Quantification of fetal microchimeric cells in clinically affected and unaffected skin of patients with systemic sclerosis

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Objective. Fetal microchimerism has been hypothesized as a potential pathogenic mechanism for systemic sclerosis (SSc). This hypothesis was based on the clinical similarities between SSc and graft-vs-host disease and the identification of microchimeric cells in affected SSc tissues. The aim of this study was to compare the quantity of microchimeric cells in clinically affected and non-affected skin of female patients with SSc.

Methods. Fluorescence in situ hybridization (FISH) and real-time PCR were employed in paired skin biopsies obtained from clinically affected and unaffected areas from five female SSc patients with diffuse cutaneous SSc (dcSSc) and 10 healthy women. All women in the study had delivered a male fetus.

Results. FISH analysis revealed the presence of male fetal cells in 1/5 SSc patients (20.0%) compared with 0/10 healthy women \((P = 0.0037)\), whereas quantification by real-time PCR revealed that all SSc samples were positive for male DNA compared with none of the controls. In the five patients with dcSSc, there were similar numbers of microchimeric cells in both affected and unaffected skin \((P = 0.4)\).

Conclusion. The presence of higher numbers of microchimeric cells in clinically unaffected SSc skin, before any clinically detectable evidence of sclerotic changes, suggests that an influx of microchimeric cells may precede the development of tissue fibrosis. This provides additional support to the hypothesis that fetal microchimerism may play a role in the pathogenesis of SSc.

Key words: Microchimerism, Systemic sclerosis, Affected skin, Unaffected skin, Diffuse.
Fluorescence in situ hybridization

Slides were washed in phosphate-buffered saline and denatured at 73°C for 5–9 min in 70% formamide/2× standard saline citrate (SSC), then dehydrated in 70, 80 and 100% ethanol for 1 min. DNA probes specific for the alpha satellite region of the X and Y chromosomes were purchased from Vysis. The X-chromosome probe was labelled with SpectrumRed™ and the Y-chromosome probe with SpectrumGreen™. The sections were incubated at 42°C overnight with 5 μl of probe. After hybridization, the sections were washed in 0.4× SSC at 73°C for 2 min to remove unbound probe, rinsed in 0.1% NP-40/2× SSC, and allowed to air-dry. The slides were counterstained with DAPI, viewed with an epifluorescence microscope, and photographed. The entire surface of tissue present on the slides from each of the five patients corresponding to skin sections from clinically affected and unaffected areas was analysed and the number of male cells was determined by counting the nuclei which had one green signal and one red signal, corresponding to the X (red) and Y (green) chromosomes. The total number of cells in each slide was corrected for the area (mm²) of the tissue evaluated. The area of tissue analysed was calculated with the Image J 1.26t program (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov).}

**Quantification of Y chromosome by real-time PCR**

DNA was extracted from 4 mm pieces of skin using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). The DNA bound to the column was eluted in 100 μl of buffer. The recovered DNA was quantified at 260 nM with a RNA-DNA spectrophotometer (Pharmacia, Piscataway, NJ, USA). Quantification of the Y chromosome in skin samples was performed with real-time PCR according to the previously published methods of Zhong et al. and Hahn et al. 2000 [7, 8]. The number of copies of the Y chromosome obtained was normalized to 100 μg of DNA in each sample. The number of male cells/100 μg DNA was determined by assuming that 6.6 pg of DNA corresponded to one cell.

**Statistical analysis**

A contingency table was used for the evaluation of the PCR and FISH results. A paired t-test or the Mann–Whitney rank sum test was used to compare the numbers of microchimeric cells, calculated from the amount of DNA extracted from biopsies using the GraphPad InStat statistical program (GraphPad Software, San Diego, CA, USA). The results are expressed as median with interquartile range (IQR).

**Results**

**Quantification of Y chromosome in skin samples**

Biopsy samples were analysed by real-time PCR to quantify the number of male microchimeric cells/100 μg DNA extracted from the lesions. Y-chromosome sequences were amplified in all samples. In patients with dcSSc, similar numbers of microchimeric cells were found in the affected and unaffected biopsies 4.73 cells/100 μg DNA; (IQR 3.76–5.63) vs 12.49 cells/100 μg DNA (IQR 3.47–13.55). These differences were not found to be statistically significant (P = 0.4).

**Fluorescence in situ hybridization (FISH) of skin samples**

The presence of male cells was confirmed by FISH in the affected and unaffected tissues. Male cells were identified by the presence of one Y chromosome (green signal) and one X chromosome (red signal) in the nuclei (Figs 1 and 2; these figures may be viewed in colour at Rheumatology Online). The results are shown in Table 2. Male cells were detected in biopsies from 4/5 patients with SSC (80.0%) compared with 0/10 in samples from healthy women (P = 0.0037, odds ratio = 63.0, IQR 2.1–1862.6).

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**Table 1. Pregnancy history and of patients with SSc included in the study**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at biopsy (yr)</th>
<th>Duration of SSc (yr)</th>
<th>Pregnancies</th>
<th>Offspring sex, miscarriage, abortion (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>0.83</td>
<td>G2, P2</td>
<td>F (1955), M (1957), M (1969), M (1972), F (1979)</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>6</td>
<td>G3, P3</td>
<td>M (1952), F (1962), M (1963)</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>2</td>
<td>G10, P6</td>
<td>F, miscarriage (year unknown), F, miscarriage (year unknown), M, abortion (year unknown)</td>
</tr>
</tbody>
</table>

G, number of gestations; P, number of live deliveries; F, female; M, male.
In affected skin, 1/5 samples were found to be positive by FISH, whereas in the unaffected skin 4/5 were positive (Table 2). Although there was no statistically significant difference, there was a tendency for a greater number of microchimeric cells/mm² in unaffected skin (median 1.5 cells/mm², IQR 0.3–1.6) when compared with affected skin (median 0 cells/mm², IQR 0–0).

**Discussion**

The presence of male microchimeric cells was analysed by FISH analysis and quantified by real-time PCR of DNA extracted from involved and uninvolved skin biopsies from five women with SSc. In affected skin, male microchimeric cells were identified by FISH in one of five patients with SSc. However, FISH is a relatively insensitive technique, and these results represent only a very small portion of the whole skin sample. In contrast, all of the samples were positive by real-time PCR. None of the patients had received a transfusion or organ transplant, but all had a male child. Therefore, these microchimeric cells were most likely derived from a previous male pregnancy. In one patient, male cells were not documented by FISH; however, the samples were positive for male DNA when examined by PCR. In agreement with results from others, microchimeric cells were not identified in skin samples from the normal women [1, 3]. The present study, however, shows for the first time that microchimeric cells were also present in uninvolved skin biopsies of women with SSc. Recently, some of the pathogenic mechanisms responsible for the alterations observed in patients with SSc have been elucidated. An increased number of CD4⁺ T cells has been found in SSc skin lesions and affected organs in the early phase of the disease. This observation suggests that cytokines secreted by T cells and/or other cells from the immune system play an important role in the pathogenesis of SSc [9]. In a recent study, Scaletti et al. [4] generated T-cell clones from the peripheral blood and skin biopsies from women with SSc of recent onset and from peripheral blood from three healthy control women. All subjects had at least one male child. They observed an increased number of T-cell clones obtained from SSc

<table>
<thead>
<tr>
<th>Patient</th>
<th>Skin</th>
<th>Tissue size analysed (mm²)</th>
<th>Number of microchimeric cells detected by FISH</th>
<th>Microchimeric cells detected by FISH/mm² tissue analysed</th>
<th>Microchimeric cells/100 μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>3.6</td>
<td>0</td>
<td>0</td>
<td>27.18</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>3.3</td>
<td>1</td>
<td>0.3</td>
<td>3.47</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>6.9</td>
<td>0</td>
<td>0</td>
<td>3.76</td>
</tr>
<tr>
<td></td>
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<td>0</td>
<td>26.03</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>2.98</td>
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<tr>
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<td>3</td>
<td>2.3</td>
<td>13.55</td>
</tr>
<tr>
<td>4</td>
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<tr>
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<td>4</td>
<td>1.5</td>
<td>12.49</td>
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<tr>
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<td>A</td>
<td>3.2</td>
<td>3</td>
<td>0.94</td>
<td>4.73</td>
</tr>
<tr>
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<td>U</td>
<td>2.5</td>
<td>4</td>
<td>1.60</td>
<td>1.21</td>
</tr>
</tbody>
</table>

A, clinically affected skin; U, clinically unaffected skin.

**FIG. 1.** Fluorescence in situ hybridization (FISH) specific for the centromeric region of X chromosome (red signal) (this figure may be viewed in colour at *Rheumatology Online*) in a biopsy of affected skin from an SSc patient. Nuclei were counterstained with DAPI and viewed with a Nikon epi-fluorescent microscope at 1000X magnification as described in Materials and methods.

**FIG. 2.** Fluorescence in situ hybridization (FISH) specific for the centromeric region of X chromosome (red signal) and Y chromosome (green signal) (this figure may be viewed in colour at *Rheumatology Online*) in a biopsy of affected skin from an SSc patient. Nuclei were counterstained with DAPI and viewed with a Nikon epi-fluorescent microscope at 1000X magnification as described in Materials and methods.
patients, which proliferated in response to autologous T cells when compared with clones obtained from the healthy women. They also demonstrated that 18% of the reactive clones from the SSC samples and 9% of the clones from healthy women were positive for the Y chromosome. Furthermore, they demonstrated that the clones from the SSC patients produced higher levels of the profibrotic cytokine IL-4, whereas the normal clones did not. This observation suggests that male fetal cells are present in the circulation and/or skin of women with SSC, are reactive against maternal major histocompatibility complex antigens, and display a TH2-oriented profile [4].

The presence of microchimeric cells in the tissues demonstrated in this study confirms previous findings by us and others [1, 3, 4]. Microchimeric cells were detected by FISH in one of five individuals analysed; however, by PCR quantification, male cells were detected in all samples. In the present study, it was also observed that clinically uninvolved SSC skin contained microchimeric cells. This finding reinforces the potential role of microchimeric cells in SSC lesions. We identified microchimeric cells in a woman approximately 50 yr after the birth of her son. It is conceivable that microchimeric cells, once established in a person, remain for the life of that individual. We believe that it is not sufficient to have microchimeric cells present, as it appears to be a frequent phenomenon in women who have carried fetuses. However, we believe that a second event (bacterial, viral or chemical) is necessary to activate the microchimeric cells, which subsequently move to the lesions. Similarly, in men or nulliparous women with SSC, it is possible that microchimeric cells are maternal in origin.

In the present study, the selection of anatomical areas for the skin biopsies was based on clinical assessment of the skin, but the unaffected skin may not necessarily become indurated over time. However, the haematoxylin/eosin and trichrome staining confirmed the presence of an increased thickness of the dermis and of the number of thicker collagen fibres in the affected tissue sections from patients with SSC when compared with unaffected tissue sections. Nevertheless, it is recognized that clinical assessment is not conclusive, as demonstrated by the presence of inflammatory and fibrotic alterations in affected and non-affected skin samples of patients with SSC [10]. These observations suggest that vascular and fibrotic abnormalities at the tissue level precede the clinical evidence of the disease. In agreement with these observations, we found in the present study that the clinically unaffected skin contained inflammatory cells, although the number of cells was lower than that observed in the affected skin. The lower number of microchimeric cells in affected skin compared with unaffected skin may be accounted for by the increased number of inflammatory cells in affected skin, as the cells were normalized to 100 μg of DNA. It is not possible to conclude whether microchimeric cells are involved in the early events that cause disease merely by their presence in the unaffected skin; however, the absence of microchimeric cells in tissues from healthy individuals gives support to the possible role that they have in the development of disease.

The present study adds substantial support to the hypothesis that microchimeric cells may be involved in the pathogenesis of SSC, as microchimeric cells were identified in SSC skin even before the tissue displayed clinically or histopathologically detectable evidence of sclerotic changes.

### Key messages

- Microchimeric cells are found in very early skin lesions.

### Acknowledgements

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The authors have declared no conflicts of interest.

### References