

Evidence of Fetal Microchimerism in Hashimoto's Thyroiditis

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ABSTRACT

Fetal microchimerism, the engraftment of fetal progenitor cells into maternal tissues, has been implicated in the etiology of autoimmune diseases. We used PCR analysis to determine whether microchimerism occurred in the thyroid glands of female patients suffering from Hashimoto's disease and thus may be involved in its etiology. PCR amplification was performed from thyroid gland specimens using primers unique to a Y-chromosomal sequence (SRY gene) and primers for a sequence that is Y/X-chromosomal homologous except for a 6-bp deletion in the X-chromosomal sequence (amelogenin). Microchimerism was detected in 8 of 17 Hashimoto patients, but in

only 1 of 25 controls (nodular goiters). Both groups were of similar age and had comparable numbers of pregnancies and numbers of sons. All individuals with microchimerism had given birth to at least 1 son. Our results show that microchimerism is significantly more common in Hashimoto patients than in patients suffering from nodular goiter. We therefore suggest that microchimerism might play a role in the development of Hashimoto's disease, although we cannot completely eliminate the hypothesis that microchimerism is just an "innocent bystander" in a process triggered by other mechanisms. (*J Clin Endocrinol Metab* 86: 2494–2498, 2001)

THE MIGRATION of fetal cells into maternal blood during pregnancy has recently become an accepted fact. Fetal-derived DNA has been found in maternal blood as early as 4 weeks gestation (1) and up to 36 yr after pregnancy (2). The chimeric cells are CD34⁺ or CD34⁺/CD38⁺ cells and may play a role in the development of allogeneic tolerance to the fetus (2). Moreover, the presence of fetal cells appears to have implications not only for maternal-fetal immunology, but also in autoimmune diseases. Independently, Nelson *et al.* (3) and Artlett *et al.* (4) detected significantly more Y-chromosomal DNA in the peripheral blood of female patients with systemic sclerosis than in a control group using PCR analysis for a Y-chromosomal sequence. The latter group also succeeded in detecting Y-chromosomal DNA in sclerodermic skin lesions. Both researchers argued, therefore, that fetal microchimerism might play a role in the etiology of systemic sclerosis by initiating an immunological reaction similar to that observed in chronic graft *vs.* host disease. Although this assumption is challenged by other researchers (5, 6), there is strong theoretical support for the first hypothesis, *i.e.* the clinical similarities of chronic graft *vs.* host disease and systemic sclerosis, the female predilection to this autoimmune disease, and, as in other autoimmune diseases, exacerbation after delivery.

Hashimoto's disease is a chronic autoimmune thyroiditis characterized by diffuse lymphocytic infiltration, thyroid follicles of reduced size containing sparse colloid, and fibrosis

replacing the thyroid parenchyma (7). There are obvious parallels to systemic sclerosis. Both diseases are chronic autoimmune diseases predominant in middle-aged women. Moreover, it is well known that Hashimoto's disease tends to be aggravated after childbirth. It is therefore not surprising that fetal microchimerism has recently been mentioned as a potential etiological factor in Hashimoto's disease (8). However, there are no data available to support or reject the occurrence of microchimerism in Hashimoto's disease. The aim of the present study was to elucidate this question.

Materials and Methods

Forty-two thyroid gland specimens were collected from the Institute of Pathology of the University Graz (17 female patients with Hashimoto's thyroiditis and 25 women with nodular goiter).

DNA was extracted from archival paraffin-embedded thyroid tissue using a commercially available kit (Puregene Kit, Gentra Systems, Minneapolis, MN) comprising a deparaffination step with xylene, proteinase K digestion, salting out of the proteins, and isopropanol precipitation with glycogen as DNA carrier. Positive and negative controls were included throughout.

Two PCR methods were chosen to detect Y-chromosomal DNA in the histological specimens. The first method is of high sensitivity yet lacks the possibility to quantitate the amount of Y-chromosomal DNA relative to the amount of X-chromosomal DNA. This approach comprised Y-chromosome-specific PCR for the SRY locus using ATA AGT ATC GAC CTC GTC GGA AG (forward) and GCA CTT CGC TGC AGA GTA CCG AAG (reverse) as primers (9, 10). Approximately 50 ng DNA were used in a 12.5- μ L assay containing 2 μ L 10 \times PCR buffer, 75 nmol/L of each primer, 200 μ mol/L of each dNTP, and 0.5 U Dynazyme DNA polymerase (Finzymes, Espoo, Finland). Cycling conditions were 94 C for 1 min, 59 C for 1 min, and 72 C for 1 min and 30 s for 45 cycles, followed by 60 C for 45 min and a 25 C hold. The amplicates were run on horizontal native polyacrylamide gels as previously described (11), and bands were visualized by silver staining.

The second approach is of relatively low sensitivity, but enables estimating the ratio of Y-chromosomal and X-chromosomal DNA. The primers applied [5'-CCCTGGGCTCTGTAAAGAATAGTG-3' (forward,

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5'tagged with ROX) and 5'-ATCAGAGCTTAACTGGGAAGCTG-3' (reverse)] prime to the amelogenin gene, which is located at both the X- and the Y-chromosome. The length of the amplified fragment, however, varies (106 bp for the X-chromosome, 112 bp for the Y-chromosome) (12). The fluorescently labeled amplicates were run on a 310 ABI automated sequencer (PE Applied Biosystems, Foster City, CA.) using fluorescence LASER detection, an approach that enables relative quantitation of the signal by comparing the area of the detected peaks.

Extreme caution was taken to avoid false positive results. All tissue samples, *i.e.* both controls and Hashimoto samples, were handled in the same manner by female technicians. The sections used for DNA extraction were prepared with meticulous care specifically for that purpose and immediately put into Eppendorf tubes. PCR and PAGE were performed in separate rooms, and negative controls were always used in every PCR reaction. Moreover, no reamplification using nested primers was attempted to further reduce the risk of falsely positive amplification, a well known risk when performing PCR from minute amounts of DNA. Levels of significance were calculated using Student's *t* test.

Results

Using the PCR for the SRY gene, Y-chromosomal DNA was detected in 8 of 17 women suffering from Hashimoto's disease (Table 1). The pherograms for the positive samples are displayed in Fig. 1. In the control group, however, in only 1 of 25 women was the SRY gene detected.

To quantitate the ratio of male/female DNA, a PCR for a fragment of the amelogenin gene that was mapped to both X- and Y-chromosomes was performed. The amplified fragments comprised a 6-bp deletion of the fragment amplified from the X-chromosome (106 bp) compared with that of the Y-chromosome (112 bp). After capillary electrophoresis and

fluorescence LASER detection, the ratio of the peak areas at 106 and 112 bp can be used as a crude estimate for the percentage of male cells to female cells. If, for example, 1 in 10 cells is of male origin, the area of the 106-bp peak should be approximately 19 times the area of the 112-bp peak. Using this approach a male contribution to the DNA extracted from the histological samples could only be identified in 4 of the 17 Hashimoto patients, but in none of the controls. (Table 1). The percentage of Y-chromosomes compared with X-chromosomes (Y/X ratio) was small. In the pherogram with the largest percentage of Y fragments, the Y signal was 0.045 times the X signal (Fig. 2), whereas in the 3 other samples values around 0.01 were observed (Table 1).

For 14 of the 17 Hashimoto patients (Table 1) and for 16 of the 25 patients in the control group an anamnesis concerning alternative sources of engraftment was possible. Neither organ transplants nor twin siblings are known for any of the patients, and only 1 person had received a blood transfusion. There is no evidence of concomitant autoimmune diseases. Both groups were of similar age [mean \pm SD, 46.8 \pm 9.4 yr (Hashimoto) *vs.* 54.4 \pm 7.4 yr (controls); *P* = 0.38], had similar numbers of children (mean \pm SD, 2.09 \pm 1.13 *vs.* 1.88 \pm 0.89; *P* = 0.68), and had similar numbers of boys (mean \pm SD, 1.23 \pm 0.83 *vs.* 0.81 \pm 0.75; *P* = 0.17). Moreover, the percentages of mothers of sons were comparable in both groups (11 of 13 Hashimoto patients and 10 of 16 controls; *P* = 0.20).

All women positive for microchimerism had given birth to at least one male child 12–46 yr before the diagnosis of

TABLE 1. Clinical history and possible sources of engraftment of the Hashimoto patients (H1–H17) and the control group (C1–C25)

Patient no.	Age (yr)	Yr of diagnosis	Age and sex of children at diagnosis	Abortus	Transfusion	Twins	Additional diseases	Microchimerism	Y/X ratio
H1	56	98	35f; 34f; 30m; 16m	+ (1965)	+ (1966)	–	Tuberculosis	Yes	ND
H2	42	98	18m	–	–	–		Yes	ND
H3	70	98	48f; 46m; 42m	–	–	–		Yes	0.045
H4	50	99	30f; 25m	–	–	–		Yes	ND
H5	56	97	29m; 33f	–	–	–	Pancreatitis	Yes	0.010
H6	50	88	12m; 7f	–	–	–	Pancreatitis	Yes	0.012
H7	53	99	35f; 32m; 29m; 19m	–	–	–	Gonarthrosis	Yes	ND
H8	59	?	?	?	?	?		Yes	0.008
H9	52	97	26m	–	–	–		No	ND
H10	37	90	8m; 5f	–	–	–		No	ND
H11	57	97	33m	–	–	–	Allergic disposition	No	ND
H12	44	94	4m	–	–	–		No	ND
H13	53	85	–	–	–	–		No	ND
H14	46	98	–	–	–	–		No	ND
H15–17	?	?	?	?	?	?	?	No	ND
C1	60	94	40m; 37f	–	–	–		No	ND
C2	49	95	27m; 26f	–	–	–		No	
C3	72	70	44f; 43f; 39m; 29m	–	–	–		No	ND
C4	52	94	30m	–	–	–		No	ND
C5	62	97	40f; 35m	–	–	–	Gall stones	No	ND
C6	57	98	–	–	–	–		No	ND
C7	60	86	24f; 22f	–	–	–		No	ND
C8	60	90	36f; 33f	–	–	–		No	ND
C9	51	98	33m; 31m	–	–	–	Diabetes	Yes	ND
C10	40	93	17m; 14m	–	–	–		No	ND
C11	53	97	29f	–	–	–		No	ND
C12	54	95	34m; 32f; 28f	–	–	–		No	ND
C13	62	92	40f; 37f	–	–	–		No	ND
C14	53	88	35m; 31f	–	–	–	Allergic disposition	No	ND
C15	42	89	14m	–	–	–		No	ND
C16	44	91	25f; 22f	–	–	–		No	ND
C17–25			Lost to follow up	–	–	–		No	ND

The presence of microchimerism and the estimated Y/X ratio are indicated. ND, Not detectable.

FIG. 1. Pherogram for the SRY gene. Lane 1, Negative control. Lanes 2 and 13, Size marker comprising fragments 89, 103, and 109 bp in length. Lane 3, DNA extracted from the blood of a healthy woman. Lanes 4–12, The nine positive specimens. Lane 14, Positive control; DNA extracted from the blood of a male individual.

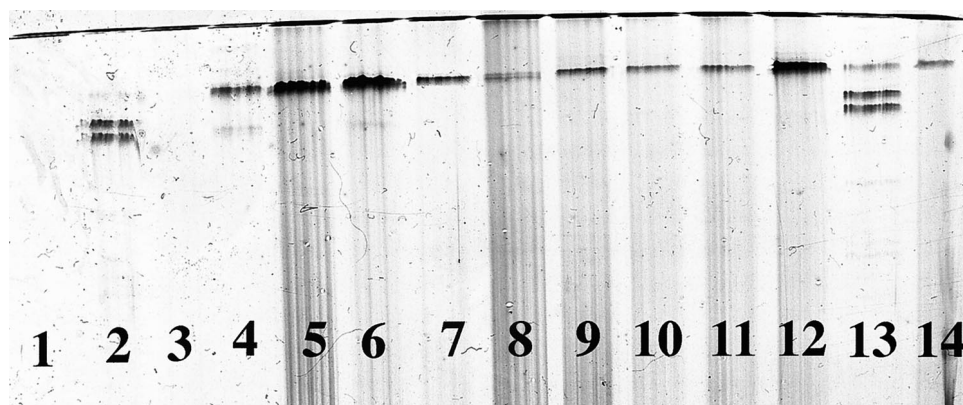
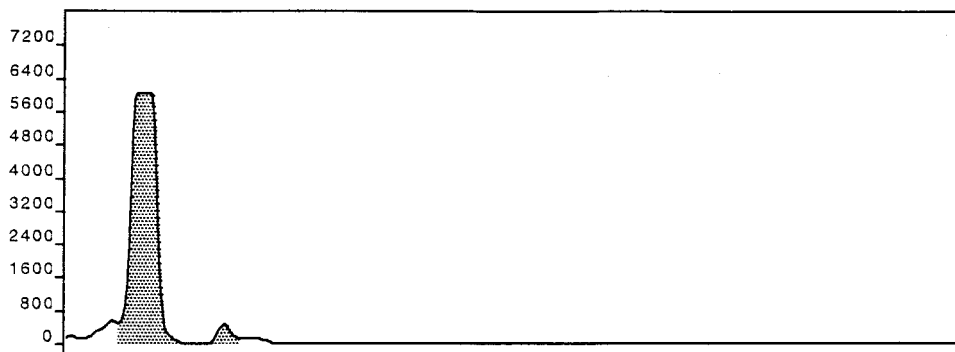


FIG. 2. Fluorescence-detected pherogram for the amelogenin locus amplified from patient 3. The peak at 106 bp was amplified from X-chromosomal DNA; the small peak at 112 bp was amplified from the Y-chromosome. The area of the Y-chromosomal peak is less than 5% of that for the X-chromosomal peak, indicating that the percentage of male cells is very small.



Hashimoto's thyroiditis. Nevertheless, there was a difference between Hashimoto patients positive for microchimerism and Hashimoto patients negative for microchimerism. Women in the first group had significantly more children in general, both sons and daughters (Table 2).

Discussion

Many autoimmune diseases, Hashimoto's thyroiditis among them, show a female predilection and tend to develop past the childbearing years. In light of the discovery of bidirectional cell traffic through the placenta (1, 2, 13), this consideration lead to the hypothesis that fetal cells persisting in the blood/tissue of mothers could contribute to the development of autoimmune diseases. For systemic sclerosis this assumption is supported by experimental data (3, 4). In the present study we investigated the presence of male cell DNA in thyroid gland specimens from female patients with Hashimoto's disease and controls by PCR analysis for a Y-chromosomal sequence (SRY) and for an X/Y homologous sequence spanning an X-chromosomal 6-bp deletion (amelogenin).

Our results for the SRY-locus show that sequences derived from the Y-chromosome are present in the thyroid glands of 8 of 17 female patients suffering from Hashimoto's disease, but in only 1 of 25 nodular goiters used as the control group. The amelogenin approach was applied to estimate the percentage of microchimeric male cells. However, using this approach microchimerism was detected in only 4 of the 8 Hashimoto patients with positive SRY-PCR and in none of the other patients. It is not surprising that this test is less sensitive in detecting minor quantities of Y-chromosomal

TABLE 2. Number of children, sons, and daughters in Hashimoto patients with and without detectable microchimerism

Patient no.	No. of children	No. of daughters	No. of sons	Microchimerism
1	4	2	2	Yes
2	1	0	1	Yes
3	3	1	2	Yes
4	2	1	1	Yes
5	2	1	1	Yes
6	2	1	1	Yes
7	4	1	3	Yes
Mean	2.57	1	1.57	
9	1	0	1	No
10	2	1	1	No
11	1	0	1	No
12	1	0	1	No
13	0	0	0	No
14	0	0	0	No
Mean	0.83	0.17	0.67	
P value	0.009	0.013	0.035	

Patients with microchimerism have significantly more children (sons and daughters) than patients without microchimerism, whereas no differences were found between Hashimoto patients and controls.

sequences, as the primers prime to both Y- and X-chromosomes (12). The signal for the minor component (Y) can therefore go unnoticed because of preferential amplification, as evidenced in the forensic practice of mixed blood stains (14). During PCR almost all primer molecules and nucleotides are consumed by amplification of the abundant X-chromosomal locus, and the Y-chromosomal locus remains unamplified. Moreover, for the amelogenin approach the number of PCR cycles was restricted to 30 to guarantee equal

amplification of both X- and Y-chromosomal alleles (compared with 45 for the SRY approach), which further decreases the sensitivity of the assay. Nevertheless, using the amelogenin approach we could demonstrate that the ratio of X- to Y-chromosomes is very small even in the 4 positive samples, ranging between 0.008 and 0.045. Although the percentage of male engrafted cells (containing one Y- and one X-chromosome) should be twice that number, it still is very small.

The highly significant difference between the Hashimoto and control groups, despite similar age and number and gender of children, is strong evidence of an etiological role of microchimerism in the pathogenesis of Hashimoto's disease. An observation of increased microchimerism in the thyroiditis group as a result of a higher number of pregnancies (with boys) and thus a higher chance for cell traffic through the placental barrier can therefore be rejected, but to what degree and in what respect could microchimerism contribute to the actual pathogenesis of Hashimoto's disease?

Three etiologic mechanisms for a microchimeric cell population have been proposed for systemic sclerosis (3). 1) Fetal cells, which are primarily sequestered in affected tissues, could act as direct effectors of damage to host tissues and initiate a graft *vs.* host reaction. The underlying mechanism might be the same as that for transfusion-associated graft *vs.* host disease (15). Transfused donor leukocytes (fetal cells) that are homozygous for one HLA antigen that is shared with a heterozygotic recipient (the mother) are tolerated by the recipient, but recognize the recipient's other haplotype (16). However, low concentrations of fetal cells found in the maternal circulation argue against a direct effector role for these cells. 2) A small population of nonhost cells could start a process by which subsequent damage is caused by host cells, *i.e.* an autoimmune reaction. 3) A small population of nonhost cells (veto cells) could down-regulate host immunoregulatory cells and thus allow damage by autoreactive host cells (17). In our opinion the same mechanisms could apply to microchimerism and Hashimoto's disease.

Moreover, autoimmune thyroiditis, not unlike other autoimmune diseases (18, 19), tends to be alleviated during pregnancy, whereas after childbirth an aggravation of the disease can be expected (8). Current hypotheses recently discussed by Davies (8) are: 1) loss of placental major histocompatibility complex-peptide complexes that were inducing T cell anergy, 2) persistence of pregnancy-induced immune changes in target organs, 3) breast feeding induction of immunologically active PRL secretion, and 4) reduced numbers of hypothetical fetal microchimeric cells leading to loss of maternal tolerance to remaining fetal cells. The last point, which assumes the engraftment of fetal progenitor cells, is supported by our study, which succeeded for the first time in demonstrating the presence of fetal microchimerism in thyroid glands of patients suffering from Hashimoto's disease. A possible mechanism for the remission of symptoms of Hashimoto's disease might therefore be that in those cases the maternal T cells appear to have become tolerant for paternal alloantigens, resulting in an immune suppression.

We should nevertheless emphasize that our data could also provide arguments against an (essential) etiological role of microchimerism in Hashimoto's disease. Firstly, in approximately 50% of our Hashimoto patients no microchi-

merism was detected. Although the percentage of detectable microchimerism in Hashimoto's disease appears comparable to that found in systemic sclerosis (3, 4), this finding could be interpreted as evidence of a heterogeneous etiology in Hashimoto's thyroiditis. In some patients microchimerism could be an essential factor, and in others (roughly 50%) it is not. Nevertheless, we cannot exclude that in these patients who appear to be negative, fetal microchimerism is not detectable using the methods applied, *e.g.* because of a lower percentage of engrafted cells or the fact that only engrafted cells with female karyotypes are present.

Secondly, our Hashimoto patients with microchimerism have a larger number of (male and female) offspring than our Hashimoto patients without detectable microchimerism. It could therefore be argued that the occurrence of microchimerism solely reflects an increased number of pregnancies. A strong argument against this assumption is the fact that we observed no difference in the number and gender of children between Hashimoto and control patients, and a similar proportion of both groups had given birth to at least one son, whereas the occurrence of microchimerism differed significantly.

Thirdly, it remains possible that the difference in microchimerism between the Hashimoto and control groups results from a difference in the leakiness of the placenta. One might therefore speculate that materno-fetal transfusion during pregnancy might be not the cause but the consequence of a preexisting subclinical autoimmune disease involving the placenta. However, at present no data supporting this assumption are available.

Fourthly, at least one of our controls and, according to the literature (1, 2), a high percentage of healthy mothers show microchimerism without autoimmune disease. It is speculated that differences (or similarities) in class II HLA compatibility might be responsible for microchimerism leading to disease or not (20). Nevertheless, the assumption that microchimerism is not an exclusive etiological factor and that the microchimeric cells are merely "innocent bystanders" in a process triggered by other mechanisms cannot be completely eliminated.

In conclusion, we successfully demonstrated the occurrence of fetal microchimerism in approximately 50% of female patients suffering from Hashimoto's thyroiditis 12–46 yr after having given birth to a son. In a control group of 25 patients with nodular goiter, microchimerism was detected in only 1 patient. Although the functional consequences of persisting fetal microchimerism are just beginning to be explored, we propose that fetal cells engrafted into maternal thyroid tissue may play a role in the etiology of Hashimoto's thyroiditis by eliciting an intrathyroidal graft *vs.* host reaction, leading to chronic inflammation, hormone release, *etc.*

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