ARTHRITIS & RHEUMATISM
Vol. 48, No. 8, August 2003, pp 2256–2261
DOI 10.1002/art.11081

Effects of Oral Cyclophosphamide and Prednisolone Therapy on the Endothelial Functions and Clinical Findings in Patients With Early Diffuse Systemic Sclerosis

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Objective. **The endothelial damage of microvascular structures in systemic sclerosis (SSc; scleroderma) is associated with increased levels of endothelial adhesion molecules and endothelium-associated cytokines, including E-selectin and thrombomodulin. Although there is still no ideal specific pharmacologic therapy for SSc, cyclophosphamide has resulted in clinical improvement in patients with SSc-related active alveolitis. This study was designed to assess the expression of E-selectin and thrombomodulin in patients with early diffuse SSc, and to investigate the effects of oral cyclophosphamide combined with prednisolone therapy on the levels of these endothelium-associated cytokines and on the patients' clinical outcomes.**

Methods. **Thirteen patients with early diffuse SSc were treated with oral cyclophosphamide (2–2.5 mg/kg/day) and methylprednisolone (30 mg/every other day) for 1 year. The outcomes were determined as clinical (skin score) and laboratory parameters (including the erythrocyte sedimentation rate, complete blood cell count, levels of C-reactive protein, antinuclear antibody, anti–double-stranded DNA, rate of creatinine clearance, and findings on pulmonary function tests, esophageal manometry, and echocardiography). The concentrations of E-selectin and thrombomodulin were measured in the pretreatment and posttreatment serum samples from the SSc patients and from 12 healthy adults as controls.**

Results. **In the patients with early diffuse SSc, pretreatment and posttreatment mean levels of E-selectin were 51 ng/ml (range 34.2–135.5) and 33.4 ng/ml (range 23–62.5), respectively ($P = 0.01$), and those of thrombomodulin were 82 ng/ml (range 35.8–120.5) and 74.6 ng/ml (range 23.3–91.3), respectively ($P = 0.016$). Clinical and laboratory parameters (the skin score and measures of pulmonary function [forced vital capacity and diffusing capacity for carbon monoxide]) were also improved ($P < 0.05$ for each) at the end of the followup period.**

Conclusion. **Combination therapy with cylophosphamide plus prednisolone is effective in the treatment of early diffuse SSc. Circulating levels of E-selectin and thrombomodulin not only demonstrate the extent of endothelial injury and/or activation, but also could be a useful marker to monitor the disease activity in SSc.**

Systemic sclerosis (SSc; scleroderma) is a systemic autoimmune inflammatory connective tissue disorder that is characterized by excessive production of extracellular matrix by fibroblasts as well as damage of the endothelium of small vessels with subsequent intimal hyperplasia and tissue ischemia, together with activation of the immune system. Endothelial cell damage and platelet activation can occur and may concurrently contribute to the peripheral ischemia of the disease. Involvement of organs such as the lung, heart, kidney, gut, and skin causes substantial morbidity and mortality in SSc.

Vascular dysfunction is a key pathobiologic element of the SSc disease process. Since vascular lesions occur early in the course of the disease and precede the development of fibrosis, endothelial cells have been

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Submitted for publication November 21, 2002; accepted in revised form March 31, 2003.

implicated in the pathophysiology of fibrosis. Endothelial cell damage is evident in SSc by the occurrence of capillary drop-out and vascular leakage, leading to formation of edema. Inflammatory cells are attracted to damaged sites and migrate to adjacent tissue. In turn, activated endothelial cells up-regulate the expression of cytokines (1).

Adherence and extravasation of immunocompetent cells depend on their interactions with adhesion molecules. One of the best-characterized endothelial cell adhesion molecules is E-selectin, which is mainly restricted to activated endothelium (2,3). Thrombomodulin is a vascular endothelial surface glycoprotein that is present on the luminal surface of endothelial cells. Soluble thrombomodulin reflects the injury to the endothelial cells, since the fragments of thrombomodulin detected in the blood were not secreted by endothelial cells under physiologic conditions (4). These endothelial parameters thus might provide a useful tool for the characterization and prediction of the activity and progression of the disease (5).

Currently, there is no ideal and specific pharmacologic curative approach for scleroderma. However, the therapeutic approach should be initiated at the early stages of SSc, before the fibrosis begins to develop. In interstitial lung disease, treatment with cyclophosphamide in SSc may improve the inflammatory aspects of the disease that occur before the development of severe fibrosis, although this approach is still in the experimental stages (6).

The aim of this study was to investigate the efficacy and the toxicity of oral cyclophosphamide and prednisolone therapy at the early stages of the disease, by examining clinical outcomes (clinical and laboratory findings) together with the levels of E-selectin and thrombomodulin, which serve as the activation markers of endothelial function.

PATIENTS AND METHODS

Patient selection and clinical evaluation. Thirteen patients with early diffuse scleroderma (11 female, 2 male; mean ± SD age 37.8 ± 11.3 years) were involved in this prospective, open trial between 1996 and 1999. The disease duration was <2 years in all patients, and all fulfilled the criteria for SSc proposed by the American College of Rheumatology (formerly, the American Rheumatism Association) (7). The initial symptoms of the patients were Raynaud's phenomenon, diffuse swelling of the hands, skin thickening, esophageal symptoms (dysphagia, heartburn, or dyspnea), and arthralgia. None of the patients was previously treated with disease-modifying antirheumatic drugs. All of them had diffuse cutaneous involvement, which is described as skin thickening proximal to the elbow and/or knee, with or without face and neck involvement (7). Patients who had severe pulmonary involvement (forced vital capacity [FVC] <50% of predicted, diffusing capacity for carbon monoxide [DLCO] <40% of predicted) and comorbidities such as congestive heart failure, chronic obstructive lung disease, or diabetes mellitus were excluded from the study. Patients who were receiving aspirin or any other nonsteroidal antiinflammatory drug that could affect endothelial cell functions were not enrolled in the study.

Physical examinations of all patients were performed by the same physician (ZO). The degree and the extent of the dermal thickening were measured by using the semiquantitative Rodnan skin scoring system (maximum score 104) (8). Laboratory assessments comprised the erythrocyte sedimentation rate (ESR), complete blood cell (CBC) count, serum biochemistry, C-reactive protein, rheumatoid factor, 24-hour urinary microprotein excretion, creatinine clearance, urine analysis, and immunologic parameters, including antinuclear (ANA), anti–double-stranded DNA, anti–Scl-70 (topoisomerase I), and anticentromere antibodies. Chest roentgenograms were obtained, and electrocardiography, echocardiography, esophagography, and pulmonary function tests (spirometry for the DLCO) were also performed to evaluate organ involvement. All of those parameters were evaluated by comparing the results at the start and the end of the study.

As mentioned, pulmonary involvement was evaluated by spirometry for measurement of the DLCO. An FVC ≥80% of predicted, a forced expiratory volume in 1 second (FEV1)/FVC ≥70% of predicted, and DLCO ≥80% of predicted were defined as normal pulmonary function. The measured FVC values <80% of predicted and FEV1/FVC <70% of predicted were accepted as the predictors of restrictive pulmonary disorders (9).

The above-mentioned clinical and laboratory parameters were reevaluated every 6 months up to 1 year. Serum levels of thrombomodulin and E-selectin were determined at the beginning and at the end of the study. Twelve healthy volunteers (4 female, 8 male; mean ± SD age 33.1 ± 10.9 years) were accepted as the control group to compare the levels of E-selectin and thrombomodulin.

Treatment schedule. All patients were treated with 2–2.5 mg/kg/day oral cyclophosphamide and with 30 mg/every other day (equivalent to 15 mg/day) oral methylprednisolone. The corticosteroid dose was tapered by 2.5 mg every 6 weeks until reaching a dose of 2.5 mg/every other day, which was then maintained.

Dose modification. Patients were followed up closely for the expected side effects of the drugs. The CBC count and urine analyses were repeated every 2 weeks. Development of leukopenia (white blood cell [WBC] counts <3,500/mm^3), serious gastrointestinal intolerance, hematuria (>5 red blood cells/high-power field), and/or proteinuria (higher than 500 mg/day) was considered to be a definitive reason to withhold cyclophosphamide. If minor side effects such as a decreased WBC count between 3,500/mm^3 and 5,000/mm^3 or gastric irritability developed, then temporary suspension of the cyclophosphamide or a reduction in the dose to half for 1–2 weeks was considered necessary. After the WBC count recovered, the drug was continued at the initial dosage schedule.

Collection of serum samples. For measurement of the levels of E-selectin and thrombomodulin, all serum samples

Table 1. The clinical and demographic features of the systemic sclerosis patients*

Feature	Value
Age, years, mean ± SD	37.8 ± 11.3
Sex, no. female/no. male (% female)	9/2 (82)
Disease duration, months, mean ± SD	11.8 ± 7.1
Followup duration, months, mean ± SD	16.7 ± 4.1
Raynaud's phenomenon	9 (81.8)
Joint involvement	5 (45.5)
Puffy hands	5 (45.5)
Nail-fold capillary dilatation	9 (81.8)
Pulmonary involvement	9 (81.8)
Esophageal involvement	6 (54.5)
Telangiectasia	1 (9.1)
Antinuclear antibody	9 (81.8)
Anti–Scl-70 antibody	8 (72.7)

* Except where otherwise indicated, values are the no. (%) of patients.

were obtained between 9:00 and 10:00 in the morning from resting subjects who had been required to fast. Care was taken to avoid platelet activation, by the use of atraumatic needle punctures, a butterfly needle, and minimal stasis with tourniquet release before blood withdrawal into 10-ml syringes. Smokers were asked to abstain from smoking since the previous night (as of 12:00 midnight). Plasma was prepared for measurement of thrombomodulin by collecting blood into 3.13% trisodium citrate, which was then centrifuged within 20 minutes of venipuncture at 3,000*g*. All samples for measurement of thrombomodulin were stored at −80°C until assayed. Blood samples for measurement of E-selectin were centrifuged at 3,000*g* for 10 minutes, and aliquots of serum were stored at −30°C until assayed.

Measurement of E-selectin and thrombomodulin. Thrombomodulin was measured with a microenzyme immunoassay technique using commercial kits (thrombomodulin immunoassay; Diagnostica Stago, Asniéres-sur-Seine, France). Circulating E-selectin was measured with a quantitative sandwich enzyme immunoassay technique using commercially available assays (R&D Systems, Oxford, UK) in accordance with the manufacturer's instructions.

Statistical analysis. All data at the end of the first year were collected and analyzed by using SPSS software, version 7.0 (SPSS, Chicago, IL). The pre- and posttreatment laboratory values of the cyclophosphamide group were compared by the paired-sample *t*-test, and pre- and posttreatment clinical values and E-selectin and thrombomodulin levels from the cyclophosphamide group were compared by using the Wilcoxon test. The comparison of the pretreatment E-selectin and thrombomodulin levels in the patients and the healthy controls was made by using the Mann-Whitney U test. A *P* value below 0.05 was considered statistically significant.

RESULTS

During the study period, 2 patients were excluded from the treatment group because of lack of cooperation. Their clinical and laboratory results were obtained at entry, but they did not comply with the given time schedule of visits. Thus, their results were not entered in the statistical analysis. The results of the remaining 11 patients were evaluated. Pretreatment demographic features of the patients, including age, sex distribution, mean disease duration, skin involvement, evidence of Raynaud's phenomenon, joint involvement, pulmonary involvement, and esophageal involvement, and ANA and anti–topoisomerase I positivity are shown in Table 1. The patients' clinical and laboratory findings before and after treatment are shown in Table 2.

The therapeutic regimen of oral cyclophosphamide and prednisolone at the given doses was well tolerated by all patients. The most common side effects appeared to be leukopenia and microscopic hematuria. Most of the side effects were readily man-

Table 2. Clinical and laboratory changes in the systemic sclerosis patients before (1) and after (2) cyclophosphamide and prednisolone therapy*

	ESR		CRP		CLcr		FVC		DLCO		Skin		WBC		E-selectin, ng/ml		Thrombomodulin, ng/ml	
Patient	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	52	10	1.8	0.0	80	78	70	85	56	75	52	24	6,600	5,000	78.0	60.0	120.5	90.1
2	49	16	2.6	0.0	60	108	88	94	93	113	43	31	9,000	6,500	34.3	30.0	103.5	102.5
3	30	11	0.9	0.0	105	150	87	101	70	80	49	32	8,900	7,000	63.5	50.0	110.8	85.5
4	90	7	1.5	0.0	70	155	85	107	67	84	31	24	10,700	4,800	51.0	23.0	90.2	84.2
5	62	9	0.8	1.1	113	137	65	77	59	75	59	63	14,800	8,300	38.1	33.4	82.0	91.3
6	78	36	2.7	1.3	62	75	57	67	52	57	30	28	8,200	7,600	70.5	34.5	45.5	38.7
7	28	15	1.0	0.8	90	95	67	82	49	63	75	67	6,000	6,800	135.5	40.7	67.3	58.2
8	90	29	0.0	0.0	70	110	77	79	70	74	49	35	5,500	3,200	48.1	31.5	72.5	74.6
9	38	18	3.0	5.0	67	70	83	80	80	86	27	30	4,800	4,500	49.4	24.8	35.8	37.2
10	27	6	0.4	0.0	78	79	78	91	57	66	48	33	6,000	6,800	34.9	32.2	47.5	31.3
11	32	33	0.9	0.0	57	78	84	86	55	67	47	39	11,400	8,700	54.0	62.5	99.2	85.5

* ESR = erythrocyte sedimentation rate (mm/hour); CRP = C-reactive protein (mg/dl); CLcr = creatinine clearance (ml/minute); FVC = forced vital capacity (% of predicted); DLCO = diffusing capacity for carbon monoxide (% of predicted); Skin = Rodnan's skin score (maximum 104); WBC = white blood cell count (cells/mm^3).

Table 3. Comparison of pretreatment and posttreatment clinical and laboratory parameters of the systemic sclerosis patients*

Parameter	Pretreatment	Posttreatment	*P*
Skin score, median (range)	48 (27–75)	32 (24–67)	0.007
ESR, mm/hour	52.3 ± 24.4	17.2 ± 10.6	0.02
WBC, mm^3	8,300 ± 3,037	6,290 ± 1,710	0.02
Creatinine clearance, ml/minute	77.4 ± 18.3	103.1 ± 31.4	0.009
FVC, mean % of predicted	76.4 ± 10.3	86.2 ± 11.3	0.001
DLco, mean % of predicted	64.3 ± 13.2	76.3 ± 15	<0.001

* Except where otherwise indicated, values are the mean ± SD. See Table 2 for definitions.

ageable by dose reduction. None of the toxic effects necessitated complete cessation of the therapy. Skin scores improved in most of the patients after cyclophosphamide therapy ($P < 0.05$). Moreover, the ESR and WBC count were significantly decreased ($P < 0.05$) and the rate of creatinine clearance as well as the FVC and DLco values were significantly increased ($P < 0.05$ for each) in the patients after the therapy (Table 3).

The median E-selectin level in the patient group prior to treatment and in the control group was 51 ng/ml (range 34.2–135.5) and 33.5 ng/ml (range 16.6–62.6), respectively ($P < 0.01$). The pretreatment thrombomodulin level was 82 ng/ml (range 35.8–120.5) in the study group and was 36.8 ng/ml (range 30.7–44.5) in the healthy controls ($P < 0.01$). After the administration of the cyclophosphamide and prednisolone therapy, the E-selectin and thrombomodulin levels in the patients with early diffuse SSc were significantly reduced ($P = 0.01$ and $P = 0.016$ for E-selectin and thrombomodulin, respectively) (Table 4).

DISCUSSION

In this study, the levels of E-selectin and thrombomodulin in patients at the early stages of scleroderma were significantly elevated, indicating endothelial activation and/or injury in SSc. The most important observation in this study is that daily oral cyclophosphamide therapy positively affects circulating endothelial cell markers in SSc. To our knowledge, this is the first study demonstrating a reduction in the overexpressed in vivo E-selectin and thrombomodulin molecules after cyclophosphamide treatment in patients with SSc.

Increased levels of E-selectin in the tissue and circulating blood of SSc patients indicate that endothelial cell activation and leukocyte adhesion take place in the pathobiology of the disease (2,10,11–23). SSc fibroblasts promote leukocyte migration across endothelial cell monolayers (24). Other adhesive molecules could also complicate the pathologically altered endothelial cell functions in SSc (25–34). The increased soluble thrombomodulin concentration indicates endothelial injury in vasculitides (21,35,36).

In previous studies, iloprost decreased thrombomodulin levels but nifedipine did not (22), and cyclosporine treatment affected the expression of adhesion molecules (37) in scleroderma. Cyclophosphamide suppresses lymphokine production and modulates lymphocyte functions by alkylating various cellular constituents, and depresses the inflammatory response via normalization of neutrophilia and healing of vascular endothelial cells. Cyclophosphamide could be effective in the early inflammation stage of SSc lung involvement, before fibrosis has developed. Nevertheless, high-dose corticosteroid therapy alone has also been shown to be efficacious in some cases of scleroderma, especially in those with neutrophilic alveolitis (38). Active interstitial lung disease could be improved after the use of oral cyclophosphamide and low-dose prednisolone (39). Furthermore, cyclophosphamide plus prednisolone combination therapy has considerably improved the effect on both cutaneous involvement and pulmonary fibrosis (40,41). Later observations confirmed the efficacy of this combination therapy on pulmonary SSc (42).

In our study, treatment of patients with early diffuse SSc with oral cyclophosphamide, before visceral

Table 4. The elevation of thrombomodulin and E-selectin levels in comparison with healthy controls, and their decrease after cyclophosphamide and prednisolone therapy in patients with systemic sclerosis*

	Control	Patients		*P*†	*P*‡
		Pretreatment	Posttreatment		
Thrombomodulin, ng/ml	36.8 (30.7–44.5)	82 (35.8–120.5)	74.6 (23.3–91.3)	<0.001	0.016
E-selectin, ng/ml	33.5 (16.6–62.6)	51 (34.2–135.5)	33.4 (23–62.5)	0.008	0.01

* Except where otherwise indicated, values are the median (range).
† The comparison of baseline levels between the patients and the healthy controls, by Mann-Whitney U test.
‡ The comparison between pretreatment and posttreatment levels in the patients, by Wilcoxon test.

involvement, was approached on the basis of its mechanism of action. Considering the side effects associated with long-term high-dose corticosteroid therapy (40), we preferred to use alternate-day oral steroid therapy in relatively low doses and we tapered the steroid dose as soon as possible. Daily oral cyclophosphamide plus alternate-day oral prednisolone therapy was well tolerated in our SSc patients.

Interestingly, in our present study, we observed that increased E-selectin levels returned to normal after cyclophosphamide combination therapy in our SSc patients. Soluble thrombomodulin concentrations also significantly improved; however, thrombomodulin levels did not normalize in the SSc patients. Expression of adhesion molecules, including E-selectin, on endothelial cells is a dynamic, ongoing process in health and disease. Up-regulation of selectins may actively contribute to the increased adherence and extravasation of leukocytes. Cyclophosphamide treatment seems to adjust that process in SSc. In contrast, soluble thrombomodulin molecules are heterogeneous fragments that mainly originate from injured endothelial membranes. Therefore, circulating thrombomodulin levels have been accepted as a significant marker of endothelial damage and injury in vasculitides (35,36). Because our SSc patients had a damaged endothelium due to their disease, cyclophosphamide combination therapy failed to produce a completely normal endothelial structure, since the thrombomodulin levels did not normalize. However, the treatment did significantly improve endothelial function, as demonstrated via decreased thrombomodulin and normalized E-selectin concentrations in our patients.

Our findings represent the basis for larger, controlled studies, which would elucidate the potential value of endothelial molecules as surrogate markers for the clinical progression or remission of SSc in relation to distinct treatments. An effective approach to clinical assessment of SSc patients might be developed if our hypotheses are supported by further investigations in that framework.

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