Experience with rituximab in scleroderma: results from a 1-year, proof-of-principle study

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Objective. To assess the efficacy of rituximab (RTX) in SSc.

Methods. Fourteen patients with SSc were evaluated. Eight patients were randomized to receive two cycles of RTX at baseline and 24 weeks [each cycle consisted of four weekly RTX infusions (375 mg/m²)] in addition to standard treatment, whereas six patients (control group) received standard treatment alone. Lung involvement was assessed by pulmonary function tests (PFTs) and chest high-resolution CT (HRCT). Skin involvement was assessed both clinically and histologically.

Results. There was a significant increase of forced vital capacity (FVC) in the RTX group compared with baseline (mean ± s.d.: 68.13 ± 19.69 vs 75.63 ± 19.73, at baseline vs 1-year, respectively, P = 0.0018). The median percentage of improvement of FVC in the RTX group was 10.25%, whereas that of deterioration in the control group was 6.45% (P = 0.002). Similarly, diffusing capacity of carbon monoxide (DLCO) increased significantly in the RTX group compared with baseline (mean ± s.d.: 52.25 ± 20.71 vs 62.23 ± 23.21, at baseline vs 1-year respectively, P = 0.017). The median percentage of improvement of DLCO in the RTX group was 19.46%, whereas that of deterioration in the control group was 7.5% (P = 0.023). Skin thickening, assessed with the Modified Rodnan Skin Score (MRSS), improved significantly in the RTX group compared with the baseline score (mean ± s.d.: 13.5 ± 6.84 vs 8.37 ± 6.45 at baseline vs 1-year, respectively, P < 0.001).

Conclusion. Our results indicate that RTX may improve lung function in patients with SSc. To confirm our encouraging results we propose that larger scale, multicentre studies with longer evaluation periods are needed.

KEY WORDS: Scleroderma, Systemic sclerosis, Rituximab, Interstitial lung disease, Fibrosis, B cells.

Introduction

SSc is a chronic systemic autoimmune disease characterized by vasculopathy and progressive fibrosis. SSc—interstitial lung disease (ILD)—is not uncommon in patients with the diffuse form of the disease and represents the clinical manifestation that dictates prognosis; this manifestation responds poorly to treatment. Therapeutic options for treating SSc-associated ILD are limited and most of the drugs tested so far have shown poor or modest results. Cyclophosphamide (CYC) has shown statistically significant but clinically questionable efficacy in the treatment of SSc-associated ILD, but is associated with immunosuppression underscoring the necessity for novel, more effective and less toxic therapies [1–4]. We and others [5–7] have employed mycophenolate mofetil (MMF) in the treatment of a limited number of patients with SSc-related ILD with encouraging, yet preliminary, results.

It has been suggested that targeting B cells could be a candidate therapy for SSc, since several lines of evidence point in the direction of B cells having a possible pathogenic role in this debilitating disease [8, 9]. B cells from tight skin mice—an animal model of SSc—exhibit chronic hyperactivity and exaggerated calcium responses after B-cell receptor (BCR) cross-linking. B cells from this animal model show augmented CD19 (an important positive BCR response regulator) signalling caused by impaired function of CD22, a negative BCR response regulator [10]. Likewise, B cells from patients with SSc overexpress CD19, compared with B cells from healthy subjects and disease control patients, and are chronically activated [11]. Furthermore, studies revealed that B-cell genes were specifically transcribed in SSc skin [12] and that B-cell infiltration was a prominent feature of SSc-associated ILD [13].

Rituximab (RTX) is a chimeric mAb against human CD20 that depletes peripheral B cells. It has been successfully introduced in the treatment of systemic rheumatic diseases and exhibits an acceptable safety profile. In animal models of SSc, administration of anti-CD20 mAb to newborn tight skin mice led to significant suppression of skin fibrosis [14]. On the other hand, there are encouraging data from the literature regarding the use of RTX in chronic graft vs host disease (GVHD) [15–18]. GVHD is a late complication of heterologous haematopoietic stem-cell transplantation and exhibits several similarities to SSc, such as scleroderma-like skin manifestations and circulating autoantibodies. Furthermore, chronic GVHD has been considered by some as a systemic autoimmune disease [19–22]. The observed microchimerism in a significant percentage of patients with SSc may further suggest pathogenetic similarities between the two entities, justifying similar therapeutic trials [23, 24]. Recently, two uncontrolled studies have explored the potential clinical efficacy of RTX in SSc. In the first one, skin fibrosis as assessed clinically and histologically improved significantly in the RTX-treated patients [25]. In the second one, even though no overt clinical benefit was observed, skin biopsies from RTX-treated patients exhibited a significant reduction in the myofibroblast score and the patients remained clinically stable throughout the study period [26]. There are also two additional reports (in abstract form) showing improvement of skin fibrosis (27, 28) and a case report of improvement of SSc-associated ILD (29). The preliminary encouraging results from the use of RTX in animal models of SSc and in humans with chronic GVHD and SSc has led us to investigate more thoroughly the potential efficacy of RTX...
in patients with SSc in an open-label, proof-of-principle, randomized, controlled study. We report herein that RTX treatment of patients with SSc and SSc-associated ILD led to improvement of lung function and was well tolerated.

Patients and methods

Patients

We enrolled 14 patients with a diagnosis of SSc, fulfilling the preliminary ACR criteria for the classification of the disease (30). Baseline demographic and clinical characteristics of the patients are presented in Table 1. All patients underwent a complete physical examination and a detailed review of their medical records prior to study enrolment. Other variables were also evaluated (full blood count, biochemistry profile, autoantibody profiles, urinalysis, ECG and cardiac ultrasound). Inclusion criteria were: (i) the detection of anti-Scl-70 autoantibodies in their sera; (ii) the presence of SSc-associated ILD as indicated by findings in either high-resolution CT (HRCT) of the chest or pulmonary function tests (PFTs) or both; and (iii) the absence of any changes in medications and/or dosage of treatment administered during the last 12 months before enrolment. All patients belonged to the diffuse variety of the disease as documented by the clinical presentation of skin involvement at the time of the study and/or its course over time since diagnosis. Moreover, all patients were anti-Scl-70 positive and had significant ILD, a feature of diffuse SSc. No changes in medication were allowed during the study.

A local (Patras University Hospital, Patras, Greece) ethics committee approved the study (which fulfilled the Declaration of Helsinki requirements) and a written informed consent was obtained from all participating individuals.

Randomization and treatment

Patients born on an even-numbered date (n=8) were assigned to the RTX group and those born on an odd-numbered date (n=6) to the control group. Patients in the RTX group received four weekly pulses of RTX (375 mg/m²) at baseline and at 6 months on top of the already administered treatment. Patients in the control group continued their previously administered treatment unchanged (details in Table 2). Four patients in the RTX group (Patients 2, 4, 5 and 6) and two in the control group (Patients 11 and 14) were on MMF therapy during study enrolment and remained so throughout the study. They have been on that therapy for at least 4 years prior to study enrolment (apart from Patient 14 who was on MMF therapy for 2 years prior to enrolment). Three patients in the RTX group (Patients 2, 3 and 4) and one in the control group (Patient 10) had received CYC in the past but were off that therapy for at least 3 years prior to study enrolment. There were no significant differences between the two patient groups as shown in Table 2.

PFT

Standard PFTs were performed, at baseline, at 24 weeks and at 1 year in all patients, including assessments of forced vital capacity (FVC), forced expiratory volume in the first second (FEV1), total lung capacity and diffusing capacity of carbon monoxide (DLCO) corrected for haemoglobin concentration. PFT parameters are expressed as a percentage of normal predicted values based on age, sex and height. All tests were performed at the same laboratory, at our institution (Patras University Hospital).

Chest HRCT

All patients had an HRCT performed at baseline and at 24 weeks using a 16 multi-detector GE CT Scan (General Electric, Waukesha WI, USA) with slice thickness of 0.625 mm. Imaging findings were interpreted separately by two experienced radiologists (C.K. and A.K.) in a blinded fashion. Acquisition parameters of tube voltage, tube current and slice thickness were 140 kilovolt and 100 milliamperes, respectively. In order to obtain contiguous images of lung abnormalities, a second low-dose scanning (120 kV, 200 mA) was performed with slice thickness of 1.25 mm, covering the whole thorax. This protocol permitted data reconstruction and evaluation of images in coronal and sagittal levels using multiplanar reformatting algorithm. The severity and extent of lung involvement was assessed according to the scoring system proposed by Warrick et al. [31] as follows: one point was assigned to ground glass appearance, two points to irregular pleural margins, three to septal/subpleural lines, four to honeycombing and five to subpleural cysts. The sum of points results in the severity score (0–15 points). Extent score
was calculated giving 1 point when involvement of one to three lung segments was present, 2 points for four to nine segments and 3 points to more than 10 segments (0–15 points). Total score was obtained by adding the two above scores (0–30 points).

**Clinical assessment of skin thickening**

The MRSS was used for clinical assessment of skin thickening at baseline and at 1 year, by an experienced assessor in a blinded fashion [32, 33].

**Skin histology**

Histological assessment of skin fibrosis was made by skin biopsies performed at baseline and at 24 weeks of evaluation (a 5-mm punch biopsy of lesional skin). Skin biopsies were performed in six patients of the RTX group and three patients of the control group (including those receiving CYC) and were performed prior to RTX administration. Biopsies were taken from lesional skin in the forearm; the second biopsy was taken from lesional skin adjacent (always < 2 cm) to the site of the baseline biopsy (supplementary Fig. 1, available as supplementary data at Rheumatology Online).

**Pathological evaluation of skin biopsies.** All skin biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin. Four micrometre-thick paraffin sections were obtained and stained with haematoxylin and eosin, and Masson’s trichrome (fibrosis evaluation). All trichrome stains of biopsies were performed at the same time, using the same staining set (04-011802-Masson trichrome, Goldner-Biopica, Milan, Italy) in order to have comparable results.

**Fibrosis quantification.** To quantify collagen accumulation in the dermis, we employed the Image J software (freely downloadable and developed by Wayne Rasband at the Research Service Branch of the NIH) according to the method described previously [34]. A similarly and simultaneously stained skin biopsy from skin keloid was analysed as well, representing a positive control for collagen deposition. Collagen deposition was examined separately for the papillary and the reticular dermis.

**Evaluation of immunostains for the presence of B and T cells.** All biopsies were examined immunohistochemically for the presence of T and B cells as described previously using the BenchMark® XT automated slide stainer (Ventana, Tucson AZ, USA) and a standard streptavidin biotin method [i view DAB-DAB MapTM Detection kit (streptavidin horseradish peroxidase detection kit), Ventana]. Antibodies used included: anti-CD4 (Neomarkers, Fremont CA, USA), anti-CD8 (Novocastra, Newcastle, UK) and anti-CD20 (Dako, Carpinteria CA, USA). As has been previously reported [35], 10 cell counts were performed manually at ×400 magnification using a 10×10 microscope grid. Numbers of immunohistochemically stained cells were determined by visual inspection of three different fields per section. The average scores then were calculated. All biopsies were evaluated simultaneously in a blinded (to treatment and date) fashion.

**Overall functional impairment**

We used the 20-item HAQ-DI [36, 37]. Clinically significant improvement in functional status was defined as a 0.2 decrease in HAQ-DI score, as previously described [38].

**Levels of circulating inter-cellular adhesion molecule-1, E-selectin, vascular cell adhesion molecule and ET-1**

Serum samples were obtained from all patients at baseline and at 24 weeks and were stored at −20°C. Serum levels of the three soluble endothelium activation/injury markers and of ET-1 were measured employing ELISA methodology, using commercially available kits, according to the manufacturer’s instructions (R&D Systems, Minneapolis MN, USA).

**Efficacy end points**

Primary end points included evaluations of (i) changes in pulmonary function as assessed by PFT and (ii) clinical assessment of skin involvement by the MRSS. Secondary outcome measures included changes in (i) skin histology including collagen deposition and lymphocytic infiltration, (ii) HRCT scores, (iii) serum levels of soluble markers and (iv) overall functional impairment.

**Statistical analysis**

Statistical analysis was performed using the SPSS software (SPSS Inc., Chicago, IL, USA), version 14. Data are presented as mean±s.d., median (upper and lower quartile values) or percentages, as appropriate. The paired Student’s t-test, Wilcoxon matched pairs test, Mann–Whitney test and Fisher’s exact test were used where indicated. Values of P<0.05 were considered as statistically significant.

**Results**

**Effects of RTX treatment on SSc-associated ILD**

PFTs and HRCT were used to assess the potential effect of RTX administration on SSc-associated ILD.

**PFTs improve following RTX treatment.** At the 1-year evaluation, there was a significant increase of FVC in the RTX group compared with baseline (mean±s.d.: 68.13±19.69 vs 75.63±19.73, at baseline vs 1 year, respectively, P=0.0018), whereas no change was noticed in the control group (mean±s.d.: 86±19.57 vs 81.67±20.69, at baseline vs 1 year, respectively, P=0.23), as shown in Fig. 1A and B. The median (upper and lower quartile values) percentage of improvement of FVC in the RTX group was 10.25% (6.19–18.65), whereas in the control group FVC deteriorated [median percentage of deterioration (upper and lower quartile values) 5.04% (4.11–11.6)]. Direct comparison of FVC changes recorded at 1 year revealed that the RTX-treated group improved significantly (P=0.002) compared with the standard treatment (control) group.

There was a significant increase of DLCO in the RTX group compared with baseline (mean±s.d.: 52.25±20.71 vs 62±23.21, at baseline vs 1 year, respectively, P=0.017), whereas no changes were noticed in the control group (mean±s.d.: 65.33±21.43 vs 60.17±23.69, at baseline vs 1 year, respectively, P=0.25), as shown in Fig. 1C and D. The median (upper and lower quartile values) percentage of improvement of DLCO in the RTX group was 19.46% (3.7–30.8), whereas in the control group the median percentage of deterioration was 7.5% (1.4–26.57) (P=0.023).

The improvement of lung function tests in the RTX-treated patients was already evident in the 24-week evaluation (mean±s.d.: 71.5±21.3 and 55.2±25.1 for FVC and DLCO, respectively).

**Effects of RTX treatment on HRCT.** HRCT scores were identical at baseline and at 24 weeks in all patients in the RTX group (mean±s.d.: 13.1±4.5). In the control group, there was a modest increase in the HRCT score that was not statistically significant (mean±s.d.: 16.4±6.4 vs 16.8±6.5, at baseline vs 24 weeks, respectively, P=0.170).

**Effects of RTX treatment on skin disease in patients with SSc**

To evaluate any potential effect of RTX on skin involvement we performed standard clinical assessment and skin biopsy analysis.
**Effects of RTX treatment on skin thickening, as clinically assessed.** Skin thickening, assessed with the MRSS, was similar in the two treatment groups at baseline (Table 1, \( P = 0.50 \)). However, at the 1-year evaluation, there was a significant decrease of MRSS in the RTX group compared with the baseline score (mean ± s.d.: 13.5 ± 6.84 vs 8.37 ± 6.45 at baseline vs 1 year, respectively, \( P = 0.0003 \)). On the contrary, no significant change in skin scores was noticed in the control group at 1 year when compared with the baseline MRSS (mean ± s.d., 11.50 ± 2.16 vs 9.66 ± 3.38 at baseline vs 1 year, respectively, \( P = 0.16 \)). The median (upper and lower quartile values) percentage of improvement in the RTX-treated group was 39.25% (27.33–64.95) compared with 20.80% (10.78–39.28) in the control group. Statistical analysis revealed that despite differences, the values were not statistically significant (\( P = 0.06 \)).

**Effects of RTX treatment on collagen deposition.** In the RTX-treated group, there was a significant reduction of collagen deposition in the papillary dermis at 24 weeks compared with baseline (mean ± s.d.: 51.75 ± 19.78 vs 31.68 ± 14.02 at Week 0 vs Week 24, respectively, \( P = 0.030 \)). The control group showed no change in collagen deposition in the papillary dermis at 24 weeks compared with baseline values (mean ± s.d., 46.53 ± 22.43 vs 46.27 ± 10.49 at baseline vs Week 24, respectively, \( P = 0.980 \)). The median (upper and lower quartile values) percentage of improvement of skin fibrosis in the RTX group was 38.33% (6.86–59.9), whereas in the control group skin fibrosis worsened (median percentage of worsening of skin fibrosis was 5.23%). Histological improvement was evident in four patients (Patients 2, 3, 4 and 6) of the RTX group and coincided with clinical improvement in these patients (Table 2). Differences were not statistically significant (\( P = 0.09 \)). Representative skin histology is shown in Figs 2 and 3.

When collagen deposition in the reticular dermis was evaluated comparatively at baseline and at Week 24, there were no differences either in the RTX (mean ± s.d.: 76.57 ± 16.04 vs 73.07 ± 9.86 at Week 0 vs Week 24, respectively, \( P = 0.758 \)) or in the control group (mean ± s.d.: 59.97 ± 28.88 vs 57.03 ± 22.63 at Week 0 vs Week 24, respectively, \( P = 0.498 \)) of patients with SSc.

Exceptionally, a striking improvement was observed in Patient 2 (RTX group), who displayed a significant reduction of skin fibrosis not only in the papillary but in the reticular dermis as well, and had clinically an almost complete resolution of sclerodermatous skin lesions (Fig. 2C and D).

**Effects of RTX on skin infiltrating B cells.** The presence of T and B cells was assessed by immunohistochemistry in all biopsies. Representation of T cells was substantially limited in all patients; these were predominantly CD8+ T cells and no differences were observed at 24 weeks, compared with baseline (data not shown). The number of B cells was relatively low as well, but they were more abundant than T cells. RTX administration significantly reduced the number of B cells in three patients (Patients 2, 4 and 6) but had no effect on the remaining three patients of the RTX group (Patients 1, 3 and 5). Patients exhibiting B-cell depletion in the skin following RTX administration were the ones with the higher numbers of B cells at baseline. All three patients with skin B-cell depletion improved histologically. However, among the three non-B-cell depletors, there was a single patient who improved histologically (Patient 3). In the control group no difference was recorded in B cell numbers at 24 weeks compared with baseline. All data and representative skin immunohistochemistry are shown in Fig. 4.

**Overall functional impairment**

There was a significant improvement in HAQ scores at 1 year compared with baseline in the RTX group [median (lower and upper quartile values), 0.687 (0.28–1.25) vs 0.312 (0.125–0.687) at baseline vs 1 year, respectively, \( P = 0.03 \)]. No change was noticed in the control group [median (lower and upper quartile values), 0.312 (0.09–0.90) vs 0.125 (0.09–0.40) at baseline vs 1 year, respectively, \( P = 0.130 \)]. In the RTX group, six patients exhibited a clinically significant improvement of functional status (as defined by a decrease of HAQ score by 0.2) and two patients remained unchanged. By contrast, in the control group one patient worsened, two remained unchanged and three patients improved.
Markers of endothelium activation/injury and ET-1

To examine any potential effects of RTX administration on endothelium, which is a key player in SSc pathogenesis, we measured serum levels of three markers of endothelium activation/injury [E-selectin, vascular cell adhesion molecule (VCAM) and inter-cellular adhesion molecule-1 (ICAM-1)] and ET-1. There was a trend towards a decrease in all three endothelium activation/injury markers and ET-1 serum levels in the RTX group at 24 weeks, compared with baseline, which did not reach statistical significance. It was worth noticing though that the patients with histological improvement in skin biopsy (Patients 2, 3, 4 and 6) were the ones who displayed a decrease in serum levels of at least three of the four markers studied. In the control group, even though no statistically significant changes were observed, VCAM and ET-1 levels showed an upward trend at 24 weeks compared with baseline (data not shown).

Adverse events

Patient 4 (RTX group) suffered a respiratory tract infection 3 months after the second cycle of RTX. The patient was hospitalized for 3 days, treated with four antibiotics and oxygen supplementation and recovered fully in a few days.

Discussion

This is the first randomized controlled study in the literature to evaluate the efficacy of RTX in patients with SSc. In this study, we report an improvement in lung function with an increase in FVC and DL_CO at 1 year, following two cycles (composed of four weekly doses each) of RTX in patients with SSc compared with the control group. Perhaps more importantly, none of the RTX-treated patients exhibited worsening of either FVC or DL_CO, whereas five out of six patients had declining FVC and DL_CO values at 1 year in the control group. These results indicate that RTX may favourably affect lung function parameters in patients with SSc. We should note, however, that patients in the control group tended to have more early disease and better lung function parameters (although not statistically different from the RTX group) making them more likely to deteriorate over the time of the study. Radiological assessment of ILD by HRCT revealed no changes in the RTX group, whereas there was a minor deterioration of two patients in the control group. It is of importance
ABSTRACT

The treatment of SSc (systemic sclerosis) is currently focused on symptomatic and supportive care. Despite the well-documented clinical efficacy of B-cell-depleting antibodies, such as rituximab (RTX), in the treatment of SSc-associated lung disease, there is limited evidence for the potential effect of RTX on skin fibrosis. We aimed to examine the effect of RTX on skin fibrosis in patients with SSc during long-term treatment. Our study is the first to report the effects of two consecutive cycles of RTX on lung function and skin thickening in SSc.

Although RTX has been successfully introduced lately in the treatment of various autoimmune diseases, its exact mechanism of action is not completely understood. Pathogenesis of SSc is largely unknown but the results of the present study indicate that B cells may play a role and RTX may have a favourable effect on the disease process. RTX targets B cells that are present in skin biopsies of patients with scleroderma and are the source of autoantibodies, some of which may contribute to pathogenesis.

In conclusion, our data support the idea that RTX treatment may stabilize skin fibrosis and possibly modify the disease process and could serve as a marker of response to RTX therapy in patients with SSc.
Rheumatology key messages

- Several lines of evidence indicate a potential role of B cells in the pathogenesis of scleroderma.
- RTX may improve lung function in patients with SSc.
- Large-scale, multicentre studies are needed to evaluate the potential efficacy of RTX in scleroderma.

Supplementary data
Supplementary data are available at Rheumatology Online.

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