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Increased fetal cell trafficking in murine lung following complete pregnancy loss from exposure to lipopolysaccharide

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Narrative abstract

To determine if chemically-induced miscarriage affects fetomaternal trafficking in a mouse model, we measured the amount of fetal DNA present in various maternal organs by PCR amplification following exposure to lipopolysaccharide (LPS). As the frequency of fetal cells and the number of animals with detectable microchimerism following LPS injection were significantly increased, particularly in lung tissue compared to controls, with no signs of an inflammatory response, we conclude that LPS-induced miscarriage results in increased murine fetomaternal cell trafficking, supporting a relationship between fetal loss and the establishment of fetal cell microchimerism.

Capsule—Chemically-induced miscarriage significantly increases fetal cell trafficking in murine maternal lung compared to controls, supporting studies in humans that suggest a relationship between fetal loss and microchimerism.

In 2001, we demonstrated that a large fetomaternal transfusion of nucleated cells occurs following elective termination of pregnancy (1). This discovery led to further investigation of specific fetal cell types that may be acquired by the pregnant woman due to the increased fetomaternal hemorrhage following termination, such as mesenchymal stem cells (2). We then performed a systematic review of all studies of fetal cell trafficking in which individual pregnancy histories (i.e. incomplete gestations as well as live births) were reported. The results showed that fetal loss may be an important factor for the engraftment of microchimeric fetal cells in maternal tissue (3). The conclusion that fetal cells may be harbored in maternal organs following pregnancy loss has also been suggested by others (4).

A limitation of the study by Khosrotehrani and colleagues (3) was that the pregnancy histories did not distinguish between fetal loss due to miscarriage versus elective termination. This distinction may have biologic significance, because in miscarriage the fetal cells may be dead or dying at the time of transfusion, whereas the fetal cells are alive at the time of termination.

Nevertheless, these and other data (5) suggest that fetal cells retained from an aborted fetus can repopulate maternal organs, and that a history of fetal loss plays a role in the development of microchimerism. However, there are obvious limitations to any prospective study of fetal cell trafficking in humans following termination of pregnancy, particularly in the assessment of solid maternal organs.

Using an animal model of mating between a wild-type female and a transgenic male that we have developed and optimized in our laboratory (6-9), our objective was to determine if fetal cell trafficking in mice increases following fetal loss, as suggested by previous studies in humans. We used the chemical lipopolysaccharide (LPS) to induce termination of pregnancy. The cytotoxic effect of LPS in mice likely occurs through the production of nitric oxide by decidual mononuclear cells (10). Subsequent research has shown that the process of apoptosis, possibly through the involvement of p53 and bcl-2, may mediate pregnancy loss in the mouse (11). Others have investigated additional characteristics of LPS exposure that result in fetal loss, such as the minimum dose of LPS required to induce pregnancy loss (12), the role of inflammatory cytokines (13), and the role of prostaglandins in the mechanism of LPS-induced fetal resorption (14). However, the association of fetal cell trafficking and termination of pregnancy caused by LPS exposure has yet to be investigated.

The Institutional Animal Care and Use Committee (IACUC) of the Tufts University School of Medicine Division of Laboratory Animal Medicine (DLAM) approved the protocol described here. All institutional guidelines regarding the ethical use and care of experimental animals were followed. The enhanced green fluorescent protein (GFP) transgenic C57BL/6-Tg(ACTB-EGFP)10sb/J (CAG)(stock no. 003291) male and wild-type C57BL/6J female mice (stock no. 000664) were purchased from Jackson Laboratories (Bar Harbor, ME). The males were hemizygous for the GFP transgene; thus, ~ 50% of the pups inherited the transgene, which was used to track the presence of fetal cells. Eight week-old virgin female mice were used for all experiments.

We analyzed maternal weight gain following mating to determine the earliest day in gestation in which this is a reliable predictor of pregnancy. Sixteen wild type female mice were weighed daily after mating with transgenic males until delivery of pups, or until approximately 21 days following mating if delivery did not occur. Maternal weight gain through pregnancy was compared as a percentage of each animal's pre-mating baseline weight (Fig 1A). Daily weight gain was used to predict pregnancy by means of linear regression analysis. We found that weight gain is predictive of pregnancy at a statistically significant level as early as day 5 of gestation ($p = 0.02$). As expected, the confidence in weight gain as a predictor of pregnancy increased with gestation. Following day 10 of gestation, weight gain was more highly predictive of pregnancy, represented by a statistically significant increase over non-pregnant weight ($p = 0.008$ at day 10, $p = 0.006$ at day 11, $p = 0.005$ at day 12), which is similar to that reported by Hau and colleagues (15). To ensure optimal reliability, we selected day 14 of gestation for subsequent injection experiments (i.e. greater than 10% weight gain).

To determine the optimal dose of LPS to result in complete fetal litter loss, a total of 14 pregnant mice were injected intraperitoneally with 0.5 mL of PBS containing LPS at a concentration of either 1.0, 3.0 or 5.0 $\mu\text{g/mL}$. The final absolute amounts of LPS injected per mouse were 0.5 μg ($n = 4$), 1.5 μg ($n = 5$) or 2.5 μg ($n = 5$), respectively. Mice were injected with LPS on approximately day 14 of pregnancy, as determined by a minimum post-mating body weight gain of 10% (Fig. 1B). While the two lower doses resulted in an immediate weight loss (i.e. at day 15 of gestation), most animals recovered and continued to gain weight after day 16 of gestation. However, the highest dose administered (2.5 μg) resulted in complete pregnancy loss, as demonstrated by all mice maintaining their post injection weight loss for up to 7 days. While at the two lower doses the animals successfully delivered at least some pups, no live

pups were delivered by mice that received the highest dose. The complete loss of pregnancy in females exposed to this dose was confirmed by the presence of aborted implantation sites within the uterus (see Supplemental Figure 1).

To determine the level of fetal cell microchimerism in maternal organs following administration of the optimal dose of LPS (i.e. resulting in complete pregnancy loss) after confirming pregnancy based on weight gain, 16 pregnant female mice were injected intraperitoneally on day 14 with either 2.5 μ g of LPS suspended in PBS (n = 8) or with PBS alone (n = 8). All mice were sacrificed either 24 or 48 hours following injection. Immediately following sacrifice, blood was obtained by cardiac puncture. Heart, lung, liver, spleen, kidney, brain and uterine tissue were obtained by dissection. With the exception of blood, all organs were separated into three fractions, and DNA was extracted from each fraction for subsequent PCR amplification. Due to the low concentration of DNA extracted from peripheral blood, PCR was performed only once. The presence of fetal cell microchimerism in peripheral blood and solid tissues was measured by PCR amplification of the *gfp* transgenic sequence as previously described (7). Negative (i.e. no template and non-pregnant female) controls were included to assess for false positive amplification. All results are expressed as pg of *gfp* DNA (mean value among three replicate experiments from each organ) in 2,500 pg total genomic DNA, as determined by PCR amplification of the *apob* sequence (7). Mixed linear regression model was applied to account for replicates obtained from each tissue. It was also used to compare logarithmically transformed fetal cell numbers in peripheral blood and organs from mice exposed to LPS compared to controls (i.e. PBS only injection), as well as to assess the effect of time.

The frequency of fetal cell microchimerism was significantly elevated only in lung tissue following exposure to LPS compared to controls (means of 26.7 and 1.2 pg of *gfp* DNA in 2500 pg *apob* DNA, respectively) (p = 0.001) (see Supplemental Table 1). All other organs and peripheral blood showed no significant difference in the level of microchimerism. The number of animals with detectable fetal cells in lung tissue was also higher in mice exposed to LPS (7 of 8) compared to controls (4 of 8). The time of sacrifice (i.e. 24 vs. 48 hours after injection) was correlated to the frequency of fetal cell microchimerism following exposure to LPS, with a higher frequency of fetal cell microchimerism observed at the earlier time point (p=0.026). As seen in studies of fetomaternal trafficking in humans, there was significant quantitative variation in the levels of fetal DNA between mice (see Supplemental Table 1).

Due to the possibility that the increased frequency of fetal cells in lung tissue from mice might result from intraperitoneal injection of LPS generating an inflammatory response (16,17), a total of 14 pregnant female mice at approximately day 14 of gestation were injected intraperitoneally with either 2.5 μ g of LPS suspended in PBS (n = 7) or with PBS alone (n = 7). Following euthanasia, lungs were either inflated with 10% formalin into the trachea and removed from the chest cavity, or removed without inflation. After removal, whole lungs were placed in 10% formalin overnight and embedded in paraffin. Sections were cut at a thickness of 5 μ m, stained with hematoxylin and eosin, and examined blindly by two pathologists (L.R. and D.R.) for signs of inflammation, such as infiltration of polymorphic nucleated cells (e.g. neutrophils). Among all specimens that were analyzed blindly, there were no significant microscopic indications (e.g. cellular infiltration) of an inflammatory response following exposure to the dose of LPS (i.e. 2.5 μ g) that results in complete loss of pregnancy.

We have shown that exposure to LPS significantly increases the frequency of fetal cell microchimerism in the maternal lung. Due to the absence of a concomitant inflammatory response in lung specimens following exposure to LPS, we conclude that the increased frequency of fetal cells in maternal lung results from increased fetomaternal cell trafficking following pregnancy loss. This supports data from studies in humans, which have shown that

a significant fetomaternal hemorrhage occurs following termination of pregnancy (1) and that fetal loss may be important for the engraftment of microchimeric cells in maternal tissue (3).

This finding of increased fetal cell trafficking in the maternal lung following fetal loss is also interesting when examined in the context of other studies that have shown that the lung is the most common site of significant levels of fetal cells during normal pregnancy. For example, we have shown that lung typically has at least an order of magnitude more fetal cells than all other organs, including spleen and liver (6,8). These results have led us to suggest that microchimeric fetal cells may originate from blood flow through the uterine vein into maternal pulmonary arteries, resulting in the highest frequency of fetal cells being found in the lung. We also hypothesized that the high frequency of fetal cells in lung tissue may also reflect the high rate of blood flow through lung tissue. It is also possible that the expansion of specific fetal cell types that results from chemical exposure may be a result of a “receptive” maternal microenvironment.

Overall, the results presented here confirm that a single, low dose of LPS induces complete pregnancy loss in mice, as reliably measured by percent change in maternal body weight, and that this pregnancy loss results in an increased frequency of fetal cells in pregnant mice, specifically in the maternal lung. Additional studies are needed to investigate the specific fetal cell phenotypes involved.

The endpoint of this study, real-time PCR amplification of *gfp*, does not distinguish between living cells and cellular debris or cell-free DNA. This is significant because the maternal lung is expected to be the first capillary bed encountered after venous drainage from the uterus, and would therefore accumulate fetal DNA from living cells and cellular debris. However, previous studies from our laboratory using stereomicroscopy and flow cytometry suggest that the DNA we have observed likely derives at least in part from living cells that are the source of microchimerism (7,9).

A potential limitation of this study is that the mouse placenta, while hemochorial, is slightly different anatomically from the human placenta. Nevertheless, all studies of microchimerism using animals with hemochorial placentas have shown similar general trends (18-21). The study presented here cannot be performed in humans. Although LPS-induced loss is different from human miscarriage and elective termination, the fact that both scenarios increase fetomaternal trafficking is likely to be biologically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Graph depicting the percent change in body weight of wild-type female mice following mating with transgenic males. A value of 1.00 on the Y axis represents each mouse's baseline weight prior to mating. Solid lines through data points indicate females that ultimately delivered live pups, while dotted lines through data points represent females that did not become pregnant. The horizontal dashed line at 1.10 represents the percent change in body weight (~10 %) that was exceeded when pregnancy was retrospectively confirmed by the delivery of live pups. **B)** Graph depicting the average percent change in body weight of wild-type female mice following mating with transgenic males and exposure to different amounts of LPS suspended in PBS. Injections were performed at day 14. Circles represent data points from females

exposed to 0.5 μg of LPS (with thick solid line), squares represent data points from females exposed to 1.5 μg of LPS (with dotted line), and triangles represent data points from females exposed to 2.5 μg of LPS (with thin solid line). The horizontal dashed line represents the percent change in body weight ($\sim 10\%$) that is exceeded when pregnancy is confirmed to occur.