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Effect of selective fetectomy on morphology of the mouse placenta

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ABSTRACT

Introduction: Placental examination is recommended when genetic mutations cause fetal lethality in mice. But how fetal death alters histomorphology of the surviving mouse placenta is not known.
Methods: Placentas were examined at E17.5 after fetectomy of 1–2 fetal mice per pregnancy at either embryonic day (E) 15.5 (N = 8; Fx-2 group) or E13.5 (N = 5; Fx-4 group), which left 12 ± 2 surviving fetuses per litter.
Results: Fetectomy caused no changes in placental weights and no increases in placental hypoxia (pimonidazole staining). The size and cell morphology of the decidua and junctional zone regions were unchanged and, in the Fx-2 group, these regions became significantly *less* hypoxic. Significant changes in labyrinth volume included a 30% increase in the Fx-2 group and in both groups, a >50% decrease in % fetal blood space and >40% increase in % labyrinth tissue. Maternal blood sinusoid volume was unchanged. Cell death in the labyrinth was significantly increased (22-fold increase in TUNEL staining) whereas placental mRNA expression of the proliferation marker *Mki67* was unchanged. mRNA expression of *sFlt1* and *Prl3b1* (mPL-II) was unchanged in the labyrinth and junctional zone tissues in the Fx-2 group and in whole placental tissue in the Fx-4 group.
Discussion: Placental examination of the junctional zone and decidual regions after spontaneous fetal death in late gestation is likely to yield useful phenotypic information and abnormalities that may contribute to fetal death. In contrast, labyrinth abnormalities including increased tissue volume and reduced fetoplacental vascularity may not be due to genetic perturbation nor predate fetal death.

1. Introduction

The placenta continues to function for days or weeks after fetal death in primates and other animals [1–3]. Nevertheless, when intrauterine fetal death is induced in humans and rats, placental abnormalities progressively emerge [4–6]. Therefore, when examining the placenta after intrauterine fetal death from unknown causes, it is important to question whether placental abnormalities predated, and potentially contributed to fetal death, or whether they were a consequence of fetal death. Currently, this question is of particular interest in mice because death occurs prenatally in ~30–50% of mice carrying lethal mutations [7,8]. A functional placenta is critical to embryonic and fetal survival [9]. Thus the recommended procedure for comprehensive phenotyping of embryonic lethal genetic mutations includes examination of the placenta by gross morphology and histology of a midline placental section [8].

In the current study, we asked how placental histomorphology in mice is altered by the loss of fetal factors caused by fetal death in a subset of the litter in wild type pregnancies. Most of the litter remained alive to model pregnancies carrying only a few embryonic lethal homozygous mutants which is typical in heterozygous intercrosses. Wild type pregnancies were used so that fetal death was the only factor responsible for changes in the placenta. Experiments were performed in late gestation (greater than embryonic day (E) 13.5), and thus pertain to the mature placenta [10]. Results of this study provide knowledge necessary to evaluate post-lethality placental phenotyping, as well as providing insight into the importance of fetal factors in promoting maturation of the placenta in mice.

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2. Methods

Experiments were approved by the Animal Care Committee of the Toronto Centre for Phenogenomics and were conducted in accord with guidelines of the Canadian Council on Animal Care. Mice were CD-1 [11] and E0.5 was the morning a vaginal plug was detected.

To induce fetectomy, two fetuses received an intracardiac injection of KCl (50 μL of 4.2 μg/μL KCl in ddH_2O) in each pregnancy at either E13.5 (N = 5; Fx-4 group) or E15.5 (N = 8; Fx-2 group) in isoflurane-anesthetized mice under ultrasound guidance (Vevo2100 VisualSonics, Canada). One centrally located fetus, which was in a suitable orientation for ultrasound imaging and fetal cardiac microinjection, was selected on the left and on the right side of the maternal abdomen. Fluorescent microspheres (Fluoresbrite YG Microspheres, Polyscience) in the cardiac injectate positively identified injected fetuses at necropsy. 85% of injected fetuses died. All that died were included in the study.

2.1. Tissue collection

After euthanasia at E17.5, fetuses and placentas were collected and weighed. From each pregnancy, a maximum of one fetectomy placenta was collected for histology and one for gene expression analysis. Littermate placentas in the same horn, not immediately adjacent to the fetectomy site, were collected as controls. In the Fx-2 group, placental anatomy was sufficiently preserved to permit microdissection and collection of flash frozen samples enriched for junctional zone or labyrinth for mRNA analysis using published methods [12]. In the Fx-4 group, whole placentas were collected for mRNA analysis. In both groups, whole placentas were immersion fixed in 4% paraformaldehyde for histology. In a separate series of pregnancies, controls were euthanized at E13.5 and E15.5 to record fetal and placental weights.

2.2. Histology

Fixed whole placentas were cut into serial 700 μm slices perpendicular to the chorionic plate, which were subsequently paraffin-embedded for 5 μm histological sections. Sections were stained for hematoxylin and eosin (H&E) to evaluate placental histomorphology, or immune-stained to detect CD34 for fetal endothelial cells (Abcam #ab8158) [13], F4/80 for macrophages (Abcam #ab6640) [14], Ly6G for neutrophils (IgG2a Cedarlane #CL8993AP) [15], and vimentin for mesenchymal cells (Abcam #ab92547) [16]. Midline sections from the Fx-2 group and from control littermate placentas were stained for terminal deoxynucleotidyl transferase (dUTP) nick-end labeling (TUNEL) (CMHD Pathology Services, Toronto Centre for Phenogenomics, ON, Canada) to detect the fragmented DNA of apoptotic, necrotic or autolytic cells [17].

Pimonidazole hydrochloride (Hypoxyprobe-1™, Chemicon, Temecula, CA; 60 mg/kg maternal bodyweight injected i.p 2 h before euthanasia) was used to detect tissue hypoxia [18] in histological sections (% area; Visiomorph, Visiopharm, Denmark).

2.3. Placental and labyrinth stereology

Volumes were estimated using the Cavalieri method (newCAST, Visiopharm, Denmark) on serial sections stained for CD34 at 40× magnification [19]. Areas were quantified using point counting on H&E stained midline sections at 10× magnification. TUNEL-stained midline sections of the placenta were analyzed at 40× to determine the number of TUNEL-positive cells per unit area (newCAST, Visiopharm, Denmark) [20].

2.4. Quantitative Real-Time PCR

RNA was obtained using the TRIzol method. DNA contamination was removed (Qiagen, #79254), RNA samples purified (Qiagen, #74204), reverse transcribed to cDNA (Life Technologies, #N8080234), and quantitative Real-Time PCR performed in triplicates (Sigma-Aldrich, #L6544). Gene expression data was normalized to the geometric mean of three internal control genes (*β-Actin, Hprt1, Tbp*) [21] using CFX Manager 2.0 (Bio-Rad Laboratories, USA) and then to the arithmetic mean of the control group.

2.5. Statistical analysis

Statistical significance was determined using one- or two-way ANOVA and Bonferroni post hoc test as appropriate (GraphPad Prism 5.0, San Diego, CA). If non-Gaussian, then the Kruskal-Wallis test was used. Statistical significance ($P < 0.05$) is indicated in figures using letters; bars that do not share the same letter are significantly different. If no significance, no letters are shown.

3. Results

Placental weight at E17.5 was not significantly altered in the Fx-2 and Fx-4 groups (i.e. groups with fetectomy either 2 or 4 days before E17.5) relative to weight at the age of fetectomy (Fig. 1A). At E17.5, fetal body weight in the Fx-2 group had significantly regressed to 43% of the weight of controls at E15.5 (Fig. 1B). In the Fx-4 group, fetal weight was not significantly changed from E13.5 ($P > 0.05$) but regressed similarly on average (−39% relative to controls at E13.5). Fx-2 fetuses were visibly smaller, tissues were white, and no blood was visible in the yolk sac or umbilical cord (Fig. S1). Fx-4 fetuses had further degenerated (not shown). The number of live fetuses per pregnancy at E17.5 was 12 ± 2 (range 8−16) as reported previously for this strain [22].

In the Fx-2 group, there were significant increases in total placental area (+18%) (Fig. 2A) and in labyrinth area (+28%) (Fig. S2) at the placental midline. There were no significant changes in decidual or junctional zone areas (Fig. S2). Volumetric analysis of the labyrinth using sections immuno-stained for CD34 (endothelial cell marker) revealed significantly increased labyrinth volume in the Fx-2 (+30%) but not the Fx-4 (+8%) group (Fig. 2B). Nevertheless, in the labyrinth, mRNA expression of the proliferation marker *Mki67* was not significantly increased in the Fx-2 group (Fig. S3 A).

Histomorphology of the labyrinth region was somewhat abnormal in the Fx-2 group and strikingly abnormal in the Fx-4 group (Fig. 3); abnormalities included collapsed fetal blood vessels (Fig. 3H,I), maternal sinusoids that were variably dilated (Fig. 3B,C), and the boundary between the labyrinth and junctional zones became less distinct (Fig. 3B,E and Fig. 3C,F).

In the labyrinth, fetectomy reduced CD34 staining, suggesting a reduction in fetoplacental endothelial cells (Fig. 4B,C), and caused fetal blood vessels to collapse (Fig. 4E,F). The percent labyrinth volume occupied by fetal blood space was significantly reduced to less than half that of E17.5 controls in both the Fx-2 and Fx-4 groups (Fig. 2C). This was balanced by a significant increase in the proportion of tissue space in the labyrinth (Fig. 2C). Tissue space appeared to include more clusters of mesenchymal cells, marked by vimentin-positive staining (Fig. 4H,I), and more spongiotrophoblast-like cells (Fig. 3F). TUNEL staining in the Fx-2 group (Fig. 5) revealed clusters of TUNEL-positive cells around fetal blood spaces (Fig. 5F). Overall, the number of TUNEL-positive cells per unit area of labyrinth was significantly increased by 22-fold (Fig. 5G). TUNEL staining was not performed in the Fx-4 group.

Maternal sinusoids in the labyrinth appeared relatively normal although some were visibly dilated, and this was more striking in