Spontaneous Chromosome Damage (Micronuclei) in Systemic Sclerosis and Raynaud's Phenomenon

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ABSTRACT. ***Objective.*** To evaluate the prevalence of spontaneous chromosome damage in cultured peripheral lymphocytes of patients with systemic sclerosis (SSc), idiopathic Raynaud's phenomenon (RP), and suspected secondary RP, by means of molecular cytogenetic analysis.

Methods. We studied 43 patients with SSc, 13 with idiopathic RP, and 16 with suspected secondary RP and 25 healthy controls. As a marker of chromosome alteration we used the micronucleus (MN) assay. All subjects were also classified for antinuclear antibodies, anticentromere antibodies (ACA), or Scl70. To identify the mechanism of MN formation, we also performed MN fluorescence *in situ* hybridization (FISH) analysis using a pancentromeric DNA probe.

Results. Patients with SSc and subjects with RP showed significantly higher MN frequencies than controls (25.9 ± 1.7 and 19.1 ± 2.15, respectively, vs 9.4 ± 2.2; $p < 0.001$). Subjects with suspected secondary RP displayed MN frequency (23.5 ± 2.7) comparable to that of SSc patients, while spontaneous MN level in idiopathic RP subjects (13.6 ± 3.0) did not differ significantly from controls (9.4 ± 2.2). ACA positive subjects showed the highest MN frequencies (32.8 ± 1.7) compared to subjects with a different antibody pattern (18.3 ± 1.6).

Conclusion. Our results show the presence of higher levels of micronuclei in circulating lymphocytes of patients with SSc and subjects with suspected secondary RP. They also suggest a possible role of ACA in determining cytogenetic anomalies. FISH analysis indicated that both aneuploidogenic and clastogenic events contributed to the formation of MN observed in SSc patients and subjects with suspected secondary RP. (J Rheumatol 2003;30:1244–7)

Key Indexing Terms:
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Scleroderma or systemic sclerosis (SSc) is a systemic connective tissue disease characterized by both progressive fibrosis of the skin and of internal organs and the presence of antinuclear antibodies (ANA) in the serum[1]. Based on the extension of cutaneous sclerosis, SSc can be distinguished as a limited or a diffused form, both presenting different serological patterns and clinical evolution[2]. In most cases Raynaud's phenomenon (RP) is an early symptom of SSc that can antedate the appearance of SSc by several years. In this case it is often possible to show the presence of specific ANA and/or of specific nailfold capillaroscopic alterations (presclerodermic RP)[3,4]. Several studies deal with the presence of elevated (spontaneous or induced) levels of cytogenetic abnormalities in peripheral lymphocytes of patients with SSc[5-9]. However, regarding RP, the evidence of an increased rate of alteration at the chromosomal level is still limited[9,10]. Beyond the discovery of a clastogenic factor in the plasma of these patients[11-16], the presence as well of a different antibody profile[17] could account, at least in part, for chromosome anomalies observed in lymphocytes of subjects with SSc. Our aim was to verify the presence of spontaneous chromosomal damage using the micronucleus (MN) test in cultures of circulating lymphocytes from patients with SSc (diffused and limited) and by RP, both idiopathic and suspected secondary. This method allows a simultaneous evaluation of chromosome breakage and chromosome loss[18]. Indeed, either chromosomal fragments without centromere (clastogenic event) or whole chromosomes in late migration during anaphase (aneuploidogenic event) that are unable to be included in the 2 nuclei of new formation can form micronuclei. Application of the fluorescence *in situ* hybridization (FISH) technique using a pancentromeric DNA probe allowed us to study the mechanism of MN formation[19]. Finally, we also evaluated the hypothesis that the extent of the cytogenetic damage could

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be correlated with specific SSc clinical subsets or associated to the presence of a specific class of autoantibodies.

MATERIALS AND METHODS

Patients. We studied chromosomal alterations in 43 patients with SSc (38 women, 5 men, mean age 52 ± 8.5 yrs, duration of illness 14 ± 7.6 yrs); 16 with suspected secondary RP (all women, mean age 47 ± 7.8 yrs, duration of RP 2 ± 0.4 yrs); and 13 with idiopathic RP (12 women, 1 man, mean age 44 ± 10.7 yrs, duration of RP 18 ± 6.8 yrs), comparing them with 25 healthy controls matched for sex and age (21 women, 4 men, median age 48 ± 6.7 yrs). RP was considered "suspected secondary" in the presence of specific nailfold capillaroscopic alterations and/or ANA[4].

Patients with SSc were further divided into those with limited sclerosis (lSSc) (33 patients, disease duration 10 ± 5.3 SD yrs) and those with diffuse cutaneous involvement (dSSc) (10 patients, disease duration 4 ± 2.3 SD yrs) according to the criteria of Le Roy, *et al*[2].

In all subjects the presence of ANA was evaluated by indirect immunofluorescence (IIF) on HEp-2 cells (Immuno-Concepts, Sacramento, CA, USA), while anti-ENA were tested by double immunodiffusion with the Ouchterlony technique and also by INNO-LIA ANA Update (Innogenetics NV, Gand, Belgium) and HEp-2 Western blot (MarDx, Carlsbad, CA, USA). According to the antibody profile, each subject was assigned to one of the following subgroups: (1) anticentromeric (ACA) positive group (36 subjects; 28 with lSSc, 8 with suspected secondary RP); (2) anti-Scl70 antibody positive group (10 subjects; 2 lSSc, 7 dSSc, one suspected secondary RP); (3) ANA positive group with nucleolar fluoroscopic pattern (4 subjects; 3 dSSc, one suspected secondary RP); (4) ANA positive group with homogeneous fluoroscopic pattern (9 subjects; 3 lSSc, 6 suspected secondary RP).

Clinical assessment was also carried out in patients with SSc and in those with suspected secondary RP. Pulmonary involvement was evaluated by chest radiography, high resolution computed tomography (HRCT), and respiratory tests, including alveolar-capillary diffusion of carbon monoxide (DLCO). Esophageal involvement was documented by barium radiography and manometry; cardiac abnormalities and pulmonary hypertension were evaluated by electrocardiograms and echo color Doppler cardiography.

The only patients receiving potentially clastogenic drugs belonged to the group with dSSc — 6 with severe pulmonary involvement were being treated with oral or intravenous cyclophosphamide (a well known anticancer drug; administered, however, at a lower dosage than usually used in cancer therapy). All patients with lSSC used vasodilators for at least 8 years and the subjects with suspected secondary RP were all being treated with vasodilators. Only 8 subjects with idiopathic RP were previously treated with vasodilators for at least 5 years. No controls were using drugs.

Identification of micronuclei. From each subject, 2 cultures of whole blood were set up in medium containing phytohemagglutinin and incubated at 37°C for 72 h. At 44 h, cytochalasin B was added. Recovery of the cells was carried out according to the classic method[18]. For each subject, the frequency of MN was expressed as the number of micronucleated cells (containing one or more MN) per 1000 binucleate lymphocytes on a total of 2000 cells scored. MN appear as roundish masses of chromatin visible in the cytoplasm of interphase cells that have completed at least one cell cycle. Treatment of cultures with cytochalasin B blocks the cytodieresis of proliferating lymphocytes, ensuring easy scoring of first-division cells for the classic binucleated appearance that they assume. In general, MN frequency in peripheral lymphocytes in healthy subjects ranges from 3 to 13 per 1000, depending on the age and sex of the subjects.

FISH analysis. Slide hybridization with a pancentromeric digoxigenin-labeled DNA probe (Appligene-Oncor, Gaithersburg, MD, USA) was performed according to the standard procedure[19]. The centromere of all human chromosomes is easily observed as a red spot in the unlabeled DNA of interphase nuclei and micronuclei counterstained in blue by the DAPI fluorochrome. MN analysis was carried out on a fluorescence microscope equipped with dual-band pass filters allowing the simultaneous visualization of red and DAPI fluorescence. MN with (C+MN) or without (C–MN) red signal are thought to contain whole chromosome(s) (aneuploidogenic event) or acentric fragment (clastogenic event), respectively.

Statistical analysis. Differences in the spontaneous levels of MN or in the frequency of C+MN/C–MN between patients and controls, and between subjects with different clinical forms and antibody profiles, were evaluated by analysis of the variance assuming statistical significance for p values < 0.05.

RESULTS

Clinical and serological assessment. Patients with diffuse cutaneous SSc (dSSc) were more severely affected due to major involvement of internal organs. Six patients had diffuse interstitial lung disease (mean DLCO = 50% of predicted); 3 of these patients also had secondary pulmonary hypertension (mean 35 mm Hg), while 3 patients had ventricular arrhythmias, and one developed chronic renal insufficiency. In the lSSc group, organ involvement was characterized by esophagopathy in 23 patients, supraventricular arrhythmias in 7, and bibasilar pulmonary fibrosis in 7 patients (mean DLCO 65% of predicted). No patient with suspected secondary RP presented visceral involvement or other signs and symptoms referable to SSc or to other connective tissue diseases.

Nailfold capillaroscopy performed in 16 subjects with suspected secondary RP showed dilatations, diffuse microhemorrhages, subpapillary edema in 12 subjects; and tortuosity, reduction of the capillary density, and megacapillaries in 4 subjects. In this group we also found ACA positivity in 8 cases, ANA positivity with nucleolar fluoroscopic pattern in one case, anti-Scl70 antibodies in one subject, and ANA positivity with homogeneous fluoroscopic pattern in the remaining 6 patients.

No control, nor any subject with idiopathic RP, showed capillaroscopic alterations and/or ANA positivity.

Cytogenetic analysis. Table 1 reports the results of the cyto-

Table 1. Results of analyses of chromosomal damage (MN test) in peripheral lymphocytes of subjects with ISSc, dSSc, and RP.

	No. of Subjects	MN Frequency, per 1000, Average ± SD
SSc	43	25.9 ± 1.7[a]
Total RP	29	19.1 ± 2.15[b]
Suspected secondary RP	16	23.5 ± 2.7[c]
Idiopathic RP	13	13.6 ± 3[d]
Controls	25	9.4 ± 2.2
ISSc	33	33.1 ± 17.0[e]
dSSc	10	19.8 ± 2.7[f]
ACA+	36	32.8 ± 1.7[g]
ACA– (Sc170, ANA+)	23	18.3 ± 1.6

[a,c,e] Significantly different from controls and from idiopathic RP ($p < 0.001$, ANOVA). [b,f] Significantly different from controls ($p < 0.001$, ANOVA). [d] Not significantly different from controls (p = NS). [g] Significantly different from ACA– ($p < 0.005$, ANOVA). ACA: anticentromeric antibody; Scl70: antitopoisomerase antibody; ANA: antinuclear antibody

genetic analysis carried out on peripheral lymphocytes of patients with SSc and RP and controls. The mean frequency of micronucleated cells observed in patients with SSc and RP was statistically higher compared to controls (25.9 ± 1.7 and 19.1 ± 2.15, respectively, compared to 9.4 ± 2.2; $p < 0.001$). Subjects with suspected secondary RP showed levels of spontaneous micronuclei (23.5 ± 2.7) statistically comparable to patients with SSc, while in subjects with idiopathic RP the frequency of micronuclei was not significantly different from controls (13.6 ± 3 and 9.4 ± 2.2, respectively). On average, patients with lSSc had higher frequencies of MN (33.1 ± 17.0) compared to the dSSc group (19.8 ± 2.7).

Table 1 also shows the basal levels of chromosomal damage in groups classified according to immunologic profile. ACA positive individuals (group 1) showed a frequency of MN of 32.8 ± 1.7, significantly higher ($p < 0.005$) than that in patients with the other antibody patterns (groups 2, 3, and 4: 19.7 ± 8.2). The results of FISH analysis are shown in Table 2. No differences in the percentage of C+MN were observed between ACA positive and ACA negative subjects compared to the control group.

DISCUSSION

An analysis of chromosomal damage was performed on peripheral lymphocytes of 43 patients with scleroderma, 16 subjects with suspected secondary RP, and 13 subjects with idiopathic RP. In the group with SSc and in the suspected secondary RP group, the level of chromosomal alterations, expressed as the frequency of micronucleated cells, was significantly higher than in healthy controls and in subjects with idiopathic RP. However, in scleroderma patients, we did not observe a correlation between the severity of disease and the presence of spontaneous cytogenetic damage. Indeed, subjects with lSSc, who presented a less severe visceral involvement, showed a higher frequency of MN compared to dSSc patients characterized by more severe organ involvement. Our results also indicate that subjects with ACA display the highest levels of spontaneous MN.

Our work confirms the results of previous studies in which high frequencies of chromosomal breakage and rearrangement, aneuploidy, or sister-chromatid exchanges were observed in patients with SSc[5-9]. The chromosome damage was associated with the presence of clastogenic factors in patient plasma. Some classes of substances were identified, among them tumor necrosis factor-α, unusual nucleosides of inosine (ITP), different cytokines, and other oxidant molecules, that are involved in lipid peroxidation, all with proven clastogenic activity[11-16]. It is likely that induction of chromosomal damage occurs through the production of highly reactive radicals of oxygen, especially the superoxide anion. Indeed, ITP-induced chromosome damage is prevented by superoxide dismutase[20].

Other studies have tried to establish the role of specific antibodies in determining chromosomal anomalies in patients with autoimmune or other connective tissue pathologies. Jabs, *et al* observed significant increases in the frequencies of aneuploidy in lymphocyte cultures of patients with scleroderma that presented antibodies against centromere[17], while patients with anti-topoisomerase I showed a prevalence of chromosomal anomalies due to clastogenic events[7]. We have described an association between early centromeric dissociation and ACA positive RP[10]. By contrast, other investigators found no significant difference in spontaneous levels of structural and numerical chromosome anomalies between ACA positive and negative patients[21]. On this issue, both ACA and anti-Scl70 antibodies could determine, respectively, the inactivation of the centromere or alterations of the activity of topoisomerase I, the enzyme involved in the initial phase of replication and repair of DNA. By these mechanisms, ACA could cause an incorrect migration of chromosomes and anti-Scl70 might determine breakage of the DNA helix. In this context, it has been suggested that IgG antinuclear antibodies can enter viable cells using mechanisms similar to those of hormones and growth factors[22,23]. On the other hand, the antibodies could also be the consequence of unrepaired lesions in DNA considered as non-self. It is also difficult to determine whether a functional relationship exists between autoantibodies and clastogenic factors whose presence in the plasma of SSc patients has been suspected to be the main cause of chromosomal alterations.

MN fluorescence analysis using a specific probe for the centromere of all chromosomes indicates that the proportion of MN containing whole chromosome (C+MN) is higher than the complementary percentage of C–MN in both the SSc and RP groups, according to the values in the healthy controls. This finding means that MN observed in lymphocytes of our patients do not follow a preferential mechanism of formation, but they are derived by both chromosome breakage and chromosome malsegregation in the same proportion as expected for subjects from a general population[19].

Our findings confirm the presence of elevated levels of chromosomal anomalies in peripheral cells of subjects with systemic sclerosis. We were able to demonstrate for the first time to our knowledge the presence as well of high frequen-

Table 2. Results of FISH analysis in peripheral lymphocytes of ACA+ or ACA– subjects.

	No. of Subjects	C+MN Frequencies, %, Average ± SD
Controls	20	66.9 ± 7.2
ACA+	18	70.7 ± 11.4
ACA– (Sc170+ ANA+)	16	71.8 ± 10.6

ACA: anticentromeric antibody; Sc170: antitopoisomerase antibody; C: centromere; MN: micronuclei; ANA: antinuclear antibody.

cies of micronuclei in patients with suspected secondary Raynaud's phenomenon. These data confirm the results of our previous studies performed by classic cytogenetic techniques. Such alterations do not seem to correlate to clinical severity, but rather, to the seroimmunological profile. In particular, anticentrome antibodies would seem to be related, in some way, to the extent of cytogenetic damage. Finally, results from FISH analysis suggest that both clastogenic and aneuploidogenic events contribute to the formation of micronuclei in subjects with SSc and suspected secondary RP.

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