Dynamic Changes in Fetal Microchimerism in Maternal Peripheral Blood Mononuclear Cells, CD4+ and CD8+ cells in Normal Pregnancy

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ABSTRACT

Objective: Cell trafficking during pregnancy results in persistence of small populations of fetal cells in the mother, known as fetal microchimerism (FMc). Changes in cell-free fetal DNA during gestation have been well described, however, less is known about dynamic changes in fetal immune cells in maternal blood. We have investigated FMc in maternal peripheral blood mononuclear cells (PBMC) longitudinally across gestation.
Study design: Thirty-five women with normal pregnancies were studied. FMc was identified in PBMC, CD4+ and CD8+ subsets employing quantitative PCR assays targeting fetal-specific genetic polymorphisms. FMc quantities were reported as fetal genome equivalents (gEq) per 1,000,000 gEq mother's cells. Poisson regression modeled the rate of FMc detection.
Main outcome measure: FMc in PBMC.
Results: The probability of detecting one fetal cell equivalent increased 6.2-fold each trimester [Incidence Rate Ratio (IRR) 95% CI: 1.73, 21.91; $p = 0.005$]. Although FMc in PBMC was not detected for the majority of time points, 7 of 35 women had detectable FMc during pregnancy at one or more time points, with the majority of positive samples being from the third trimester. There was a suggestion of greater HLA-sharing in families where women had FMc in PBMC. FMc was detected in 9% of CD4+ (2/23) and 18% of CD8+ (3/25) subsets.
Conclusions: FMc in PBMC increased as gestation progressed and was found within CD4+ and CD8+ subsets in some women in the latter half of gestation. A number of factors could influence cellular FMc levels including sub-clinical fetal-maternal interface changes and events related to parturition. Whether FMc during pregnancy predicts persistent FMc and/or correlates with fetal-maternal HLA relationships also merits further study.

Bi-directional trafficking of cells between the fetus and mother occurs routinely during normal pregnancy with long-term persistence of fetal cells in the mother and maternal cells in the child [1–3]. These small cell populations are known as microchimerism (Mc) and may also arise from cell transfer between twins or following transfusion, transplantation, or abortion [4–6]. How a pregnant woman maintains tolerance is only partially understood and is of further interest in that a pregnant woman already harbors Mc from her own mother prior to acquiring Mc from her fetus (fetal Mc; FMc). Acquisition of a new cell population (FMc) could potentially induce an adverse response similar to graft-versus-host disease following a donor leukocyte infusion in a transplant patient depending on cell numbers and phenotypes [7]. Cell-free fetal DNA (cffDNA), derived from apoptotic trophoblast debris, lacks surface human leukocyte antigens (HLA); however, cellular FMc can be expected to express incompatible HLA molecules.

Although the kinetics and quantity of cffDNA in maternal peripheral blood over the course of gestation have been well described, dynamic changes in cellular FMc during pregnancy and postpartum are less well known [8]. cffDNA is detectable by 4–5 weeks [9], as one might expect from a trophoblast source, increases in quantity during pregnancy and is rapidly cleared within hours

after delivery [10,11]. While levels of cffDNA may be considered a measure of placental size and/or function due to syncytio-trophoblast shedding, the source of cellular FMc is less clear. If trafficking into maternal blood occurs through microscopic tears in the maternal–fetal trophoblast interface, cellular FMc might increase later in gestation. Alternatively, fetal cells might increase earlier in gestation if they differentiate from pluripotent cells transferred during placentation. Although the precise composition of cellular FMc during pregnancy is not known, early pioneering studies first identified FMc in lymphocytes isolated from the peripheral blood of pregnant women [12–14]. In adults, FMc that persists after delivery has been identified in hematopoietic cells of all lineages (including T cells) and in many organs expresses a tissue-specific phenotype (i.e. hepatocytes, myocytes) [15,16].

In this study, we focused on quantifying cellular FMc in PBMC collected longitudinally before, during, and after normal pregnancies, employing real-time quantitative polymerase chain reaction (qPCR). Identifying FMc in this study involved individually identifying a unique genetic polymorphism in the fetus distinct from its mother and grandmother, which we could target by selecting from a panel of qPCR assays developed in our laboratory. The panel includes multiple HLA-specific qPCR assays, multiple qPCR assays for non-HLA genetic polymorphisms and a qPCR assay for a Y-chromosome specific sequence. We also assayed FMc within CD4+ and CD8+ T cell subsets isolated from maternal peripheral blood. We further examined the hypothesis that cellular FMc might be more prevalent when the fetal paternally-inherited HLA allele was similar to either of the mother's HLA alleles or to either of the grandmother's HLA alleles, the latter representing a previously acquired source of Mc in the pregnant woman's circulation. To prevent confusion when discussing HLA relationships in a three-generation family, we refer to the pregnant woman as the proband and her mother as the mother of the proband (MP).

1. Methods

1.1. Study Population

We prospectively recruited pregnant women and women planning a pregnancy between November 1995 and December 2008. The study was approved by the institutional review board of Fred Hutchinson Cancer Research Center and informed consent was obtained from all subjects. Blood draws were requested from all subjects prior to pregnancy, each trimester, and after delivery. Cord blood was obtained at delivery. Mouthwash samples, buccal swabs, or blood draws were requested from the MP and all prior children for DNA extraction and genotyping. After delivery, medical records and/or a self-reported questionnaire were reviewed for demographic variables, ethnicity, reproductive and transfusion history, dating of the pregnancy, pregnancy complications, and maternal and fetal outcomes. Because the intent of this study was to describe changes in cellular FMc during the course of normal pregnancy, exclusionary criteria were carefully applied after delivery in order to restrict study results to women with a normal pregnancy. Women anticipating a normal pregnancy were recruited. Exclusion criteria were applied after final delivery records were obtained and included multiple gestation, preterm birth (spontaneous or indicated), gestational hypertension, preeclampsia, diabetes, placenta previa, or placental abruption. Eight women were excluded, mainly for preterm birth. Additionally, if DNA could not be obtained from family members or a unique qPCR assay could not be identified to distinguish fetal cells from the mother or cells of any siblings, the pregnancy was not eligible for inclusion. Some women enrolled in our study for more than one pregnancy; for these subjects only samples from the first pregnancy were included in the current analysis.

1.2. Isolation of PBMC, CD4+ and CD8+ Subsets

Peripheral venous blood samples were drawn into acid citrate dextrose solution A-vacutainer tubes. PBMC were isolated from whole blood by Ficoll Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation at a density of 1.077 g/ml. CD4+ and CD8+ cells were positively selected with magnetic-assisted cell sorting separation columns (Miltenyi Biotech, Auburn, CA). Purity of depleted cells for CD4+ or CD8+ was determined by staining aliquots with anti-CD4 PE and anti-CD8 FITC (Becton Dickinson, San Jose, CA) and analyzed on a BD FACScan Flow Cytometer (97% of CD4+ subsets were ≥98% pure, 88% of CD8+ subsets ≥95% pure). The two CD4+ subsets with purity <98% were 96% and 94% pure. The three CD8+ subsets with purity <95%, were all 94% pure.

1.3. DNA Extraction

Genomic DNA was extracted from whole blood, PBMC, CD4+ or CD8+ cells using Wizard Genomic DNA Purification Kits (Promega, Madison, WI) according to manufacturer's instructions. DNA was extracted from mouthwash specimens using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN) or from buccal swabs using the BuccalAmp DNA Extraction Kit (Epicenter Biotechnologies, Wisconsin, USA).

1.4. HLA Typing and Quantification of Microchimerism by Real-time qPCR

A panel of HLA-specific and other genetic polymorphism-specific qPCR assays were employed to identify and quantify Mc in this study, which were developed and validated in our laboratory [17–19]. In order to choose an assay to detect FMc in a particular family, DNA-based HLA typing was conducted to determine DRB1, DQA1, DQB1, and B alleles, and results were used to identify a target unique to the fetus for amplification and quantification of FMc [17]. For some study subjects, DQA1 alleles were inferred based on allele-level typing results for both DRB1 and DQB1. Other genetic polymorphisms were typed by conventional PCR including glutathione S-transferase Mu 1 (GSTM1), glutathione S-transferase theta 1 (GSTT1), antithrombin III long (AT3-Long) antithrombin III short (AT3-Short), thyroglobulin insertion (Tg-insertion) and thyroglobulin deletion (Tg-deletion) [18].

HLA-specific qPCR assays used in this study have been previously reported including: DRB1*01, DRB1*15/16, DRB1*03, DRB1*07, DRB1*08, DRB1*14, DRB4*01, DQA1*03, DQA1*05 DQB1*02, DQB1*03, DQB1*06, and HLA-B*44 [19]. Sensitivity was determined by testing dilutions of the equivalent DNA of cells homozygous for the HLA-specific polymorphism in a background of cells negative for that HLA poly-morphism. All HLA-specific primers had a similar sensitivity of 1 cell in a background of ∼20,000 negative cell equivalents (sensitivity 0.005%). Specificity of the HLA-specific assays was confirmed by testing primers and probes against an extended panel of well-characterized HLA cell lines from the 13th International HLA Working Group representing all HLA–DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, and B allele groups. The non-HLA qPCR assays target sequences specific to GSTM1, GSST1, the long and short allelic polymorphisms of ATIII, and the thyroglobulin gene insertion and deletion [18]. Alternatively, the Y-chromosome-specific DYS14 assay was used to identify FMc from the first known male child [17]. All our qPCR assays, HLA-specific, non-HLA genetic polymorphisms and DYS14 have been developed to the same level of sensitivity and are capable of detecting one cell equivalent of Mc in 20,000 cells tested.

Six to twelve aliquots of DNA from PBMC and cellular subsets were tested from each blood draw, with each aliquot not exceeding the DNA genome equivalent (gEq) of 25,000 cells. For ease of expression, DNA quantities were reported as the DNA genome equivalent number of microchimeric cells per 1,000,000 host cells by using a conversion factor of 6.6 pg DNA per cell [20]. To normalize results, total DNA tested was quantified, in the same plate, using primers targeting the β-globin house-keeping gene. Standard curves for β-globin (obtained from standard human genomic DNA [Promega]) and HLA or other genetic polymorphism-specific qPCR were run in parallel with each experimental sample. This ensured that the quanti-fication by β-globin for a known number of cell equivalents would give the same result irrespective of the primer used. Because each well is assumed to have a Poisson distribution of Mc, the genome equivalent number of Mc cells was aver-aged combining information across aliquots of DNA as previously described [17]. Total host DNA tested per sample was considered acceptable if greater than 30,000 gEq and samples with fewer were excluded from this analysis. The mean total gEq tested was 104,372 (range 32,245 to 156,998).

1.5. Precautions to Minimize Contamination risk

False positive results are a concern with PCR-based methods, and risk of contamination may be of special concern for assays that target male DNA. A female technician conducted all experiments for which FMc would be detected by the DYS14 assay including DNA extraction and qPCR assays. DNA extractions and qPCR preparations were done under an ultraviolet (UV) light equipped safety hood, with UV run for 30 min between experiments. Aerosol-resistant pipette tips and clean gloves were always used. Isolation of DNA and setup of amplification reactions were accomplished in separate locations. The use of dUTP instead of dTTP and the addition of uracil-N-glycosylase in TaqMan Universal Master Mix were used for the prevention of carryover PCR product. Negative controls were included in each qPCR plate with no DNA and with background DNA from an individual not carrying the HLA polymorphism tested or a nulligravid female if testing for male DNA. Negative controls were consistently negative across all experimental plates. The optical detection system of the 7000 Sequence Detector also obviates the need to reopen reaction tubes after amplification.

1.6. Statistical Analysis

Each sample obtained during pregnancy was assigned a gestational age and trimester in pregnancy (1st trimester: <14 weeks, 2nd trimester: 14–28 weeks, 3rd trimester: >28 weeks). Each subject's expected delivery date (EDD) was determined from the physician's best clinical estimate, which was either based on the subject's