Chimerism in health, transplantation and autoimmunity

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Chimerism in childhood lupus nephritis

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Abstract

Chimerism, defined as the presence of cells from one individual into another, may play a role in the development of autoimmune diseases. From an experimental mouse model it is known that chimeric cells can induce a proliferative glomerulonephritis that resembles human lupus nephritis. Y chromosome-positive chimeric cells are significantly more often present in kidneys of women with lupus nephritis than in kidneys of normal women, pointing towards a possible role of chimeric cells in this disease. The presence of these Y chromosome-positive chimeric cells is thought to result predominantly from pregnancies.

Lupus nephritis also occurs in children, although less frequently. We investigated whether in childhood lupus nephritis Y chromosome-positive cells are present and determined whether they are present more often in lupus nephritis specimens than in normal kidney specimens of children. We compared the results to our findings in adults.

In situ hybridization of the Y chromosome was performed on 36 kidney biopsies of 29 girls (age: 4-16 years) with SLE and on 11 control kidney autopsy specimens of girls without SLE (age: 1-11 years). Y chromosome-positive chimeric cells were found in 12 of 36 biopsies with lupus nephritis and in 4 of 11 control kidney specimens. This difference was not statistically significant.

We demonstrated, for the first time, the presence of Y chromosome-positive chimeric cells in childhood lupus nephritis. The presence was not significantly different from the presence of male chimeric cells in kidney tissue of control girls. These findings are different from our previous findings in adults. Chimeric cells in children and adults may be from different origins and may therefore have different pathogenic potential.

Introduction

Chimerism is defined as the presence of cells from one individual in another individual, resulting in tissues of diverse genetic constitution. Chimerism is commonly found in blood and tissues of individuals,[1-4] although the number of chimeric cells found is usually very low. Hypotheses have been postulated about the possible effect chimeric cells may have on the immune system of the host. A proposed role of chimeric cells is that they contribute to the pathogenesis of autoimmune diseases.[5] This hypothesis resulted from observations of similarities between graft-versus-host disease seen after stem cell transplantation and certain autoimmune diseases, in particular systemic sclerosis.[5] Pregnancy is considered the main cause of chimerism, and in this light, it is interesting that autoimmunity predominantly occurs in women during or shortly after their fertile years.

Systemic lupus erythematosus (SLE) is an immune-mediated disease that has a variety of clinical symptoms affecting many organs. SLE is characterised by the presence of autoantibodies, especially autoantibodies directed toward nuclear components.[6,7] One serious manifestation of SLE is renal involvement, leading to a proliferative glomerulonephritis also called lupus nephritis, occurring in up to 60% of patients with SLE.[8] SLE occurs in adult women and men at a ratio of approximately 9:1.[9] Despite extensive research, the etiology of SLE is still unknown.

Although the clinical features of SLE show not as much similarities with graft-versus-host disease as systemic sclerosis does, a role for chimerism in the pathogenesis of SLE was presumed from findings in an experimental mouse model. In this model, the injection of parental (i.e., chimeric) cells in (DBAxC57BL) F1 mice initiated a graft-versus-host response resulting in manifestations of an SLE-like disease including the production of autoantibodies directed against nuclear antigens (e.g. anti-double-stranded DNA) and the occurrence of a proliferative glomerulonephritis.[10,11] This SLE-like disease only occurred when specific strain combinations were used. To determine the role of chimerism in human SLE, we previously investigated the presence of chimerism in kidney biopsies of adult women with lupus nephritis and found that chimerism occurs twice as often in lupus nephritis as in normal kidney specimens.[12]

SLE also occurs in children, although the incidence is much lower than in adults. Twenty percent of all cases of lupus are diagnosed during the first two decades of life.[13] Furthermore, there is a difference between the male/female ratio in adult SLE and that in childhood SLE. Adult SLE affects predominantly women, whereas in childhood SLE males and females are affected almost equally.[14] Presuming that chimerism is involved in the development of SLE, the male/female ratio difference between adults and children could be explained by a higher chance of adult women to become chimeric due to pregnancies. We hypothesized that if chimeric cells are really essential for the development of lupus nephritis, they must be present in children with lupus nephritis as well. Therefore we investigated the presence of chimerism in kidney biopsies of children with lupus nephritis. To be able to compare our findings with those of our previous studies in adults, we detected chimeric cells by using in situ hybridization of the Y chromosome.

Patients and methods

Patients and biopsies

All archived renal biopsies from girls from 1 to 16 years old, obtained in the period 1988 to 2004, were selected. They came from three University Medical Centers, namely Leiden University Medical Center, University Medical Center Utrecht and Erasmus Medical Center Rotterdam. If a definitive histologic diagnosis of lupus nephritis had been made or if the findings were very suspect for lupus nephritis and clinically or in a later biopsy the diagnosis SLE or lupus nephritis was confirmed, biopsies entered the study. Thirty-six renal biopsy samples of 29 girls with lupus nephritis (age range at biopsy 4-16 years) were included. Multiple biopsies were available from six patients (two samples obtained from five girls and three samples from one girl). The biopsy samples were evaluated for lupus nephritis according to the most recent modification of the WHO classification by the ISN/RPS (International Society of Nephrology/Renal Pathology Society).[15]

As controls, kidney specimens obtained at autopsy performed at the LUMC of 11 girls were included. The age of the control group at the time of death ranged from 1 to 11

years. Causes of death were a viral infection in two girls, cardiac arrhythmia in one girl, neurodegenerative disease in one girl, four girls died as a result of congenital defects and cause of death was unknown in three girls.

In situ hybridization targeting the Y chromosome

Archived paraffin-embedded tissues were cut into 4-µm sections, and deposited onto Superfrost Plus glass slides (Menzel-Glaser, Braunschweig, Germany). The sections were dried overnight at 37ºC. A Y chromosome-specific DNA probe[16] was labeled with digoxigenin (DIG) according to the standard Nick-translation protocol. After labeling, the probe was precipitated, dried and dissolved in a hybridization mixture (50% deionized formamide, 0.05 M sodium phosphate buffer pH 7.0, 0.3 mol/L NaCl, 30 mmol/L Na citrate [2 × SSC] and 10% dextran sulphate). To prevent nonspecific binding of DNA, salmon sperm DNA, transfer RNA and Cot-1 DNA were added to the hybridization mixture.

Paraffin was removed by placing slides in xylene. Samples were rehydrated by serial passage through ethanol/water mixtures, followed by a distilled water rinse. The sections were pretreated with 0.01 M citrate buffer (pH 6.0) at 80ºC for 80 minutes, rinsed in distilled water at 37º, and treated with 0.5% Pepsin (Serva Electrophoresis GmbH, Heidelberg, Germany) in 0.01 M HCL at 37ºC for 20 minutes. Slides were then dehydrated in an ethanol series and air-dried. Slides were covered with a 30-µL hybridization mixture containing 5 ng/µl labeled probe. DNA was denatured by placing the slides on a metal plate at 80ºC for 10 minutes, followed by incubation at 37ºC overnight.

The following day, the sections were washed three times in 2 × SSC/0.1% Tween at 37ºC, and three times in 0.1 × SSC at 60ºC. To visualize the DIG-labeled probe, sections were incubated consecutively with a mouse-anti-DIG monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA), rabbit-anti-mouse immunoglobulin-HRP (Dako, Glostrup, Denmark), and swine-anti-rabbit immunoglobulin-HRP (Dako) at room temperature. Finally, sections were developed with Nova Red Vector for ten minutes. Hematoxylin staining served as a background.

A tissue sample from a male subject served as a positive control for the in situ hybridization of the Y chromosome; this sample was characterized by red-brown dots, confirming a positive signal in 58% of the nuclei. By nested polymerase-chain-reaction (PCR) and sequencing we confirmed that the probe was specific for the Y chromosome [described in reference [3]]. As a negative technical control, a tissue sample from a male subject was used, on which the complete in situ hybridization protocol was performed, except that instead of the hybridization mixture with the Y chromosome probe, only the hybridization mixture was added. This negative control yielded consistently negative results.

Scoring

All specimens were scored by two observers who were blinded to the clinical information of the study subjects. Strict criteria were applied in the scoring, in which a cell was scored positive for the Y chromosome only if there was a dot inside the nucleus; this dot had to have a similar size and staining intensity as those found in the nuclei of male control samples. The size of the investigated area of kidney biopsy tissues from both patients and controls was measured by digital image analysis, using the Image Tool program (University of Texas Health Science Center, San Antonio, USA).

Statistics

Categorical variables were compared with the use of the Fisher's exact probability test. Continuous variables were compared with the Student's t-test. To determine the predictive value of age and WHO class for the presence of chimerism, we performed a logistic regression analysis.

Results

Thirty-six biopsies with lupus nephritis of 29 girls were available. Age distribution at time of biopsy is given in Figure 1. The biopsy specimens from girls with lupus nephritis covered a median area of 5.31 mm^2 (range 0.30 - 111.86 mm^2). Y chromosome-positive cells were found 12 of 36 biopsies (33%) with lupus nephritis. In the first renal biopsy

specimen of 10 of 29 girls with lupus nephritis (34%) Y chromosome-positive cells were found, and in 11 of 29 girls (38%) at least once male cells were found. Results are summarized in Table 1. The presence of Y chromosome-positive cells in relation to lupus nephritis class is shown in Table 2 and the relation to age is shown in Figure 2. Age was not a predictor for the presence of chimerism in biopsies of girls with lupus nephritis (OR = 0.8, CI = 0.6 - 1.1, P = 0.31).

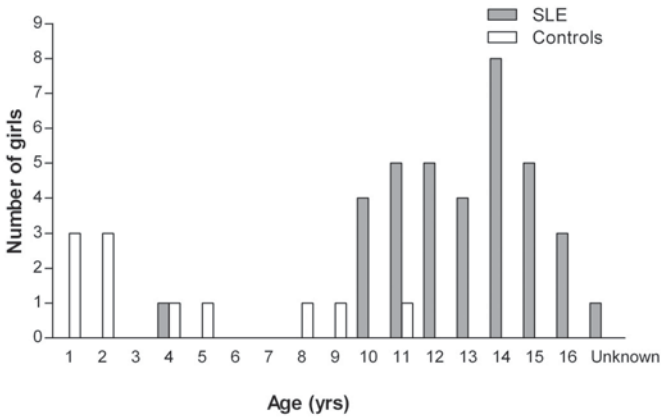

Figure 1. Age distribution of girls with lupus nephritis and control girls. Grey bars indicate girls with lupus nephritis, white bars indicate control girls.

Table 1. Presence of Y chromosome-positive cells in patient and control renal specimens

	Total number	Chimerism present	Chimerism absent
Biopsies with lupus nephritis	36	12	24
Patients with lupus nephritis			
only first renal biopsy included	29	10	19
all renal biopsies included	29	11	18
Controls	11	4	7
Controls categorized by cause of death:			
viral infection	2	1	1
complications after congenital defect surgery	3	0	3
complications as a result of congenital defect	1	0	1
cardiac arrhythmia	1	1	0
neurodegenerative diseases	1	0	1
no cause found	3	2	1

There were no significant differences

Table 2. Presence of chimerism according to Lupus-class

Lupus-class	Total number	Chimerism present	Chimerism absent
I	1	0	1
II	3	2	1
III	12	5	7
IV	16	4	12
V	4	1	3
VI	0	0	0

Lupus-class was scored according to the ISN/RPS classification

In five girls, two biopsy samples were assessed for Y chromosome-positive cells: neither sample was positive for chimeric cells in four of the girls, and one girl had a negative result in the first sample and a positive result in the second. In one girl, three biopsy samples were assessed: the first had a positive result, whereas the second biopsy sample had a negative result, and the third biopsy was positive again.

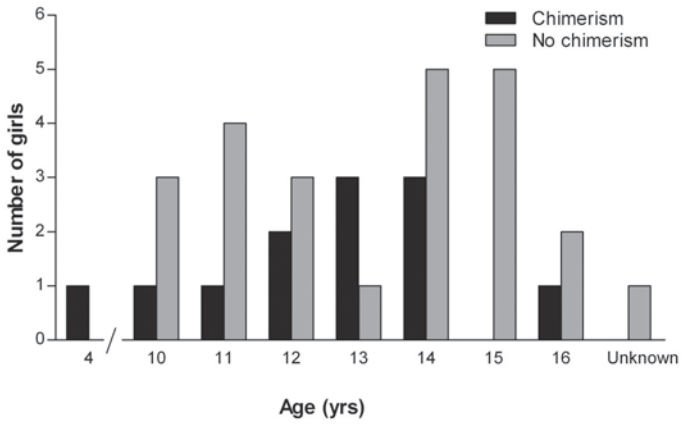

Figure 2. Distribution of chimerism according to age in biopsies of girls with lupus nephritis. Black bars indicate samples with chimerism, grey bars indicate samples without chimerism.

Eight lupus nephritis renal tissue specimens contained one Y chromosome-positive cell, three specimens contained two Y chromosome-positive cells, and one specimen contained four Y chromosome-positive cells. Taking all renal specimens of patients with lupus nephritis together, on average one Y chromosome-positive cell was found per

area of 15.8 mm^2. Within the renal tissue specimens, there was no predilection site where most of the chimeric cells were present. An example of a Y chromosome-positive cell in a tubule of the kidney of a 13-year-old girl with lupus nephritis is shown in Figure 3A. In Figure 3B an example is shown of a male cell in a glomerulus of a four-year-old girl with lupus nephritis. Chimeric cells were not typically found in areas of active inflammation. Surface area did not predict for the presence of chimerism in biopsies with lupus nephritis (OR = 1.1, CI 0.9 - 1.2, P = 0.53).

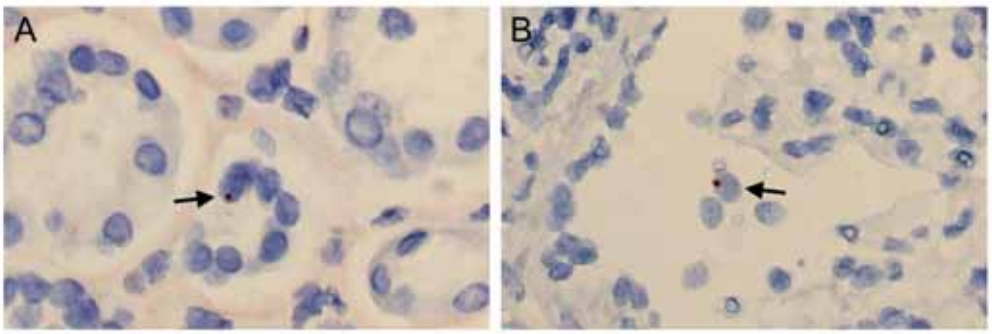

Figure 3. Results after in situ hybridization of the Y chromosome on renal biopsies of girls with lupus nephritis. Y chromosome-positive cells in kidney tissue of a 13-year-old girl (A) and a 4-year-old girl (B).

In the control group, 11 renal specimens of 11 girls were investigated. Age distribution at time of death is given in Figure 2. A randomly chosen area of 58 mm^2 was scored in all samples. Four of 11 renal specimens (36%) contained Y chromosome-positive cells. Three specimens contained one Y chromosome-positive cell and one specimen contained two Y chromosome-positive cells. Taking all control specimens together, on average one Y chromosome-positive cell was found per area of 127.6 mm^2. The presence of chimerism in relation to cause of death is shown in Table 1. When comparing the two groups, the results showed that there is no significant difference between the occurrence of chimerism in girls with lupus nephritis compared to control kidney specimens (P = 1.0).

Discussion

Chimerism has been considered to play a role in the development of autoimmune diseases. In two previous studies we investigated the presence of Y chromosome-positive chimeric cells in kidney specimens of women with the autoimmune disease SLE and found that chimerism was present in 51% and 46% of biopsies with lupus nephritis, respectively.[12,17] This is significantly more often than in control kidney specimens of women (25%). If chimerism indeed plays a role in lupus nephritis, chimeric cells are expected to be or to have been present in children with lupus nephritis.

In the present study we investigated the presence of Y chromosome-positive chimeric cells in 36 biopsies of girls with lupus nephritis and found them in 12 (33%) of the 36 biopsies. This was not significantly different from the occurrence of Y chromosome-positive chimeric cells in both groups of adult women with lupus nephritis ($p = 0.16$) we investigated previously. There was also no significant difference with the occurrence of chimerism in normal renal tissue specimens from control girls. From these results it can be concluded that male chimerism in renal specimens of girls with lupus nephritis can be present, but that their presence is not unique for girls with SLE. Also in our previous study, we found that the presence of chimerism as such is not unique for SLE. However, in our adult studies, chimerism occurred twice as often in renal biopsies of patients with SLE as in controls. In the present study, on average, one Y chromosome-positive cell was found per area of 128 mm^2 in controls, and of 16 mm^2 in patients. We assume that this difference is partly based on sampling error, and therefore, the clinical significance is uncertain.

In the present study, we set out to investigate the presence of male chimerism in females who have never been pregnant. Since lupus nephritis is rarely diagnosed before the age of 10, most of our patients included were youngsters of whom some may have had sexual intercourse which could have led to pregnancy. Because we are unaware of these data, they may have influenced our results. In entering patients into this study, we excluded one female patient of 14 years old with SLE who was pregnant. Although only exemplarily, cases like these raise the question of the possible importance of sexual development and the occurrence of SLE in youngsters.

In girls, male chimeric cells are presumed to derive mainly from twin-twin transfusion in utero between (vanished) twins of opposite sex and transplacental transmission of male cells present in the mother before pregnancy, i.e. as the result of a previous male pregnancy or blood transfusion.[18] In fertile women, next to the above mentioned sources, pregnancy is an important source of chimeric cells. We assume that adult fertile women have a higher incidence of pregnancies than fertile girls. Therefore adult women have a higher change of being chimeric from fetus-derived cells than girls. During pregnancy, also cells from the mother enter the fetus, leading to maternal chimerism. Contemplating on the possible sources of chimerism, it seems likely that in children most chimerism results from cell transfer from mother to child during pregnancy, leading to a pool of predominantly maternal chimeric cells at birth. A drawback of the present study is that maternal chimerism could not be investigated. Momentarily we are exploring ways to investigate the presence of maternal chimeric cells to determine their role in childhood SLE.

Maternal chimerism is difficult to investigate in girls due to the absence of a sex-difference between mother and child. However, from studies that investigated maternal chimerism by targeting maternal HLA alleles not shared by the child, we know that maternal chimeric cells are present in 22% of healthy females aged 13-62 years.[18] It has also been demonstrated that these maternal cells could have an immunological phenotype[19] and therefore also maternal chimerism may be involved in the development of SLE.

The presence of Y chromosome- positive cells in organs of girls has been reported in 2005 by Guettier et al.[20], who investigated male chimerism in liver tissue of ten fetuses and six girls and compared the results to their findings in liver tissue of 29 adults. Male DNA was found in the liver specimens of seven out of ten (70%) fetuses and five out of six girls (83%) compared to 27 of the 29 (93%) adult liver specimens, both normal ones and diseased ones (no autoimmune diseases). They did not observe any relationship between male chimerism and the presence and intensity of liver disease. They concluded that chimeric fetal cells may be present in a large proportion of female subjects and be able to cross generations. We are the first to report the presence of male chimerism in kidney specimens of girls. Although we found a lower occurrence of male chimerism in

kidneys than Guettier et al.[20] found in livers, our results are comparable with regard to the absence of a relationship between the presence and intensity of disease.

Because the origin of the male chimeric cells in women and girls is different, it is possible that they behave differently as well. Perhaps chimeric cells derived from a child are better tolerated than cells from a full sibling. In line with this theory, chimeric cells from different sources may have different pathogenetic potential. In sum, we have shown for the first time that male chimerism occurs in renal specimens of girls with lupus nephritis. The occurrence of chimerism differs not significantly from the occurrence in normal kidney specimens of girls. This finding is different from our results in adults, in which we found that chimerism occurs twice as often in renal specimens with lupus nephritis as in normal kidney specimens. Our results indicate that the presence of male chimerism alone is not sufficient to induce lupus nephritis in children. The immunological capacities of the chimeric cells and the occurrence of female chimeric cells need to be investigated before conclusions can be drawn on the pathogeneity of chimerism in childhood lupus nephritis.

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