Fetal microchimerism is not involved in the pathogenesis of lichen sclerosus of the vulva

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INTRODUCTION

Fetal cells pass into the maternal circulation during pregnancy and they have the potential to invade maternal tissues. Persistence of fetal cells in the maternal circulation has been found for decades postpartum (Bianchi et al., 1996; Bianchi, 2000). All pregnant women have detectable fetal cells in their blood and as pregnancy advances, the passage of fetal cells increases. However, a term pregnancy is not required for fetomaternal cell trafficking (Bianchi, 2000; Skinner et al., 2001). Recently it was suggested that, in particular, fetal loss is significantly associated with the presence and engraftment of microchimeric cells in maternal tissue (Khosrotehrani et al., 2003).

Persistent fetal microchimerism has been associated with certain autoimmune disorders. Systemic sclerosis was the first autoimmune disease with female predisposition where male DNA was identified in affected skin (Artlett et al., 1998; 1999) and in peripheral blood samples (Artlett et al., 1998; Nelson et al., 1998). The hypothesis that fetal cells might contribute to the pathogenesis of autoimmune diseases was based on the clinical and histological similarities of systemic sclerosis with chronic graft versus host disease (cGvHD), which is induced by chimeric cells.

Artlett et al. (1998) proposed that fetal chimeric CD3 cells cause an antimaternal reaction similar to that seen in cGvHD. More recently, microchimeric fetal cells were found to differentiate into host tissue in endocrine tissue (Srivatsa et al., 2001) and viral infected organs (Johnson et al., 2002) probably initiating tissue repair. It was also demonstrated that microchimeric cells exhibit tissue specific markers in maternal organs indicating multilineage potential of these cells (Khosrotehrani et al., 2004).

Lichen sclerosus is a chronic inflammatory disease of the skin, which most commonly affects the anogenital area and may progress to squamous cell carcinoma (SCC). The highest incidence occurs between the fifth to sixth decade in women, and it is six to ten times as frequent in women as in men. The pathogenesis is unknown, but there is growing evidence that an autoimmune (Oyama et al., 2003) or infectious mechanism may be involved (Powell and Wojnarowska, 1999). Lichen sclerosus of the vulva is associated with diseases of the liver and the thyroid, which are target organs where microchimeric cells migrate. Its cutaneous manifestation shares clinical features with those observed in the cutaneous manifestation of systemic sclerosis.

The aim of this study was to determine whether fetal microchimerism might be involved in the pathogenesis of lichen sclerosus of the vulva. We tested...
this hypothesis by using fluorescence in situ hybridization (FISH) of cells within chronic inflammatory cell infiltrates in paraaffin-embedded biopsy specimens with X- and Y-chromosome specific DNA probes. To confirm these findings, we used fluorescent PCR amplification of sequences located on chromosomes X- and Y (AMXY and SRY) to detect male DNA in paraaffin-embedded vulval skin biopsy samples.

MATERIAL AND METHODS

Our gynaecopathological database was reviewed and 23 paraaffin-embedded vulval skin biopsy samples diagnosed as lichen sclerosus were selected (from 1996 to 2002). The original slides were reviewed and the diagnosis of lichen sclerosus was confirmed in all cases. We could reach 18 women for an interview regarding pregnancy history, autoimmune diseases, previous blood transfusions, current medication and therapy. All of them gave informed consent and 15 women who gave birth to at least one son were selected for our study. Those samples originating from three women who had never been pregnant were included in the control group as well as six vulval specimens without pathological finding originating from autopsies and seven male gingival specimens.

Fluorescence in situ hybridization

Five 4-µm thick paraaffin-embedded tissue sections, originating from skin biopsies 5 mm in diameter, were mounted on positively charged slides. Pretreatment of the tissue sections was achieved by using an automated paraaffin pretreatment instrument as described by Jacobson et al. (2000) (VP 2000 Processor, Vysis, Inc, Downers Grove, IL, USA). After deparaffinization in xylene the samples were rehydrated with an ethanol series, followed by soaking in hydrochboroethanol. The slides were placed into the pretreatment reagent (Vysis Inc, Downers Grove, IL, USA) at 80°C for 30 min and protease digested at 37°C for 10 min. The specimens were refixed in 10% neutral buffered formalin, rinsed in distilled water and dehydrated in graded ethanol.

Pretreated specimen slides were placed on a Hybrite (Vysis Inc, Downers Grove, IL, USA), set on 37°C holding temperature and 10 µL of the hybridization mixture (CEP X Spectrum-Orange and CEP Y Spectrum-Green, Vysis, Inc.) was applied to each section, coverslips were affixed and sealed with rubber cement (Fixogum, Marabu, Germany). The tissue DNA and DNA probes were denaturated at 82°C for 5 min and hybridized at 37°C for 18 h. After hybridization, the rubber cement was removed and the slides soaked in 2 × Saline Sodium Citrate (2 × SSC)/0.1% NP 40 at room temperature until the coverslips were removed. The slides were then immersed in 2 × SSC/0.1% NP 40 at 72°C for 2 min and allowed to dry in the dark. The nuclei were counterstained with Dapi II (Vysis, Inc.) and viewed with a fluorescence microscope (Zeiss Axiosplan, Oberkochen, Germany) equipped with Dapi/green/orange/triple bandpass, Dapi/green dual pass, Dapi/orange dual pass filter sets (Vysis, Inc.).

Fluorescent PCR

Five 5-µm paraaffin-embedded tissue sections were placed in propylene tubes and the paraaffin extracted with three washes in xylene and ethanol. After removal of the ethanol, the pellet was dried at 37°C. DNA was extracted by use of a QIAamp DNA mini kit (Quiagen, Valencia, CA, USA).

PCR was performed for the first intron of the X/Y homologous gene amelogenin (AMXY) using CCC TGG GCT CTG TAA AGA ATA GTG (forward) and ATC AGA GCT TAA ACT GGG AAG CTG (reverse) as primers. Additionally, the sex-determining region SRY located on chromosome Y was amplified using TGG CGA TTA AGT CAA ATT CGC (forward) and CCC CCT AGT ACC CTG ACA ATG TATT (reverse) as primers (Oswel, Southampton, UK). PCR amplification was performed in a total volume of 25 µL, containing extracted genomic DNA (5µL), 200 µM dNTPs, 5–20 pmoles of each primer, 1X Taq polymerase buffer (3 mM MgCl₂), and 1.5 U Taq polymerase (both Promega, Madison, WI, USA). After initial denaturation at 94°C for 5 min, hot start PCR was done for 25 cycles at 94°C for 48 s, 60°C for 48 s, and 72°C for 1 min. Final extension was done at 72°C for 5 min. The PCR products (3 µL) were mixed with 2.6 µL gel loading buffer and 0.4 µL Genescan-500 Rox (Applied Biosystems Inc, Warrington, UK), containing the reference molecular-size standard. Electrophoresis was performed on a 373 DNA sequencer (Applied Biosystems Inc., Warrington, UK) using a 6% denaturing polyacrylamide gel. The amplification products were analyzed and their relative fluorescent intensities calculated using Genescan 672 software (Applied Biosystems, Inc., Warrington, UK) as described by Pertl et al. (1999, 2000).

To determine the sensitivity of the PCR assay, serial dilutions using decreasing concentrations of male DNA in female DNA were performed.

RESULTS

We studied 18 patients affected with lichen sclerosus of the vulva. 15 women gave birth to at least one son; 7 of them reported an abortion and 2 a blood transfusion. Three women had never been pregnant. Associated diseases found in our study group were diabetes in one case and thyroid disorders in four cases.

Nucleated cells containing Y chromosomes were neither detected within the inflammatory cell infiltrates nor within the epithelial layers of the vulval lichen sclerosus samples by screening five tissue sections. More than 90% of the nuclei had two detectable X chromosome signals (Figure 1). We also failed to identify male microchimeric cells in the skin sections from three women without a history of male progeny, as well as in the vulval skin samples without pathological findings.
originating from six autopsies. To confirm these findings, Y-chromosome specific sequences (AMXY, SRY) were amplified from DNA extracted from paraffin-embedded vulval lichen sclerosus and normal vulval skin samples. Y-chromosome specific alleles were not detected; only one allele specific for chromosome X at the AMXY locus was found in all samples (Figure 2).

To test the hybridization efficiency and PCR reaction, seven paraffin-embedded tissue samples originating from male gingival biopsies were used as male controls. In more than 90% of the nuclei two hybridization signals, a red signal for chromosome X and a green signal for chromosome Y, were found. In none of the samples, intact nuclei with 2X-chromosomes were detected indicating the presence of female microchimerism. Analysis of the PCR amplification products showed two alleles at the AMXY locus and one fragment at the SRY locus indicating a male control sample (Figure 3).

The sensitivity of the PCR assay was determined by serial dilutions of male DNA in female DNA. Male DNA was constantly detected using 0.1 ng of male DNA mixed with 100 ng of background female DNA as templates.

**DISCUSSION**

In our study, we were not able to demonstrate the presence of intact male cells or Y chromosome specific DNA sequences in the skin lesions of lichen sclerosus of the vulva and in normal unaffected vulval skin specimens. This result may be related to the small size of the tissue biopsies. However, many studies performed in this research area have successfully identified microchimeric cells in biopsies by using FISH (Artlett et al., 1998; Fanning et al., 2000; Schöniger-Hekele et al., 2002). In order to enlarge the area of the tissue section, we investigated five sections per patient. In contrast to many studies on microchimerism, we used fluorescent PCR as an additional molecular technique to confirm our negative result. We are aware of the low sensitivity of fluorescent PCR compared to other PCR techniques, but it was used as a complementary method with the advantage of a low false-positive rate. Like many authors, we did not investigate tissue samples known to contain microchimeric cells as positive controls; thus we have not demonstrated the detection of single microchimeric cells. However, since our FISH showed a hybridization rate of 90% in both female and male tissues we consider our result as ‘true negative’. A similar hybridization rate of >90% for FISH was recently reported by Khosrotehrani et al. (2005).

Regauer et al. (2004) performed a study investigating different types of vulval skin samples for microchimerism using PCR. Y-chromosomal DNA was found with decreasing frequency in Paget’s disease, normal vulval skin, lichen sclerosus and SCC. A heterogeneous distribution of Y-chromosome positive samples was identified in concomitant and successive biopsy samples of the same patient probably with respect to disease stage and activity. According to this study,
our negative result might be related to the activity of disease since many histological sections of our specimens showed significant lymphocytic cell infiltration. An inflammatory response might be responsible for the destruction of microchimeric cells, but it is unlikely that neither a single cell nor residual male DNA is detectable.

Fetal microchimerism was further identified in systemic sclerosis (Artlett et al., 1998; Johnson et al., 2001a), SLE (Johnson et al., 2001b), PBC (Fanning et al., 2000; Schöniger-Hekeley et al., 2002; Tanaka et al., 1999) and autoimmune thyroiditis (Klintschar et al., 2001; Srivatsa et al., 2001), but was also found in normal liver samples (Tanaka et al., 1999) and in samples obtained from women with viral hepatitis (Johnson et al., 2002).

These studies demonstrated a heterogeneous prevalence and number of male microchimeric cells. Interestingly, microchimeric cells were distributed randomly or differentiated to male functional units within the female tissue (Srivatsa et al., 2001) and were morphologically indistinguishable from the tissue they were invading (Johnson et al., 2002). However, the definite role of fetal microchimerism in the development and progression of disease or in tissue repair needs to be elucidated in further studies. Another issue to be addressed is the immunologic environment that is needed for microchimeric cell infiltration and differentiation.

Lichen sclerosus of the vulva has been associated with a number of immune mediated conditions including primary biliary cirrhosis (PBC), SLE, lichen planus and myositis. The association between lichen sclerosus and autoimmune immunity became evident with the detection of organ-specific autoantibodies to thyroid and gastric parietal cells (Goolamali et al., 1974) and to a specific extracellular skin protein (Oyama et al., 2003).

Since microchimeric cells were found in PBC, SLE and autoimmune thyroiditis, we hypothesized that microchimerism may contribute to the pathogenesis of lichen sclerosus. However, in contrast to the aforementioned studies we could not detect intact male cells or Y-chromosome specific DNA sequences in the investigated specimens. On the basis of our results, we suggest that persistent male fetal cells or allogeneic cells originating in blood transfusion or even from sexual intercourse are not involved in the pathogenesis of lichen sclerosus.

REFERENCES