measurements were carried out using a reticule in the eyepiece with a known width between divisions, at 50x. The absolute count of lymphocytes, both morphologically and by immunohistochemistry, and of plasma cells was divided by the number of endothelial cells surrounding the vessel lumen in order to correct for its calibre and orientation.

**Serum IgG purification**

IgG fractions were purified from serum of SSc patients by a 2-step process:

1. Serum was mixed with 20% (v/v) of a solution of sodium sulfate for 1h at 4°C and then centrifuged at 10,000g at 4°C for 15 minutes.
2. The supernatant was stored at 4°C and the pellet was resuspended with 20% (v/v) of a solution of sodium sulfate.
3. This procedure was repeated twice.
4. The final IgG fraction was dialyzed against physiological saline at 4°C for 24h.

**Immunohistochemistry**

Immunohistochemistry was performed on sections from frozen tissue using the following antisera:

- CD3
- CD4
- CD8
- CD20
- PDGFR
- PDGFR receptor
- CD79a
- CD45RA
1. affinity chromatography on protein A/G-sepharose column (Pierce, Rockford, IL); 2. size-exclusion chromatography to remove trace amounts of contaminating cytokines.

**Cell lysis and immunoblotting**

Adherent fibroblasts were lysed with cold RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM sodium orthovanadate, 2 μg/ml aprotinin, 1mM PMSF) and processed for immunoblotting as described (28). Immunoblots were incubated with antibodies against pERK, HaRas and β-actin (Santa Cruz, Dallas Texas), α-SMA (Sigma-Aldrich, St. Louis MO).

**Protein immunoprecipitation**

Total mouse fibroblast lysates expressing human PDGFRα (Fα) or devoid of any PDGFRα (F−/−) were immunoprecipitated with IgG purified from serum of SSc patients (SSc IgG). Immunocomplexes were isolated by protein A/G and immunoblotted with a rabbit anti-human PDGFRα antibody (Santa Cruz Dallas Texas) (14).

**Functional bio-assay for autoantibodies against PDGF receptor (PDGFR)**

SSc IgG were tested for the presence of stimulatory anti-PDGFR autoantibodies by a functional bioassay as described (14). The level of intracellular reactive oxygen species (ROS) was determined in adherent fibroblasts preincubated with SSc IgG (15 min) and then loaded with dichlorofluorescein diacetate (DCF-DA) (Life technologies, Carlsbad, CA) for 15 minutes at 37°C (14).

**Expression of type I collagen genes**

Expression levels of human Col1A1 and Col1A2 genes were detected by real-time PCR as described (29). Total RNA was isolated from fibroblasts with Pure Link RNA Minikit (Life Technologies Carlsbad, CA) and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer. Gene expression was quantified by SYBR Green real-
time PCR in a iCycler iQ™real-time PCR Detection System (Bio-Rad Hercules, CA). Specific primer pairs for each gene were designed with the Universal ProbeLibrary Assay Design Center by Roche Applied Science and were as follows: type I collagen (COL1A1) 5'-AGGGCCAAGAC-GAAGAC- ATC-3' (forward), 5'-AG-ATCACGTCATCGCACAACA-3' (reverse); α-SMA 5'-AGCCAA-GCACTGTCAGGAATC-3' (forward), 5'-AGCCATTGTCACACACCAA GG-3' (reverse); GAPDH 5'-TGCAC CACCTGCTTAGC-3' (forward), 5'-TGCCATTTCCATGGATGACAA GC-3' (reverse). GAPDH was used to test the quality of cDNA and as a housekeeping gene in real-time PCR. The threshold cycle (Ct) was used to detect the increase in the signal associated with an exponential growth of PCR product during the loglinear phase. The relative expression was calculated using the 2^{-ΔΔCt} formula. The ΔCt validation experiments showed similar amplification efficiency for all templates used (difference between linear slopes for all templates less than 0.1).

**B Lymphocyte count**

The count of peripheral blood CD19^+ B cells was assessed monthly by flow cytometry analysis. Depletion of B cells in peripheral blood was considered positive when CD19^+ cell count was lower than 5 cells/μl.

**Clinical assessment**

Clinical evaluation was performed according to the guidelines for clinical trials in SSc (30, 31). A complete physical and laboratory examination was performed at baseline, then monthly. The extent of skin involvement was assessed using the modified Rodnan skin score (MRSS) (32) performed as described (33). Visual Analogue Scale (VAS) for global wellness and Health Assessment Questionnaire (HAQ) were evaluated monthly (34). Antinuclear, anti-topoisomerase I and anti-centromere antibodies were tested prior to entry the study and at month 6 using current assays (35).

**Tolerability and safety**

Laboratory parameters, including peripheral blood cell count, serum liver enzymes, serum creatinine and urine dipstick were measured before the beginning of treatment, then monthly. Additional examinations were performed at
baseline, then every 3 months (electrocardiogram) or at month 6 (echocardiography with evaluation of pulmonary arterial pressure; pulmonary function tests with DLco; high-resolution CT scan of the lungs; esophagogastroduodenoscopy in one patient with heartburn).

Adverse events were reported and investigated for their possible correlation with the experimental drug.

### Statistical analysis

All the clinical outcomes and the biological evaluations are expressed as mean ± SE and analysed with the Friedman non-parametric test. When making multiple comparisons, the Dunn’s multiple comparison test using Prism4 (GraphPad Software, Inc.) was applied. *p*-values less than 0.05 were considered statistically significant.

### Results

#### Histological examination of skin

With regard to the structural features, thickness of the papillary and the reticular dermis, number of parallel elastic fibers of the reticular dermis and percentage of periadnexal fat around the sweat gland did not show any significant improvement after rituximab (data not shown).
Six months after rituximab the perivascular lymphocyte infiltrates in the superficial and reticular plexuses, expressed as lymphocyte-endothelial cell ratio, was significantly reduced compared to baseline (4.29±2.53 vs. 1.86±0.65; p=0.004) (Fig. 1a). A depletion of plasma cell count was also evident although not statistically significant (0.274±0.24 vs. 0.075±0.09; \( p=n.s. \)) (Fig. 1b). One representative histological evidence of lymphocyte depletion is shown in Fig. 1c and 1d.

Given the low number of B lymphocytes in the skin biopsies, we use other pan-B cell marker, such as CD79a and CD45RA. Although not statistically significant, CD79a suggested B cell depletion in the superficial and deep reticular dermis due to rituximab better than the other two pan-B markers (Fig. 2a). The reduction in the CD3 cell count around the superficial and deep reticular plexuses (Fig. 2b) did not reach significance.

B cell depletion in peripheral blood and levels of anti-PDGFR antibodies

B cell depletion, defined as CD19+ lymphocytes <5 elements/µl, was present in all patients three months after the end of treatment with rituximab. B-cell depletion was still observed at month 6 in 3 patients, whereas in the other three patients the CD19+ B cells were 12, 15 and 88/µl, respectively. Mean values of CD19+ cells were 128.7±71.70 at baseline, 0.33±0.81 at month 3 (\( p=0.001 \) compared to baseline) and 19.67±34.06 at month 6 (\( p=ns \)) (Fig. 3a).

Since stimulatory anti-PDGFR antibodies found in SSc patients are able to stimulate type I collagen gene expression inducing the Ha-Ras-ERK1/2 and ROS cascade (14, 29), we wondered whether B cell depletion was associated with a reduction of their serum levels.

Anti-PDGFR stimulatory antibodies serum level were measured using a cell-based functional bioassay (14). The mean stimulation index was 0.62±0.20 at baseline, 0.25±0.20 at month 3, 0.40±0.11 at month 6 (\( p=0.012 \) and n.s., respectively, when compared to baseline values) (Fig. 3b). These findings were validated by immunoprecipitation experiments. IgG fractions purified from serum samples taken from SSc patients at baseline and six months after rituximab were used to immunoprecipitate PDGF receptor from mouse fibroblasts expressing the human PDGF-\( \alpha \) subunit (Ftx cells). Control cells were mouse embryo fibroblasts derived from PDGF receptor knock-out embryos (F-/- cells). Figure 3c shows that the amount of human PDGF\( \alpha \) immunoprecipitated by post-treatment IgG was remarkably decreased compared to baseline IgG (Fig. 3c).

With regard to the serum levels of SSc specific autoantibodies, no significant variation was detected 6 months after rituximab.

**Down-regulation of the Ha-Ras-pERK-ROS-Collagen loop triggered by anti-PDGFR autoantibodies**

We have demonstrated that T lymphocytes as well as fibroblasts derived from SSc patients spontaneously generate high levels of reactive oxygen species, which mediate and maintain an autocrine loop, involving Ras and ERK 1/2, responsible for increased expression of collagen and \( \alpha \)-SMA (28, 29, 36, 37). Thus, we have evaluated these molecular markers in skin fibroblasts taken at baseline and after treatment.

Interestingly, a significant decrease in the spontaneous ROS production by fibroblasts isolated from a forearm skin biopsy was observed in all patients after rituximab treatment. Figure 4a shows that fibroblasts had reduction of spontaneous ROS generation at months 3 and 6 after rituximab (171.7±76 and 158.5±75, respectively (\( p=0.005 \) when compared to baseline 317.5±74).

Immunoblotting of total proteins extracted from SSc fibroblasts grown in vitro from biopsies taken before and after rituximab showed a lower amount of Ha-Ras and pERK proteins six months after treatment (Fig. 4b). In the same fibroblasts, human Col1A1, Col1A2 and \( \alpha \)-SMA gene expression was significantly decreased three months after treatment, as shown by quantitative real-time PCR (Fig. 4c). Reduced protein expression of \( \alpha \)-SMA after rituximab was confirmed by immunoblotting (Fig. 4d).

**Skin score and clinical outcome**

The median MRSS was 23.5±4.1 at baseline. Six months after rituximab treatment the median MRSS was significantly reduced compared to baseline values (16.5±2.17; \( p=0.027 \)). A MRSS decrease ≥20% was detected at month 3 in 3 patients, in the remaining three patients MRSS improved after month 3. Four patients maintained skin improvement until month 6, whereas two patients showed MRSS increase after month 4 (data not shown). Intraobserver agreement was found according to Landis and Koch guidelines, with a weighted \( \kappa \) at 0.70 (95% CI 0.51–0.96) (38).

The HAQ score markedly improved in five patients and was unchanged in one (data not shown). The VAS for self reported wellness showed a trend towards improvement (data not shown).

Lastly, visceral involvement remained stable in all patients.

Rituximab infusion was well tolerated and no major side effects were observed. A slight increase (x 1.5) of gamma-glutamyltransferase was observed from month 2 to month 6 in one patient, and a transient, mild elevation of both aminotransferases was seen in another patient one month after the last infusion.

**Discussion**

Recent uncontrolled studies (21-26) and a retrospective analysis (39) have reported improvement of skin fibrosis in SSc patients treated with anti-CD20 monoclonal antibodies rituximab. In a further clinical trial Lafyatis et al. reported a significant reduction of myofibroblasts in skin biopsies from patients treated with rituximab, although there was no improvement of the MRSS (40).

This is the first study to demonstrate that B cell depletion is associated with a downregulation of the molecular markers of fibroblast activation and provides an experimental proof of the mechanism responsible for the clinical amelioration of skin fibrosis in RTX-treated patients.

B cells may facilitate the pathogenesis of fibrosis in SSc patients by multiple mechanisms including amplification of the immune response towards autoanti-
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gens, release of cytokines and growth factors, and production of autoantibodies (for review see ref. 41). With regard to the latter, in recent years several putative pathogenic autoantibodies have been described (42). Their presence and role are still a matter of debate. In the present work we have focused on anti-PDGFR receptor stimulatory autoantibodies which we described in a cohort of SSC patients (14). In vitro experiments demonstrated that these auto-antibodies can convert normal human fibroblasts into SSc-like cells, characterised by overproduction of ROS and collagen through PDGFR stimulation, suggesting that they may contribute to the pathogenesis of SSc (14). Their presence has not been confirmed by other authors due to the different IgG purification and readout methods employed (43–45), as addressed elsewhere (46). However, more recently, further demonstration and precise detection of anti-PDGFR IgG in the serum of SSC patients has been made by cloning monoclonal and anti-PDGFR auto-antibodies from the immune repertoire of SSC patients (47). In the present work we show that following rituximab treatment anti-PDGFR autoantibodies reduction corresponded with the decrease of ROS generation by fibroblasts as well as with the expression of Ha-Ras, pERK1/2 and type I collagen genes. This implies that the effect of rituximab on fibroblast activation is due, although other mechanisms cannot be ruled out, to the depletion of agonistic anti-PDGFR autoantibodies producing B cells. Of note, as already shown by Lafyatis et al., the titer of anti-centromere and anti-topoisomerase autoantibodies did not change significantly (40).

This finding is reminiscent of what occurs in patients with systemic lupus erythematosus, in whom autoantibodies that are secreted by short-lived plasma cells correlate with disease activity and are depleted by treatment with anti CD20 monoclonal antibody (48).

Interestingly, the biological effect of rituximab was short lived. At month 6 there was evidence of increase of the number of circulating B cells, of anti-PDGFR autoantibodies. The absence of CD20 on pro-B cells, which allows the regeneration of circulating B lymphocytes from the bone marrow, can explain the time-limited effect of anti-CD20 monoclonal antibody. Periodic reinfusion of anti CD20 may be thus required to allow the stabilisation of clinical and biologic efficacy in SSc.

It remains to be established whether these results can be applied to fibroblasts from other sites than the skin and in patients with longer disease duration. In the latter situation it has been suggested that at some stages of disease progression, fibroblasts may become constitutively activated engaging a ROS-mediated self-perpetuating loop. ROS amplified collagen production inducing DNA damage and oncogene expression (49-51).

In conclusion, we herein report the biological and clinical efficacy of B cell blockade with rituximab in 6 patients with systemic sclerosis. Anti-PDGFR autoantibodies were markedly decreased and activation of skin fibroblasts was markedly reduced after rituximab treatment. Controlled, randomised trials (52) are required to verify whether rituximab therapy may be more indicated in some subgroups of SSC patients.

References
2. JIMENEZ SA, DERK CT: Following the molecular pathway toward an understanding of the pathogenesis of systemic sclerosis Ann Inter Med 2004; 140: 37-50.


