Possible contribution of microchimerism to the pathogenesis of Sjögren’s syndrome

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Abstract

Objectives. Microchimerism of foetal cells occurs during most pregnancies. Two autoimmune diseases, systemic sclerosis (SSc) and Sjögren’s syndrome (SS), have many clinical and pathological similarities to chronic graft-vs-host disease (GVHD). These findings suggest that anti-maternal graft-vs-host reaction by foetal cells may be involved in the pathogenesis of the diseases. To explore this hypothesis, we examined foetal DNA in peripheral blood of 59 women and in salivary glands from 28 women.

Methods. DNA extracted from peripheral blood and the affected minor salivary glands was analysed for the Y-chromosome-specific gene using a nested polymerase chain reaction (PCR) test. In the minor salivary gland specimens, the Y-chromosome-positive foetal cells were identified by in situ hybridization with a Y-chromosome-specific DNA probe.

Results. In peripheral blood, there was no significant difference between controls and patients with SSc or SS. In salivary glands, foetal DNA was detected in 11 of 20 women with SS but in only one of eight normal controls using PCR test. Additionally, foetal cells were clearly detected in three out of eight women with SS by the use of in situ hybridization.

Conclusions. The identification of foetal cells in salivary glands suggests that anti-maternal GVHD may be involved in the development of SS.

Key words: Systemic sclerosis, Sjögren’s syndrome, Foetal cells, Microchimerism, Y-chromosome.

It is well recognized that there is traffic of cells between maternal and foetal circulation during pregnancy, and that low levels of chimerism occur during most pregnancies [1–4]. In a study of women with sons, it was demonstrated that the amount of male DNA was significantly higher in peripheral blood in patients with systemic sclerosis (SSc) than in healthy controls [5]. The results of these studies appear to indicate the possibility that SSc may be a graft-vs-host disease (GVHD) induced by foetal cells in mothers.

Such a hypothesis is based upon the recognition of clinical similarities between SSc and chronic GVHD, a chimeric disorder that develops in the recipients of allogeneic stem-cell transplants [6, 7]. Sjögren’s syndrome (SS), an autoimmune rheumatic disease of the exocrine glands [8], also has many clinical and pathological similarities to chronic GVHD [9]. The strong female predilection and increased incidence of SS after childbirth [8, 10] suggests a relationship between SS and pregnancy. These observations have led us to the hypothesis that foetal microchimerism may be involved in the pathogenesis of SS as well as SSc.

To investigate whether microchimerism is associated with the post-partum development of SSc or SS, we examined the presence of male-specific Y-chromosome DNA as a marker of foetal cells in female patients who had delivered at least one son. Initially, we investigated DNA extracted from peripheral blood and affected minor salivary glands using a polymerase chain reaction (PCR) test. Next, we performed an in situ hybridization assay to confirm the existence of Y-chromosome-positive cells in the affected salivary glands.

Materials and methods

Subjects

All study subjects, including both the patients and the healthy control individuals, were Japanese women. The main clinical information is summarized in Table 1. In the patients with son(s), it was confirmed that the disease had been diagnosed after the birth of the son(s). Blood collection and lip biopsies were performed with informed consent. We extracted DNA from whole blood of the women with SSc (n = 23), SS (n = 6) and healthy controls (n = 20), and salivary glands of the women with SS (n = 20) and controls (n = 8). All these women had given birth to at least one son. DNA was also
extracted from whole blood of the women with SSc (n = 4) and healthy women (n = 6) who had no sons. All SSc patients satisfied the criteria of the American College of Rheumatology, and all women with SS fulfilled the criteria of the Japanese Ministry of Health and Welfare for SS [11]. Of the minor salivary glands in the control group, five were obtained from normal marginal regions excised from labial cancer specimens and three were biopsy material that had proved to be free from SS. In the control group, DNA was extracted from male peripheral blood, male minor salivary glands and fibroblasts from females. The DNA from peripheral blood was prepared using a DNA extraction kit (Stratagene, La Jolla, CA, USA). The DNA from all minor salivary glands was isolated from formalin-fixed, paraffin-embedded 50 μm thick tissue specimens. New batches of paraffin were used to embed the specimens. All procedures were performed by the female author of this study (YE).

**PCR analysis**

A specific Y-chromosome sequence [sex-determining region Y (SRY)] was detected by amplifying DNA in a nested PCR. The first amplification was performed using the primers SRY-1 (5'-CAGTGTGAAACGGAGGAACAGT-3') and SRY-2 (5'-TATAAGTATCGACCTCGTCGGAAAG-3') as described previously [12], and the second amplification was done with primers designed by our laboratory: SRY-3 (5'-AAA-GGCAAAGTCCAGGATAGAGTG-3') and SRY-4 (5'-ATTCCGGTATTTTTCTCTGTGCACT-3'). Thirty cycles of PCR (94°C for 2 min, 55°C for 2 min and 72°C for 2 min) followed by a second series of 30 cycles of PCR (94°C for 1 min, 55°C for 90 s and 72°C for 2 min). The first amplification was conducted in 2 mM MgCl₂ and 1 U of Taq polymerase (Roche, Foster City, CA, USA), and the second was done in 1 mM MgCl₂ and 1 U of Taq polymerase. All PCR analyses contained DNA extracted from both the female fibroblasts as a negative control and from the male salivary gland and peripheral blood as a positive control. Serial dilutions showed that 100 pg of male DNA could be detected in this SRY PCR assay. As an internal control, glyceraldehyde-3-phosphate dehydrogenase-specific DNA was amplified with an upper primer (5'-CCACCATGGCAATTCCATGGCA-3') and a lower primer (5'-TCTAGACGGCAGTGGTCCACC-3'). The PCR products were fractionated in 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. To confirm whether the PCR products were derived from Y-chromosome, 10 μl of each sample was digested with XbaI or PvuII (New England Biolabs, Beverly, MA, USA), and the fragments were separated in 2% agarose gel.

**In situ hybridization assay of minor salivary gland specimens**

The eight biopsy specimens of minor salivary glands in which the Y-chromosome-specific bands had been detected by PCR were analysed further using an *in situ* hybridization technique. Paraffin-embedded sections of 4 μm thickness were mounted on silane-coated slides. The sections were deparaffinized in xylene twice for 10 min and rehydrated stepwise in 99, 95, 90 and 80% ethanol for 10 s each, washed twice in distilled water and treated with 0.1% pepsin (Sigma-Aldrich-Chemie GmbH, Steinheim, Germany) at 37°C for 2 min. They were dehydrated in 95 and 100% ethanol twice for 3 min each and hybridized with 2 ng/μl of digoxigenin-labelled Y-chromosome-specific probe mixture (Boehringer Mannheim, Mannheim, Germany), of which length distribution shows a maximum of 200–500 bases, at 48°C overnight. The slides were washed successively at 48°C in 2 × saline sodium citrate twice for 10 min, in 0.1 × saline sodium citrate (1 × saline sodium citrate = 0.15 M sodium chloride and 0.015 M sodium citrate) for 10 min and at 55°C in a stringent wash solution (Dako, Glostrup, Denmark). The signal was detected immunohistochemically using an alkaline phosphatase-conjugated anti-digoxigenin (Boehringer Mannheim) according to the manufacturer’s instructions. The reaction product was developed with the substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl (NBT/BCIP) (Boehringer Mannheim) and sections were counterstained with nuclear fast red solution (Kreatech Diagnostics, Amsterdam, The Netherlands).

**Results**

**PCR analysis of peripheral blood and salivary gland specimens**

Our initial investigation involved peripheral blood of female patients with SSc (n = 23) or SS (n = 6) and 20 healthy women who had given birth to at least one boy (Table 1). Data representative of PCR analyses are shown in Fig. 1a. A stretch of 222 base pairs (b.p.) of SRY-specific sequence was amplified by PCR in blood from three of 23 SSc patients (13%) in SSc, two of six (33%) patients with SS and five of 20 (25%) normal controls (Table 2). SRY sequence was not detected in

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<th>Table 1. Clinical characteristics and pregnancy history of the patients and controls</th>
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In all *in situ* hybridization analyses, a male skin specimen was used as the positive control and labial mucosa of the patients as the negative control.
peripheral blood samples from four women with SSc (data not shown) and six healthy women (Fig. 1a) who had not given birth to a son; lanes 21–26 show DNA from healthy women who had not given birth to a son; lane + shows DNA from male peripheral blood; lane – shows DNA from a female fibroblast; lane M is a size marker. Upper lanes show SRY and lower lanes the internal control (GAPDH amplification). (b) PCR products were digested with XbaI (left) or PvuII (right). Lanes 1–4 correspond to the PCR products from lanes 5, 12, 14 and + in panel (a). UC, uncut original 222-b.p. fragments from a normal male sample; M, size marker.

**Fig. 1.** Representative results of PCR analyses of the peripheral blood. (a) Lanes 1–13 show DNA from peripheral blood of women with SSc; lanes 14–20 show DNA from healthy women who had delivered at least one son; lanes 21–26 show DNA from healthy women who had not given birth to a son; lane + shows DNA from male peripheral blood; lane – shows DNA from a female fibroblast; lane M is a size marker. Upper lanes show SRY and lower lanes the internal control (GAPDH amplification). (b) PCR products were digested with XbaI (left) or PvuII (right). Lanes 1–4 correspond to the PCR products from lanes 5, 12, 14 and + in panel (a). UC, uncut original 222-b.p. fragments from a normal male sample; M, size marker.

**Table 2.** Results of PCR analysis and *in situ* hybridization assays

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<th>Peripheral blood</th>
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<td>No.</td>
<td>Y-positive:</td>
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<tr>
<td>SSc</td>
<td>23</td>
<td>3 (13)</td>
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<tr>
<td>SS</td>
<td>6</td>
<td>2 (33)</td>
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<tr>
<td>Normal controls</td>
<td>20</td>
<td>5 (25)</td>
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<tr>
<td>Salivary glands</td>
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<tr>
<td>SSc</td>
<td>20</td>
<td>11 (55)a</td>
</tr>
<tr>
<td>Normal controls</td>
<td>8</td>
<td>1 (13)a</td>
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ND, not done.

*a P < 0.05.*

In situ hybridization assay of salivary gland specimens

We attempted to identify foetal cells *in situ* in the minor salivary glands from which the SRY sequence had been detected by PCR. Mononuclear cells bearing a Y-chromosome were observed clearly in three out of 8 SS patients (Table 2). Dot-like positive signals were detected clearly in mononuclear cells infiltrating...
around the ducts of the affected minor salivary gland (Fig. 3a). Cells positive for the Y-body were not observed in indigenous tissues, such as the epidermis of the patients’ labial mucosa (Fig. 3b). Figure 3c shows male epithelium.

**Discussion**

Various types of foetal cells can cross the placenta to the maternal circulation [1–4]. Transferred foetal haematopoietic stem cells can be detected in the circulation of women up to 27 yr after delivery [13]. In addition, persisting foetal stem cells are able to differentiate into mature immune-competent cells, such as lymphocytes, monocytes and natural killer cells [14]. These cells may recognize mother-specific antigens and be activated under certain conditions. It seems reasonable to suppose that foetal cells persisting in the maternal circulation or tissues might mediate a GVHD, leading to the development of autoimmune...

Fig. 2. Representative results of PCR analyses of affected salivary glands from patients with SS. Lanes 1–8 show DNA from salivary glands of patients with SS and lanes 9–13 show DNA from salivary glands of normal subjects. Lane + shows DNA from normal male salivary glands; lane − shows DNA from female fibroblasts; lane M is a size marker. Upper lanes show amplified SRY fragments; lower lanes show an internal control (GAPDH).

Fig. 3. *In situ* hybridization assay for Y-chromosome in the affected minor salivary glands from patients with SS. (a) Affected salivary glands of the women with SS. Y-chromosome-positive cells (arrowheads) were present in the mononuclear cells infiltrating the area surrounding the ducts of the affected minor salivary gland. (b) The labial mucosa of the patient shown in panel (a). Y-chromosome-positive signals were not observed in indigenous tissues, such as epidermis of the patients’ labial mucosa. (c) Male positive control. Dot-like positive signals were observed in the nuclei of epithelium. Original magnification, × 200.
disease. Considered together with the fact that SSc and SS have many clinical features similar to those of chronic GVHD, the above concept may explain why the diseases have a strong female predilection with a peak incidence in the years after childbearing. It is of great interest that maternal–foetal HLA compatibility was susceptible to SSc [5, 15], because blood transfusions from donors who were homozygous for HLA antigens increased the risk of GVHD in recipients with immunodeficiency [16].

In our study, the Y-chromosome-specific sequence was detected in the peripheral blood of normal females as frequently as in women with SSc or SS. The frequency of circulating chimeric cells in SSc patients was lower than has been reported previously [5]. One possible explanation is that there might be a difference between our subjects and those of earlier reports, and another is the sensitivity of the assays. The presence of foetal cells in peripheral blood does not seem to be a specific phenomenon, at least for Japanese women with SSc or SS. Murata et al. also reported that microchimerism in peripheral blood lymphocytes from Japanese women with SSc was not different that in healthy women [17]. Therefore, it is hard to conclude that circulating chimeric cells by themselves directly play a pivotal role in the pathogenesis of SSc or SS. On the other hand, Y-chromosome fragments were identified at a significantly higher rate in the affected salivary glands than in the normal controls. Furthermore, we demonstrated that the foetal mononuclear cells had actually infiltrated into the minor salivary glands. These cells are considered to participate directly or indirectly in the acinic destruction of salivary glands. Artlett et al. found specific Y-chromosome sequence in active skin lesions from 11 of 19 SSc women (58%) by amplifying DNA in a nested PCR, and fluorescence in situ hybridization confirmed the male chromosome in all seven samples examined [18].

Recently, maternal cells have been detected in 40% of umbilical cord blood samples [19], and microchimerism of maternal origin has been also found in adults [20]. Microchimerism of maternal cells may account for the cases of SS or SSc in men and children. In this study, we could not detect the Y-chromosome-specific sequence in nine of 20 affected salivary glands from SS women. There is a possibility that the GVHD might have been induced by the microchimerism established by either the maternal cells or the daughter’s cells in these cases.

Although the cause of autoimmune disease is frequently ascribed to an intrinsic breakdown of self-tolerance, such as the presence of autoantibodies and/or autoreactive lymphocytes [21], the precise mechanism(s) of the initiation of autoimmune response remains unclear despite numerous studies. Our findings may support the possibility that microchimerism is involved in the inflammation of salivary glands in SS. Further research to determine whether microchimerism is a disease-specific or a ubiquitous phenomenon in certain diseases could have great clinical importance.

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References


