Concise Report

Male microchimerism and HLA compatibility in French women with scleroderma: a different profile in limited and diffuse subset

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Objectives. Male microchimerism (Mc) persisting from pregnancy has been found at greater frequencies and/or higher quantities in women with scleroderma (SSc) compared with controls, suggesting a possible role in disease development. Moreover, women with an HLA-compatible child have a higher risk to develop SSc. We tested the hypothesis, on our French SSc cohort, that women with lcSSc and dcSSc, two distinct clinical subsets, have a different profile in terms of Mc and HLA compatibility in families.

Methods. We studied 98 women (52 lcSSc and 46 dcSSc) for male Mc, by real-time PCR, in their whole blood and/or peripheral blood mononuclear cells (PBMC). Similarly, 91 matched healthy women were analysed. Complete HLA-DRB1 typing was obtained for 58 SSc and 68 control families (proband/children).

Results. Women with lcSSc (N=50) had male Mc more often in their whole blood than women with dcSSc (N=40, 20 vs 5%, P=0.038), but not significantly more than controls. By contrast, women with dcSSc (N=36) hold Mc more often in PBMC (25 vs 9%, but not significantly and have greater quantities than controls (N=82, P=0.048). This contrast is also visible in fetomaternal HLA-DRB1 compatibility, which was increased only among women with lcSSc (N=33) compared with controls (N=68, P=0.003).

Conclusion. For the first time, we showed that women with lcSSc and dcSSc hold male Mc in different blood compartments. Furthermore, a distinct pattern between the two SSc subtypes is observed for fetomaternal HLA-DRB1 compatibility. These results suggest a different mechanism behind each type of disease.

Key words: Microchimerism, Limited cutaneous scleroderma, Diffuse cutaneous scleroderma, Blood, Peripheral blood mononuclear cells, Y-chromosome.

Introduction

SSc is a complex autoimmune disease manifested by extensive collagen deposition in the skin, fibrotic changes in internal organs and abnormalities in the vascular system [1]. In clinical practice, two different forms of disease are defined by the degree of skin and internal organ involvement: lcSSc and dcSSc. ACA or anti-topoisomerase I (ATA) antibodies are generally found in patients who have, respectively, lcSSc or dcSSc disease, and are almost always mutually exclusive [2].

The clinical similarity between SSc and chronic graft-vs-host disease, a known state of chimerism, led to the hypothesis that chimerism may be involved in the development of SSc [3]. The recent knowledge that fetal cells and/or DNA could persist for decades in the maternal circulation in small quantities after delivery [4] initiated studies on detection and quantification of fetal microchimerism (Mc) in women with SSc. Pioneer North American studies reported increased frequency and/or levels of chimeric cells or DNA in peripheral blood and/or peripheral blood mononuclear cells (PBMC) in comparison with healthy women (for review see [5]). Moreover, HLA-DRB1 compatibility of a child is associated with increased risk of subsequent SSc in the mother [6].

By contrast, some other studies showed either a marginal difference or no difference for Mc between patients and controls [7–9]. Discrepancy in results may come from technical differences in Mc detection assays, pregnancy history, ethnics or clinical distinction between patients.

In the current study based on a French cohort of patients and controls, we propose testing the hypothesis that presence of male Mc is primarily dependent on clinical subsets and blood compartments tested. In parallel, we analyse fetomaternal HLA-DRB1 compatibility according to clinical status.

Patients and methods

Subjects

Patients were enrolled in collaboration with five French hospitals. All patients met the requirements of LeRoy for SSc [10]. In parallel, healthy women with no family history of autoimmune disease were locally recruited in the Centre d’Examen de Santé de l’Assurance Maladie (CESAM), Marseille, France. This study received the approval from the French Ethical Committee Marseille 2 and is registered at the INSERM under the Biomedical Research Protocol number RBM-04-10. Written consent forms obtained according to the Declaration of Helsinki [11] were signed.

Questionnaires with detailed information about history of transfusion, history of pregnancy, older brother (as a possible
source of male Mc) were filled in for each participant of the study. For one patient, we could not obtain all information.

**Subjects for male Mc analysis**

Altogether, 52 women with lcSSc and 46 with dcSSc were tested for male Mc in their whole peripheral blood ([WPB] respectively \(n = 50\) and \(n = 40\)) and/or in their PBMCs (\(n = 33\) and \(n = 36\)). Thirty-one women with lcSSc and 30 with dcSSc were analysed in both compartments.

In parallel, male Mc was quantified for 91 control healthy women, in their WPB (\(n = 49\)) and their PBMCs (\(n = 82\)). Twenty-six healthy women were analysed in both compartments. Patients and controls were matched for their mean age, respectively 55 [31–78] and 52 yrs old [37–69], mean number of children (\(n = 2\)) and sons (\(n = 1\)). The percentage of women with at least one son and no blood transfusion (45 and 52%, respectively), of nulligravid women (10 and 7%) and of women transfused (18 and 12%) was not statistically different between patients and controls. The majority of women were Caucasians with 81% of the patients and 93% of controls. Patients were diagnosed at a mean age of 49 yrs old (\(n = 97\), range: 12–74 yrs) and had a mean duration of disease of 8 yrs (\(n = 97\), range: 0–41 yrs). Among the 52 women with lcSSc, 39 had ACA, 1 ATA, 11 negative for both ACA and ATA, 1 unknown. Among the 46 patients with dcSSc, 30 had ATA and 16 had neither ACA nor ATA.

**Subjects for HLA compatibility analysis**

From our cohort of SSc patients, we obtained complete information on HLA typing from 58 women with SSC (25 with dcSSc and 33 with lcSSc) and their children. Since the hypothesis is that having at least one HLA-compatible child could be a risk factor for subsequent SSC in the mother, only women for whom children were born before the onset of the disease and all children typed were included. From our cohort of healthy controls we obtained complete HLA-DRB1 typing from 68 women and their children.

**DNA extraction from whole blood and PBMCs**

Whole blood was taken in EDTA vacutainer tubes. An aliquot of 350 μl of WPB was taken and DNA was extracted with EZ1 DNA Blood Kit (Qiagen, Hilden, Germany) on a BIOROBOT® EZ1 according to the manufacturer’s instructions. The remaining WPB was processed by Ficoll Histopaque 1077 gradient centrifugation (Sigma-Aldrich, St Louis, MO, USA) to isolate PBMCs. DNA from PBMCs was extracted with EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) on a BIOROBOT® EZ1.

**Quantification of male Mc**

Male Mc was quantified by real-time PCR for a Y-chromosome-specific sequence DYS14 as previously described [12]. The only change was the use of a Light Cycler® and Light Cycler® Fast Start DNA MasterPLUS Reaction kits (Roche, Indianapolis, IN, USA) instead of an ABI 7700 (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Data were analysed on LightCycler® Software 3.5.3. Sensitivity of the DYS14 assay was of 1 genome equivalent (gEq) male cell in a background of 20 000 gEq female cells. Inter- and intra-assay consistency was maintained in each assay by running a point from the DYS14 standard curve (i.e. standard point 10 gEq) and reporting its value to the previously registered standard curve. Each DNA sample from PBMCs or from WPB was tested for DYS14 in 10 aliquots of DNA equivalent of 20 000 cells. As a conservative estimate of the quantity of male DNA, we required that a sample exceeds the threshold in at least two wells out of 10. The amount of male DNA was expressed as the number of genome equivalent male cells per million of female cells (gEq/M).

**HLA-DRB1 typing**

All subjects were HLA-DRB1 typed in our laboratory by SSO HLA-DRB1 typing kits (Dynal, Invitrogen, Bromborough, UK) according to the manufacturer’s protocol. All family members were contacted to send us mouthwash samples (Eludril®, gift from Fabre Laboratory, Castres, France) from which DNA was extracted with High Pure PCR Template Preparation Kit (Roche) and used for SSO HLA-DRB1 typing. HLA-DRB1 allelic subtyping was done by Etablissement Français du Sang (EFS), Marseille, France.

**Statistical analysis**

Comparisons in frequency (male Mc or HLA compatibility) were performed using either the chi-square with Yates or Fisher’s exact test when appropriate. Quantities of male DNA between two groups were compared by \(t\)-test. Results were considered as significant when \(P\)-values were <0.05.

**Results**

**An opposite pattern of male Mc in WPB and PBMC samples from women with lcSSc and dcSSc**

Our first analysis tested whether women with lcSSc or dcSSc were more frequently positive than healthy women for any male foreign DNA, whatever the origin of male Mc (Table 1, all women). Feasibility of such overall analysis was assured by matched patient and control groups for age, number of children, sons and women who had received a blood transfusion. In whole blood, women with lcSSc were more often positive for male Mc than women with dcSSc (20 vs 5%, \(x^2 = 4.33, P = 0.038\)). However, quantities were not significantly different in the two groups (respectively, 1.4 and 0.5 gEq/M, respectively). Neither frequencies nor quantities of Mc

### Table 1. Male Mc frequencies and quantities in WPB and PBMCs in women with dcSSc and lcSSc and controls

<table>
<thead>
<tr>
<th>Analysed group</th>
<th>Sample type</th>
<th>Subjects (%)</th>
<th>Positive for Mc (%)</th>
<th>(P)-value</th>
<th>Mean quantity male Mc (gEq/Ma)</th>
<th>(P)-value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All women</td>
<td>Whole blood</td>
<td>lcSSc (50)</td>
<td>20.0</td>
<td>0.038</td>
<td>1.4 (3.29)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dcSSc (40)</td>
<td>5.0</td>
<td></td>
<td>0.5 (2.37)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls (49)</td>
<td>12.2</td>
<td>NS</td>
<td>0.7 (2.62)</td>
<td>NS</td>
</tr>
<tr>
<td>PBMC</td>
<td>lcSSc (33)</td>
<td>9.1</td>
<td>0.082</td>
<td></td>
<td>0.2 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>dcSSc (36)</td>
<td>25.0</td>
<td>1.0 (2.16)</td>
<td>0.048</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls (82)</td>
<td>15.9</td>
<td>NS</td>
<td></td>
<td>0.4 (1.05)</td>
<td></td>
</tr>
<tr>
<td>Women with at least one son, no blood transfusion</td>
<td>Whole blood</td>
<td>lcSSc (24)</td>
<td>25.0</td>
<td>0.062</td>
<td>1.7 (3.79)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>dcSSc (21)</td>
<td>4.8</td>
<td>NS</td>
<td></td>
<td>0.6 (2.77)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Controls (27)</td>
<td>14.8</td>
<td>NS</td>
<td></td>
<td>0.8 (2.86)</td>
<td>NS</td>
</tr>
<tr>
<td>PBMC</td>
<td>lcSSc (14)</td>
<td>14.3</td>
<td>NS</td>
<td></td>
<td>0.4 (0.99)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>dcSSc (16)</td>
<td>31.3</td>
<td>NS</td>
<td></td>
<td>1.0 (1.84)</td>
<td>0.099</td>
</tr>
<tr>
<td></td>
<td>Controls (44)</td>
<td>18.2</td>
<td>NS</td>
<td></td>
<td>0.4 (1.02)</td>
<td></td>
</tr>
</tbody>
</table>

Ma (gEq/M): Mc quantity expressed in genome equivalent of male cells per million of host cells. NS: non-significant. Values in bold indicate significant results.
were significantly different in WPB between lcSSc or dcSSc in comparison with controls.

In PBMCs, an inverted pattern appeared. Women with lcSSc had lower frequencies and lower quantities of male Mc in PBMCs than women with dcSSc (frequencies: 9 vs 25% and mean quantities: 0.2 vs 1.0 gEq/M). Nevertheless, no statistical significance was reached. A marginal difference was observed between quantities of male Mc in women with dcSSc and controls, with 1.0 and 0.4 gEq/M, respectively ($P = 0.048$).

Then, to exclude confounding sources of male Mc and study primarily the effect of male DNA originating from pregnancy, we restricted the analysis to women who had never been transfused and with at least one son (Table 1). The pattern of opposite Mc distribution between dcSSc and lcSSc was confirmed. However, due to small numbers, results did not reach significance in restricted analyses. It is of note that no relationship was found between blood transfusion or history of older brother with the presence of Mc in either in whole blood or PBMC.

**Dichotomy between women with lcSSc and dcSSc for HLA-DRB1 compatibility with their children**

HLA-DRB1 compatibility was first analysed between mothers (patients or controls) and their children for the basic HLA-DRB1 families from HLA-DRB1*01 to HLA-DRB1*14, without allelic discrimination (Fig. 1). Women with lcSSc had an HLA-DRB1-compatible child significantly more often than healthy women (46 vs 18%, $\chi^2 = 8.8$, $P = 0.003$). This difference was mostly due to HLA-DRB1 children identical to the mother (i.e. mother: HLA-DRB1*11, *07 and child HLA-DRB1*11, *07) with 33% of lcSSc vs 7% of healthy women ($\chi^2 = 11.3$, $P = 0.0008$). In addition this difference was still significant at the allelic level of HLA-DRB1, 36% of women with lcSSc ($n = 31$) had a HLA-compatible child vs 6% of healthy women ($n = 63$), ($\chi^2 = 13.2$, $P = 0.0003$).

On the contrary, no difference was observed for women with dcSSc compared with controls (24 vs 18%). Also, no statistical difference was observed between women with lcSSc and dcSSc.

No correlation was found between the presence of Mc and HLA-DRB1 compatibility between subjects and their sons (data not shown). Indeed, in whole blood analysis, among the 14 women who had at least one HLA-compatible son, only two were positive for Mc compared with 9 out 27 women with incompatible sons (Fisher’s exact test, two-tailed: $P = 0.28$). Therefore, having an HLA-compatible son is not associated with Mc in whole blood.

Similarly, having an HLA-compatible son is not associated with Mc in PBMC (1/10 vs 11/33, Fisher’s exact test, two-tailed: $P = 0.24$).

**Discussion**

Pioneer North American studies on Mc in SSc described higher frequencies and greater quantities of male Mc in patients compared with controls, whereas others showed marginal or no difference between patients and controls [7–9]. A French report analysing feto-maternal Mc in CTDs, similarly, did not find a difference between SSc patients and controls [9]. We tested male Mc in women with SSc according to their clinical subsets on the largest number ever reported in two blood compartments. In WPB, we found a statistically higher frequency of women with lcSSc positive for male Mc compared with women with dcSSc. On the contrary, in PBMCs, women with dcSSc were more often positive for male Mc than women with lcSSc and controls, although not significantly. Additionally, women with dcSSc had significantly higher quantities of male Mc in PBMCs than controls. Of note, none of our results would have been significant if all the patients had been counted as a whole (without lcSSc and dcSSc distinction) and compared with controls. Therefore, controversies in literature are probably due to the heterogeneity of SSc subset analysed and blood compartment tested. To illustrate this, a Spanish and a Japanese study testing mainly women with lcSSc did not find differences between patients and controls in PBMC samples [7, 8], whereas North American studies testing mostly patients with dcSSc did [12]. Interestingly, another small Japanese study showed, although on a small number of patients, higher prevalence of male Mc in PBMCs from patients positive for ATA (hallmark of diffuse SSc) [13]. A systematic review on Mc with regard to disease subsets would be necessary to confirm this trend, but this is beyond the scope of the current study.

The divergence between clinical subsets of SSc is even more striking in HLA compatibility. In our cohort, almost half of women with lcSSc have HLA-DRB1 compatibility with their child from their perspective, vs only 18% of healthy women and 24% of women with dcSSc. Thus, having an HLA-DRB1-compatible...
child confers an increased risk to develop subsequent lcSSc and not dcSSc in the mother.

Taken together, results on male Mc and HLA-DRB1 compatibility highlight distinction between lcSSc and dcSSc and suggest a different mechanism behind each clinical subset. Decreased frequency of Mc in PBMC samples from women with lcSSc, without parallel decreased frequency in whole blood, indicates that microchimeric cells could be of other phenotype than PBMCs. Interestingly, we found one patient with lcSSc, positive for Mc in whole blood and negative in PBMC, to also have Mc in CD15-positive polynuclear cells (data not shown). Possibly, HLA compatibility between children and the mother would influence women with lcSSc to generate, from microchimeric haematopoietic stem cell precursors, granulocytes instead of PBMCs. However, HLA-compatibility is not mandatory to the presence of Mc. Finally, increased male Mc in PBMCs from patients with dcSSc could reflect more tissue damage and release of Mc mononuclear cells from inflammation sites into circulation. One could expect that HLA compatibility, and thus ‘HLA-invisibility’ of fetus would favour engraftment of its cells into the maternal organism as seen for maternal Mc in fetuses [14]. However, such correlation was not found for fetal Mc [6], and our analysis does not support it.

In conclusion, lcSSc and dcSSc differ in terms of male Mc prevalence in blood compartments and feto-maternal HLA-DRB1 compatibility and should not be regarded as a single disease.

Rheumatology key messages

- The lcSSc and dcSSc patients do not hold male Mc in the same blood compartment.
- Feto-maternal HLA-DRB1 compatibility is frequently observed among women with lcSSc but not among women with dcSSc.

Acknowledgements

We are thankful to Sylvie Parlier, Nathalie Pero and Fanny Benjamin for coordination of patients in, respectively, St Louis Hospital, Paris; INSERM U639, Marseille; and St Antoine Hospital, Paris. We deeply thank Elisabeth Eynier for all the administrative management, Dr A. Dieudonné Loundou and Dr Nathalie Balandraud for statistical advice and Laboratoire Fabre for the generous gift of Eludril® samples, which facilitate DNA collection of family members. Finally, we are thankful to all participating subjects and their family members.

Funding: This study was supported by PRO-A INSERM (Grant # ASE06017ASA), Association des Scérodermiques de France (GFRS).

Disclosure statement: The authors have declared no conflicts of interest.

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