IGF-I Overexpression Causes Fetal Loss during Placentation in Mice

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Abstract. To understand the role of IGF-I in murine pregnancy, we studied the reproductive performance of IGF-I overexpressed mice. Fetal loss occurred only in the transfected uterine horn during Day 10–15 of pregnancy. The placenta appeared healthy until Day 10 of pregnancy. From Day 12, the decidua basalis of the transfected horn increased in thickness. The vascular lumen was expanded, and most of embryos were dead. Uterine Natural Killer cells did not undergo apoptosis from Day 10 to Day 15 when they usually go through apoptosis. Thus, it is likely that IGF-I plays a role in the decidual formation through regulation of uNK cells. This is the first report to demonstrate that IGF-I overexpression can cause fetal loss during murine placentation.

Key words: Insulin-like growth factor-I, Fetal loss, Overexpression, Uterine natural killer cell, Apoptosis

In rodents, decidualization occurs in response to the implanting conceptus or to artificial stimuli. The decidual reaction involves a spatially coordinated progression of proliferation and differentiation of endometrial stromal cells [1, 2, 3]. Information is limited on the role of insulin-like growth factor (IGF) -I in decidualization, but the pattern of IGF-I expression in the mouse uterus during peri-implantation correlates with the maternal placenta development and the fetal growth [4, 5]. Between Day 5 and Day 6 of pregnancy, there is a rapid induction of IGF-I mRNA in uterine stromal cells underlying the myometrium [4]. IGF-I has metabolic, mitogenic and differentiative actions in the fetal tissues and the placenta [5]. These results suggest that IGF-I plays a role in differentiation of the endometrial stroma cells to the decidual cells. Decidual cells are known to produce various factors including IL-15, TGF-β, HGF and extracellular matrix that regulate the maternal placenta development [6, 7, 8]. On the other hand, uterine NK (uNK) cells, specific lymphocytes found throughout pregnancy, differentiate with the progress of placental development and are known to have significant function in the maintenance of pregnancy through stimulating angiogenesis in the placenta [9, 10]. The uNK cells differentiate in situ from bone marrow-derived precursor cells that are small and agranular [11, 12]. Following implantation, the precursors increase in number and differentiate to mature granular cells [13, 14]. However, mechanism of uNK differentiation remains to be fully elucidated. We found that IGF-I overexpression caused fetal loss in pregnant mice, and that uNK cells looked unusual in the placenta with fetal loss. We address here the reproductive performance in IGF-I overexpressed mice and the histology of their placentae.
Materials and Methods

Animal
ICR virgin mice (CLEA JAPAN, Osaka) of 10 to 14 week old were used for the cDNA transfection to the uterus. Female mice were paired with ICR males. In all experiments, the morning of vaginal plug detection was designated as day 0 (D0) of pregnancy.

Tissue collection
Transfected mice were sacrificed on D7, D10, D12 and D15 of pregnancy under anesthesia condition, and fixed by perfusion of Bouin’s solution for morphological and immunohistochemical analyses. For PCR and real-time PCR, the placenta was separated into three parts; metrial gland (MG), decidua basalis (DB), and labyrinthine zone (LZ).

IGF-I cDNA transfection
Mouse IGF-I cDNA (cloned in our laboratory) was inserted into pcDNA3.1/V5-His vector (Invitrogen, Carlsbad, CA, USA) and transformed into E. coli using pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen). Purified plasmid DNA was diluted into 100 ng/µl with sterilized distilled water, which was these mixed with the Effectene Transfection Reagent (QIAGEN, Tokyo, Japan). On D3 of pregnancy mice received 30 µl IGF-I cDNA solution were injected into the left uterine horn of pregnant and non pregnant mice. pcDNA3.1/V5-His vector without IGF-I cDNA was used as control.

PCR
To confirm if the plasmid DNA was properly transfected, polymerase chain reaction (PCR) was performed using RNA samples extracted from IGF-I cDNA transfected, untransfected or intact uterine horn. RNA was obtained from tissue samples using Isogen (WAKO, Osaka, Japan). Samples of placental RNA were used as template for the synthesis of a multiple cloning site of the vector containing inserted IGF-I cDNA, following a standard protocol for reverse transcription-polymerase chain reaction (RT-PCR) using PCR High (TOYOBO, Osaka, Japan). The primers, 5’-ATTATGCTGAGTGATATC-3’ (T7 promoter) and 5’-TAGAAGGCACAGTCGAGG-3’ (BGH; bovine growth hormone reverse primer) were used. One microliter of reverse-transcribed cDNA was used. Thirty cycles under the following thermocycling conditions were used: 94 C for 1 min, 60 C for 1 min, and 72 C for 2 min. The amplified DNA fragments were analyzed by electrophoresis.

Real-time quantitative PCR
To provide a quantitative measure, we evaluated the level of IGF-I mRNA by reverse transcription and real-time PCR in the gene transfected, untransfected and intact placenta of D10 of pregnancy. Total RNA was isolated from the transfected, untransfected and intact uterine horns of D10 of pregnancy using Isogen. RT-PCR was performed using PCR High (TOYOBO, Osaka, Japan). Real-time quantitative PCR was carried out with the GeneAmp 5700 Sequence Detection System using SYBR Green Core Reagent (Applied Biosystems, Tokyo, Japan) as the detection format. The reactions were carried out for 40 cycles under the appropriate parameters for the primers and the fluorescence was measured every 15 sec at the end of each cycle to construct the amplification curve. The primers for mouse IGF-I were 5’-ACAGGCTATGGCTCCACGATCAT-3’ and 5’-TCCAGTCTCCTCAGATCACGC-3’.

Histological analysis
For light microscopy, paraffin sections (4 µm) were processed using periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin.

Detection of apoptosis by anti-ssDNA antibody
Detection of apoptosis using an antibody against single-stranded (ss) DNA was performed as previously described [15]. Deparaffinized sections were treated with 0.3% hydrogen peroxide at room temperature for 30 min. After washing with 10 mM PBS, the sections were incubated with 2% normal goat serum at room temperature for 15 min, followed by incubation overnight at 4 C with a rabbit polyclonal antibody against ssDNA (DakoCytomation, Kyoto, Japan). Biotinylated goat anti-mouse IgG (Vector, Burlingame, CA, USA) was used at a dilution of 1: 500 in 2% normal goat serum-PBS at room temperature for 30 min. After a 10 mM PBS wash, the sections were reacted with avidin-biotin complex (ABC Kit; Vector, Burlingame, CA, USA) at room temperature for 30 min and reacted with 3,3’-diaminobenzidine (Wako, Osaka, Japan) in 50 mM Tris-HCl containing 0.2% hydrogen peroxide. The sections
were counterstained with hematoxylin.

**Result**

**Confirmation of the IGF-I cDNA transfection and the overexpression**

The PCR revealed that IGF-I cDNA inserted vector was expressed only in the decidua basalis and the labyrinthine zone of the transfected uterine horn (Fig. 1A). Besides, the real-time quantitative PCR showed that IGF-I transcripts increased notably only in the transfected uterine horn (Fig. 1B), indicating IGF-I overexpression in the murine uterus. The same result was also confirmed by *in situ* hybridization (data not shown).

**Fetal loss in the IGF-I overexpressed mice**

Fetal loss was observed in mice uteri that had been transfected with the IGF-I expressed plasmid on D3 of pregnancy. The fetal loss occurred with the progress of pregnancy (Fig. 2). In the untransfected uterine horn (right side), fetal loss was not observed and embryos developed normally. On D7 of pregnancy, there were scarcely abortion sites but each implantation site was larger in size in the transfected horn than the untransfected horn (Fig. 3A). On D10 of pregnancy, some abortion sites were observed in the transfected horn (left side) (Fig. 3B). Furthermore, on D12 of pregnancy, most embryos were dead in the transfected horn (Fig. 3C), whereas fetal loss did not occur in the mice transfected with vector only (Fig. 3 D).

**Histological changes in the transfected site**

In the transfected site on D12 of pregnancy, the decidua basalis was thickened and blood vessels in the decidua were expanded (Fig. 4A). However, the placenta looked healthy, i.e., the placenta constituted of the metrial gland, the decidua basalis and the labyrinthine zone. The uterine artery was well developed. Such abnormal pathological changes were not observed in the untransfected horn (Fig. 4B) and intact horn (Fig. 4 C).

**Detection of apoptosis**

uNK cells did not undergo apoptosis in the decidual basalis of the transfected site on D13 of pregnancy (Fig. 5A, C), though uNK cells usually show apoptosis after D12. Indeed, uNK cells underwent apoptosis in the untransfected site (Fig. 5B, D).

**Discussion**

This study showed that fetal loss was induced by IGF-I overexpression from D10 to D15 of pregnancy. Fetal loss occurred only in the
transfected uterine horn. Between D10 and D12 of pregnancy, the decidua area of the transfected horn was thickened, the blood vessels were expanded, and the uNK cells did not undergo apoptosis.

IGF-I is known to play a role in the regulation of fetal and placental growth [2, 3]. Gene knockout experiments in mice have shown that Igf-1–/– mice have restricted growth [16]. It has been reported that IGF-I prevents apoptosis in rabbit blastocysts [17]. However, on the contrary, a high concentration of IGF-I is reported to trigger apoptosis in the mouse blastocyst [18]. The present study suggests that IGF-I accelerates proliferation of endometrial stromal cells and uNK cells in early pregnancy, and that IGF-I inhibits apoptosis of uNK cells in mid-late pregnancy. Each implantation site in the transfected horn on D7 of pregnancy increased in size, due to proliferation of endometrial stromal cells including uNK cells. uNK cell differentiation starts at the same time as decidualization [19, 20]. If IGF-I was one of the differentiation factors for uNK cells, IGF-I overexpression would cause the abnormal differentiation of uNK cells. Indeed, in the treated horn on D13 of pregnancy, uNK cells did not undergo apoptosis but increased in number. uNK cells play a crucial role in the modification and

**Fig. 2.** The number of implantation sites in the transfected and untransfected uterine horn. The fetal loss becomes apparent with the progress of pregnancy. Vertical bar shows mean ± SD. *p<0.05, vs. untransfected site.

**Fig. 3.** Pregnant uteri from the IGF-I overexpressed mice. On D7 of pregnancy, each implantation site was larger in size in the transfected uterine horn (left side) than the untransfected horn, and fetal loss is not observed (A). On D10 of pregnancy, some of the abortion sites (arrowheads) could be seen in the transfected horn (B). On D12 of pregnancy, most of embryos were dead in the transfected horn (arrowheads), while healthy embryos developed in the untransfected horn (C). Fetal loss did not occur in mice transfected with vector only (D).
development of the spiral artery and the decidua vessels [21]. Therefore, IGF-I overexpression during implantation could directly alter the characteristics and functions of the uNK cells, resulting in the expansion of the spiral artery and the vascular lumen. Alternatively, IGF-I overexpression may affect the decidual stromal cells. Decidual cells, stimulated by IGF-I, could produce some factors including TGF-β and extracellular matrix, which can change the characteristics of uNK cells [7, 22, 23].

This is the first report to demonstrate that IGF-I overexpression can cause fetal loss during murine placentation.

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References


