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Fetal Microchimerism in Women with Breast Cancer

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Abstract

Fetal microchimerism (FMc) describes long-term persistence of small numbers of fetal-derived allogeneic cells in the mother. Although FMc has been implicated as a mechanism of autoimmune disease, it may confer a beneficial effect with immune surveillance of malignant cells. We hypothesized that allogeneic FMc imparts a protective effect against breast cancer. Two observations provided a rationale for the study hypothesis. First, allogeneic cells convey risk reduction for recurrent malignancy in hematopoietic cell transplantation. Second, reduced risk of breast cancer is well recognized among parous compared with nulliparous women. As an initial test of the hypothesis, we investigated 82 women, 35 with breast cancer and 47 who were healthy, for male DNA in peripheral blood, presumed from a prior pregnancy with a male fetus. The prevalence and levels of male DNA were determined by real-time quantitative PCR for the Y chromosome–specific gene *DYS14* in DNA extracted from peripheral blood mononuclear cells. FMc was found significantly more often in healthy women than women with breast cancer (43% versus 14%, respectively). Considering the absence of FMc as a risk factor, the odds ratio was 4.4 [95% confidence intervals (95% CI), 1.34–16.99; P = 0.006]. Restricting analysis to women known to had given birth to a son, the odds ratio was 5.9 (95% CI, 1.26–6.69; P = 0.01). Our findings indicate that allogeneic FMc may contribute to reduction in risk of breast cancer. Further studies are indicated and, if confirmed, extended studies to examine whether allogeneic immune surveillance from FMc is deficient in women with breast cancer. [Cancer Res 2007;67(19):9035–8]

Introduction

Fetal cells routinely enter the maternal circulation during normal pregnancy (1) and may persist in the peripheral blood of the mother for years after pregnancy completion, referred to as fetal microchimerism (FMc; refs. 2–6). FMc is usually semi-allogeneic to the mother and because autoimmune diseases are more prevalent in women, FMc has been investigated recently in several autoimmune diseases. Initial studies described significantly increased levels of FMc in the peripheral blood of women with systemic sclerosis compared with healthy women (7). Although FMc is not limited to autoimmune disease, some studies have found a significant increase of FMc in Sjogren's syndrome (in labial salivary glands), systemic lupus (in kidney), and Hashimoto's disease (in thyroid; refs. 6, 8, 9). In functional studies of fetal T-cell

clones derived from maternal blood, FMc reactive to maternal HLA was observed more frequently in women with systemic sclerosis than normal women (10). FMc, however, is found commonly in the peripheral blood of healthy women (22–75%; refs. 2, 3, 5) and could also have beneficial effects, such as tissue regeneration (4) and allogeneic immune surveillance for malignant cells. Fetal cells represent a naturally acquired source of allogeneic immune cells, and in prior studies, the prevalence of T, B, natural killer (NK), and antigen-presenting cells (APC) of fetal origin from healthy women ranged from 30% to 58%, 45% to 75%, 44% to 62%, and 36% to 58%, respectively (3, 11). In general, fetal cells are expected to be tolerized to noninherited maternal antigen (also known as "NIMA" effect) when exposure occurs *in utero* in the thymus as shown in human organ transplantation (12). However, findings from haploidentical hematopoietic cell transplantation suggest that for fetal effectors that have transited into the maternal circulation, encounters with maternal antigens could result in immune surveillance against malignancy (13). Alternatively, fetal-derived APC (based on paternally inherited HLA) could present maternal antigens to maternal effectors and also result in effective priming rather than tolerance.

A compelling disease in which to investigate a potentially beneficial role of naturally acquired allogeneic FMc is breast cancer, especially because of the previously established protective role of parity (14, 15). Prior studies of parity and breast cancer have focused on the role of pregnancy hormones in the induction of terminal differentiation of breast epithelium (16). Priming of the maternal immune system to cancer neoantigens resembling common breast cancer antigens expressed by the developing fetus is another mechanism by which parity may influence breast cancer development (17). Because allogeneic immune cells provided during stem cell transplantation in humans and animal models in remission from malignant conditions often impart significant immune surveillance (known as the graft-versus-tumor response; refs. 18, 19), we reasoned that naturally acquired allogeneic immune cells in the form of FMc might correlate with protection from development of breast cancer. We examined women with breast cancer and healthy women for male DNA as a measure of FMc, presumably originating from a prior pregnancy with a male fetus. We report an overall reduction of FMc in breast cancer patients compared with healthy controls consistent with the hypothesis that allogeneic FMc provides a protective advantage against breast cancer.

Materials and Methods

Women with breast cancer were prospectively recruited from the Seattle Cancer Care Alliance breast cancer specialty clinic. Thirty-five women met study inclusion criteria from among 42 recruited subjects; 2 were excluded because the pathologic diagnosis did not confirm breast malignancy and 5 because insufficient blood sample was available for testing. The median number of days from the diagnosis of breast cancer to study participation was 51 days and ranged from 7 to 2,646 days (Table 1). Whole blood (20 cc)

Table 1. Clinical characteristics of breast cancer patients and healthy controls

Characteristic	Breast cancer (*n* = 35)	Healthy (*n* = 47)
	n (%)	*n* (%)
Obstetrics history		
Parous	26 (74)	34 (72)
Nulliparous and nulligravid	6 (17)	5 (11)
Nulliparous and gravid	3 (9)	8 (17)
Male children		
Yes	22 (63)	29 (62)
Median age at first live birth (range)	28 (19-38)	28 (16-34)
Stage (0–IV)		N.A.
In situ (0)	8 (23)	
Invasive (I–IV)	27 (77)	
Median days from diagnosis to phlebotomy (range)	51 (7-2646)	N.A
Chemotherapy		N.A.
Yes	8 (23)	
No	27 (77)	

Abbreviation: N.A., not applicable.

was collected into heparinized tubes and peripheral blood mononuclear cells (PBMC) were isolated using standard Ficoll density gradient methods, within 24 h of phlebotomy. Isolated PBMC were extracted for total genomic DNA using the Wizard kit (Promega) according to the manufacturer's instructions. DNA was stored at −20°C. Forty-seven healthy control women were recruited as part of other ongoing studies of FMc and were known to have no prior diagnosis of cancer (or autoimmunity). Detailed information about obstetric and other medical history was requested of study participants. The Fred Hutchinson Cancer Research Center Institutional Review Board approved the human research activities described. All participants provided informed consent.

We previously described development and validation of quantitative PCR (Q-PCR) specific to the *DYS14* gene to identify male DNA in women (5). The sensitivity of the assay is approximately one male cell detected in 100,000 female cells. Six aliquots of genomic DNA from PBMC were tested for each subject. Two additional DNA aliquots were amplified concurrently for β-*globin* to define the test sample DNA concentration and the Y chromosome–specific calibration curve was run on each plate. Q-PCRs were done on an ABI Prism 7000 running the manufacturer's software (SDS version 1.2.3). Raw fluorescence data generated were plotted on simultaneously run calibration curves for *DYS14* and β-*globin* genes by the Q-PCR software to determine absolute quantity of male and test sample DNA. A correction factor was applied to account for the monoallelic *DYS14*. For inclusion, it was required that a minimum of 30,000 total cell equivalents was tested and to be considered positive, it was required that at least two aliquots from a sample show results above threshold. The amount of microchimerism was expressed as the number of male genome equivalents detected within a sample containing 100,000 female cell equivalents [male genome equivalent cells per 100,000 female cells (gEq/100,000)]. The mean total number of cell equivalents tested was similar for the two groups, 103,150 and 92,726 gEq, cases and controls, respectively.

Rigorous precautions against PCR contamination were taken as described previously (5). Multiple negative controls were included in each Q-PCR plate including no DNA and DNA from a nulligravid female (known negative). Negative controls were consistently negative across all experimental plates. Female technicians did all assays.

Odds ratios with exact 95% confidence intervals (95% CI) were estimated to describe the association between FMc and breast cancer occurrence (EpiInfo). Odds ratios were reported treating absence of FMc as a risk factor for breast cancer. FMc quantities were reported standardized to a ratio of male genome equivalents detected per 100,000 maternal genome equivalents as described above. Both authors contributed to the design, analysis, and preparation of the manuscript.

Results

Patient characteristics. Patient characteristics for the women studied for FMc are provided in Table 1. Twenty-six (74%) patients and 34 (72%) controls were parous. Six (17%) patients and five (11%) controls were nulliparous and nulligravid. Three (9%) patients and eight (17%) controls were nulliparous and gravid. Age at the time of phlebotomy was somewhat greater in the breast cancer patients compared with healthy women (median, 50 versus 42; range, 31–73 and 31–68, respectively).

Twenty-two (63%) patients and 29 (62%) controls had given birth to at least one son. Among women with sons, the ages at first and subsequent pregnancies were very similar in the two groups and the number of births was also not substantially different in the two groups. Among the 22 patients, the median ages at births were 28, 29, 32, and 34.5 for first (*n* = 22), second (*n* = 15), third (*n* = 5), and forth (*n* = 2) births, respectively. Among the 29 controls, the median ages at births were 28, 29, 31, and 34 for first (*n* = 29), second (*n* = 22), third (*n* = 12), and fourth (*n* = 5) births, respectively. One control had a fifth birth at age 38 years.

Twenty-seven patients had a diagnosis of stage I to IV invasive cancer. Eight had stage 0 (ductal or lobular carcinoma *in situ*). Nine patients had prior systemic chemotherapy.

Detection and quantification of male DNA in PBMC. A total of 82 women were studied, 35 with breast cancer and 47 who were healthy, for quantitative assessment of male DNA within DNA extracted from PBMC, presumed from prior pregnancy with a male fetus (Table 2). Among women with breast cancer, 5 (14%) had male DNA compared with 20 (43%) healthy women. When absence of FMc was treated as a risk factor for breast cancer, the odds ratio was 4.4 (95% CI, 1.34–16.99; *P* = 0.006). When analysis was restricted to women who had a live born son, FMc prevalence was 14% (3 of 22) in the breast cancer patients compared with 48% (14 of 29) in the controls (odds ratio, 5.9; 95% CI, 1.29–36.69;

Table 2. Prevalence of FMc (male DNA) in breast cancer and control women

	n (%)	Odds ratio (95% CI)	*P*
Total cohort			
Breast cancer	5/35 (14)	4.4 (1.34–16.99)	0.006
Healthy controls	20/47 (43)		
Prior male birth			
Breast cancer	3/22 (14)	5.9 (1.26–36.69)	0.01
Healthy controls	14/29 (48)		
No chemotherapy			
Breast cancer	3/27 (11)	5.9 (1.40–28.77)	0.01
Healthy controls	20/47 (43)		
Prior male birth, no chemotherapy			
Breast cancer	1/16 (6)	14 (1.53–327.79)	0.01
Healthy controls	14/29 (48)		

P = 0.01). When only breast cancer cases with no prior exposure to chemotherapy were compared with control women, FMc prevalence was 11% (3 of 27; odds ratio, 5.9; 95% CI, 1.40–28.77; P = 0.01). For women with breast cancer, a known prior male birth, and no exposure to chemotherapy compared with control women with known prior male birth, FMc prevalence was 6% (1 of 16; odds ratio, 14; 95% CI, 1.53–327.79; P = 0.01). The subjects' age at the time of phlebotomy, age at first or subsequent births, and total cell equivalents tested did not differ significantly between cases and controls.

The levels of FMc, expressed as the genome equivalent number of male cells per 100,000 subject cell equivalents (gEq/10^5) ranged from 0 to 1.47 and from 0 to 4.02 in breast cancer patients and controls, respectively, both overall and among those who had sons (Table 3). Median values were 0. The number of positive results in the breast cancer group was not sufficient for meaningful analysis for quantitative differences in patients and controls. The positive results in breast cancer patients were found in 3 women with sons (0.60, 0.99, and 1.47 gEq/10^5), 1 woman who was nulliparous but had an induced abortion (0.12 gEq/10^5), and 1 nulligravid woman (0.17 gEq/10^5). All patients with FMc had invasive cancer. The two highest levels (0.99 and 1.47 gEq/10^6) were found in women who had given birth to sons, one of whom also had a miscarriage and the other an induced abortion and both had exposure to chemotherapy.

Discussion

We identified a decreased prevalence of FMc, as assessed by male DNA in peripheral blood mononuclear cells, in a prospectively recruited cohort of women with breast cancer (stage 0–IV) compared with healthy women. Our principal finding is consistent with the hypothesis that allogeneic FMc provides a protective effect against breast cancer. Most of the women in the current study were parous and when analysis was restricted to women who had given birth to sons, the magnitude of difference between patients and healthy women increased and results remained significant. The effect of chemotherapy on FMc prevalence and levels has not been directly addressed. The inclusion of women with prior chemotherapy could potentially bias our hypothesis. Analysis was therefore done excluding women with chemotherapy and the magnitude of difference between patients and controls increased both when all women were considered and when restricted to women with a known prior male birth.

Because parity is known to decrease risk of breast cancer, these observations raise the question of whether parous women who develop breast cancer are deficient in effective allogeneic immunity from FMc. In certain autoimmune diseases, pregnancy is a risk factor and alloimmunity derived from pregnancy might be contributory. For instance, fetal microchimeric T-cell clones derived from women with systemic sclerosis can be expanded against maternal antigens and represents a potential pathobiological mechanism (10). By analogy, FMc could be primed in parous healthy women to recognize maternal cancer antigens and impart allogeneic immune surveillance. Alternatively, in women who develop breast cancer, fetal immune tolerance to maternal antigens could result in failure of allogeneic immune surveillance. Direct evidence in breast cancer is not presently available, but there is evidence for a similar phenomena in the stem cell transplantation field where HLA disparity of the recipient from the donor's perspective correlates with reduced risk of recurrent malignancy (while being associated with graft-versus-host disease;

Table 3. Levels of FMc (male DNA) in breast cancer and control women

	Concentration (gEq/10^5)*
Total cohort, median (range)	
Breast cancer cases	0 (0–1.47)
Normal controls	0 (0–4.02)
Prior birth of a son, median (range)	
Breast cancer cases	0 (0–1.47)
Normal controls	0 (0–4.02)
Cancer cases with FMc (n = 5)	
Parous, known male child	0.60, 0.99,[†] 1.47[†]
Nulliparous, nulligravid	0.17
Nulliparous, gravid	0.12
Controls with FMc (n = 20)	
Parous, known male child	0.02, 0.02, 0.03, 0.06, 0.06, 0.06, 0.07, 0.11, 0.14, 0.20, 0.32, 0.43, 0.56, 4.02
Nulliparous, nulligravid	0.05
Nulliparous, gravid	0.06, 0.18, 0.89, 1.11, 1.59

*gEq/10^5 = genome equivalent number of male cells per 100,000 subject cell equivalents.

[†] Chemotherapy exposure before phlebotomy.

refs. 18, 19). If analogous to transplantation, excessive child-mother HLA sharing could result in FMc with less allogeneic immunity than when FMc is HLA disparate, and this subject merits exploration.

There are several limitations to our study. Although the approach provides quantitative results, which is important in studies of FMc as healthy women often have FMc, there are other possible sources of male DNA besides pregnancies that result in the birth of a son. We found previously that women with miscarriages or induced abortion and sometimes nulligravid women have male DNA in peripheral blood (20). In nulligravid women, the most likely source is an unrecognized miscarriage. Other possibilities include from a vanished male twin or a blood transfusion (21, 22). None of our subjects had a known twin. Blood transfusion history was not available for all study subjects (none reported a transfusion, but a bias toward controls would not be expected). Another possibility is acquisition of cells from an older brother passed from an earlier to a subsequent fetus via the maternal circulation; this has not been described but would not be expected to explain differences observed in the current study. Nevertheless, a better attribution of the origin of male DNA would be achieved by Q-PCR for genetic polymorphisms known to be specific to a woman's children. We have described recently this approach (23) but it requires comprehensive familial polymorphism genotyping, which was not available (or possible) for the current studies. There was no suggestion in the current study that results were confounded by differences in reproductive history. Patients did not differ significantly from controls for parity or type of gravidity. Women in the two groups did not differ significantly for miscarriages and induced abortions, and age of women at the time of births, which is an established variable in breast cancer risk, was very similar in patients and controls. Breast cancer patients were somewhat older than controls. There was no suggestion that age was a confounding

Cancer Research

factor for prevalence or levels of male DNA in the current study. However, some control women may be expected to develop breast cancer and additional studies are indicated, as well as studies that include breast-feeding history, another recognized factor in breast cancer risk (15).

In summary, allogeneic persistent fetal cells have not been considered previously in protection from breast cancer, and the current studies serve as an initial test of whether the concept merits exploration. We investigated FMc for correlation with protection from breast cancer based on the observation that allogeneic cells in hematopoietic stem cell transplantation are capable of cancer immune surveillance. We found a significant decrease of FMc prevalence in breast cancer patients when compared with healthy women, consistent with the study hypothesis. Whether allogeneic immunity might result from fetal-

derived effector cells (e.g., T or NK cells) or processing of maternal antigens by fetal-derived APC (macrophages or dendritic cells) is a subject of potential future interest. To our knowledge, the current results provide the first indication that allogeneic FMc could impart a protective effect against breast cancer.

Acknowledgments

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