ISOLATION OF DEDUCED IMPLANTARION INDUCIBLE FACTORS IN MURINE UTERUS

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Introduction

Pregnancy is a complex process that abundant factors derived from the mother and the fetus cross each other. Dynamic morphological changes including invasion of trophoblasts, maternal vasculogenesis, and/or proliferation and differentiation of maternal lymphocytes occur in every mammalian uterus during pregnancy. Especially, the murine placentation process shows the most drastic time-dependent changes in morphology because they have very short gestation periods.

Murine pregnancy starts with inflammation reaction in the endometrium of the uterus in advance of the attachment of the blastcyste stage of the fetus to the uterine epithelium. Cytokines produced by lymphocytes should relate to the attraction of blastcystes to some specific regions of the uterus [1]. However, it remains unclear not only what cytokines are predominant during implantation window, but also what cell types of lymphocytes are present in this period. Among lymphocytes, uterine natural killer (uNK) cells are well defined in their appearance and disappearance during pregnancy. Progenitors of uNK cells are detectable in the uterus of 3-week old immature mice. These cells initiate proliferation and differentiation accompanied with this inflammation reaction in the endometrium. The uNK cell are one of the most potent candidates to synthesize the implantation inducible factors since these cells are the most dominant cells in the endometrium among all lymphocytes. However, it should be noted that uNK cells are under differentiation during implantation window.

Leukemia inhibitory factor (LIF), conoly-stimulating factor-1 (CSF-1), insulin-like growth factor (IGF-1), and epidermal growth factor (EGF) et al.[2-5] have predominated as implantation regulators. Later studies shown that these molecules are quite important for successful pregnancy. Restricted to implantation period, disruption of LIF gene caused the failure of attachment of the blastcyst to the uterine epithelium. On the other hand, LIF expresses in endometrium including uNK cells, not indicating specific localization. These has assumed that LIF was indispensable factors for implantation, however, do not directly attract the blastocyst. Therefore, implantation inducible factors have not been isolated yet and, not necessarily induced implantation of the blastcyst artificially.

In this study, exhaustive collection of implantation inducible factors was tried to isolate using the delayed implantation model (DIM) of mice by subtractive DNA hybridization and two-dimensional polyacrylamide gel electrophoresis with following proteome analysis. Then, the expression patterns of isolated molecules were examined by fluorescent-based quantitative PCR and *in situ* hybridization methods.

Materials and Methods

Preparation of the DIM

Ten-week-old C57BL/6J mice were used throughout this study. Eight females are treated under pentobarbital anesthesia. Ovariectomy was performed in all females on the day 0 (D 0) when the vaginal plug was recognized after mating. First injection of progesterone was carried out subcutaneously at D 0 at noon. Every 12 hours (12 h), the same amount of progesterone was administered. Twelve hours after the final injection of progesterone at noon of D 4, estrogen was injected intraperitonealy only to the experimental group (Half of the mice was treated with vehicle only (the control group).). Six hours later, uterus was excised from mice of the control and experimental group. Uterus from the mice belonging to each group were discriminated and mixed together. Mixed uterus in two groups were frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until use.

Subtractive DNA hybridization

Subtractive DNA hybridization was performed according to the technical manual from the supplier (PCR select cDNA subtraction kit, Clontech, CA). After final PCR, products are subcloned into the 1 ZAPII phagemid vector. For differential screening of subtractive DNA hybridization, probes derived from reverse-transcribed single-stranded DNA from mRNA of the control and the experimental groups were used.

Two D electrophoresis

For the 2D analysis, samples from the control and the experimental group were homogenized and sonicated in a sample buffer containing 5 mg/ml leupeptin, 200 mM PABSF, 1 mM pepstatin, and 1mM EDTA. The subsequent procedures of 2D gel electrophoresis were performed according to the technical manual of the supplier of the electrophoretic apparatus used (IPGphor and Multiphor 2, Amersham-Pharmacia). The differentially expressed spot by Coomasie staining was excised and followed by protein extraction, trypsin digestion, and MALDI-TOFMS. Then, measured molecular weight of the peptide fragments were searched for identification of the isolated spot at the internet site, *ExPASy Molecular Biology Server* (http://www.expasy.ch/)

In situ hybridization

The method for sense and anti-sense cRNA probe preparation has been described previously[6]. Deparaffinized sections were treated with 4% paraformaldehyde/0.1 M phosphate buffer, 0.1 M glycine/0.2 M Tris-HCl (pH 7.6), and then with 8 mg/ml proteinase K/50 mM Tris-HCl (pH 7.5)/2 mM CaCl$_2$, for 10 min each. Subsequently, sections were hybridized for 16 h at 45°C in the following solutions: 50% formamide, 5X standard saline citrate (SSC), 1X Denhardt's solution, 100 mg/ml heparin, 10 mM dithiothreitol, 100 mg/ml yeast tRNA, 10% dextran sulfate, and 5 mg/ml cRNA probe. Sections were then rinsed three times in 2X SSC for 30 min at 50°C and three times in 1X SSC at 50°C for 30 min. Sections were then incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody for 1 h at room temperature (RT) and then visualized using an enzymehistochemical method.

Results and Discussion

Novel three clones up-regulated in the uterus of the experimental group were isolated by subtractive DNA hybridization (Each clone is called C1, C2, and C3.). Time-dependent changes in amount of mRNA corresponding to these clones were examined among virgin mice, D 3, and D 3.5. All three mRNAs increased and reached maximum in amount at D 3.5. These mRNAs were already cloned, but biological analysis has never carried out. *In situ* hybridization shows that two mRNAs were localized specifically between mesometrial and anti-mesometrial region of the placenta. The C1 and C2 mRNA showed intriguing localization. Figure 1 in this script shows the distribution of C1 mRNA at the D3. Strong signals were recognized in the uterine glands and epithelium.
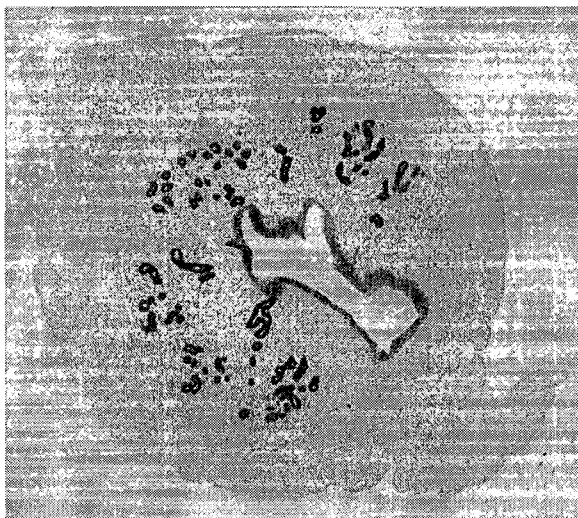
In the endometrium, progenitors of uNK cells shows a positive reaction. In this case, no uterine gland are present at the anti-mesometrial region. All uterine glands are positive. On the other hand, The C2 mRNA showed unique distribution. The C2 mRNA was localized in the same components of the uterus as C1 mRNA, except for the epithelium (no reaction was detectable in C2 mRNA). Notably, from transitional area between mesometrial and anti-mesometrial region, on the anti-mesometrial region, the signal of C2 mRNA gradually diminished. C2 protein might be a strong candidate to regulate the movement of the blastcyst. The period of the opening of murine implantation windows are from D3 to

D4. Production of C1 and C2 protein by uNK cells appears to be less than uterine gland. However, other molecules of uNK cells could regulate the production of C2 protein by the uterine glands. The analysis of uNK cells under differentiation remains to be determined. Further studies are required.

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