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Regulation of LIF Gene Expression by Interleukin-1 in the Mouse Peri-implantation Embryos and Uterine Endometrial Cells

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생쥐의 착상시기 배아와 자궁내막세포에서 IL-1에 의한 LIF 유전자 발현 조절

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이정복[1,2] · 김종월[1] · 오은정[2] · 양혜영[1] · 류형은[1] · 이지연[1]
계명찬[3] · 윤현수[1,2] · 김문규[1]

연구목적: 포유류의 착상은 배아가 모체의 자궁벽에 매몰되는 현상으로 부착과 침투 과정을 거쳐 진행되며, 이 과정은 스테로이드 호르몬, 성장인자, 세포점착분자, 그리고 cytokine 등의 상호작용으로 이루어진다. 이 시기에 Interleukin-1 (IL-1)과 leukemia inhibitory factor (LIF) 등이 발현되는 것으로 알려져 있다. 본 실험에서는 이들의 발현이 착상과정에 어떠한 역할을 하는지 그 상관관계를 알아보고자 하였다.

재료 및 방법: 착상 전후의 배아와 자궁내막세포에서 LIF 유전자의 발현양상과 IL-1β와 IL-1 receptor antagonist (IL-1ra)를 처리한 후 LIF 유전자의 발현양상을 역전사중합효소연쇄반응 (RT-PCR)을 통해 비교하였다.

결 과: 배아에서의 LIF 유전자 발현은 *in vivo*와 *in vitro* 모두에서 상실기와 포배기에 발현되었고, 자궁내막에서는 임신 1일과 4일째에 발현되었는데, 상실기보다는 포배기에, 그리고 임신 1일보다는 착상시기인 4일째의 자궁내막세포에서 발현양이 많은 것으로 나타났다. 자궁내막세포를 배양한 경우 LIF 유전자는 *in vivo*에서의 발현양상과 동일하게 임신 1일과 4일에 발현되었으며, 배양액에 IL-1β (500 pg/ml)를 처리하였을 경우 LIF 유전자가 초기 임신 (1～5일) 중 발현되는 것으로 나타났다. 2-세포기 배아의 배양시에 IL-1β를 처리한 경우 8-세포기부터 LIF 유전자가 발현되었으며, 또한 IL-1ra (60 ng/ml)를 배양액에 첨가하였을 경우에는 임신 1일째 자궁내막에서는 LIF 유전자가 발현되지 않은 반면, 임신 4일째의 자궁내막세포와 상실기, 포배기 배아 모두에서 LIF 유전자 발현이 감소하는 경향을 보였다.

결 론: 이러한 결과는 착상 전후 배아와 자궁내막세포에서 IL-1에 의해 LIF 유전자 발현이 조절되며, 그 결과 착상에 영향을 줄 수 있다는 것을 의미한다. 또한 배아와 자궁내막세포에서 IL-1이 LIF 유전자 발현에 영향을 주는 것으로 보아 착상을 위해 IL-1과 LIF의 상호작용이 중요한 요인이라는 것을 확인할 수 있었다.

Key Words: Implantation, IL-1, LIF, RT-PCR

Implantation begins when the loss of the zona pellucida leads to attachment of the blastocyst to the uterine epithelium. Thereafter, the trophoblast passes through the epithelium and into the underlying the stroma, described as invasion.[2,3,8] Embryo-endometrium interaction leading to implantation requires continuous and synchronous dialogue between these two compartments involving endocrine and paracrine regulators.[4,8,12~14,16,22,29]

It had been reported that various cytokines are involved in the immune response, implantation, decidualization, steroid secretion, and embryo development. Especially, it is becoming increasingly evident that cytokines play a crucial role in the process of implantation. The factors involved in the regulation of implantation in mice have been not fully understood, while recent studies were strongly suggested a critical role for several autocrine/paracrine factors produced in the uterus, including colony stimulating factor-1 (CSF-1),[17,27] granulocyte-macrophage colony stimulating factor (GM-CSF),[11,19,27] interleukin-1 (IL-1),[22] interleukin-6 (IL-6), and leukemia inhibitory factor (LIF).[1,24]

LIF is a 45- to 56-kDa secreted glycoprotein[7,18,23] has multiple activities on various cell types *in vitro*.[1,25,28] In mouse, the principal sites of LIF expression are the endometrial glands of the uterus. Expression in the uterus is temporally restricted, with peak levels occurring at just prior to the onset of blastocyst implantation. LIF expression is under maternal control and not dependent upon the presence of the embryos in the uterus.[1,21] Direct evidence that LIF is an absolute requirement for implantation was obtained by the derivation of mice deficient in a functional LIF gene.[24]

IL-1β mRNA that regulated by ovarian steroids (esterogen, progesterone)[5] has been detected from 4-cell to blastocyst stage embryos, and 8-cell stage embryos cultured *in vitro*.[26] But IL-1β mRNA was detected from 4-cell stage embryos co-cultured with the uterine epithelial cells.[10]

In the uterine endometrium of pregnant mouse, IL-1β mRNA was increased from day 3 of pregnancy to make a peak at the time of implantation on day 4.[5]

In case of the LIF mRNA, a strong signal was detected in the cells of the endometrial glands located in the uterine endometrium on day 4 of pregnancy. Weaker signals were seen in the endometrial layer lining the uterine lumen on day 1-3, with a slightly stronger signal present on day 1.[1,15,29]

LIF production is regulated by steroid hormones and other cytokines including IL-1 and TNF-α. In human synovial fibroblasts,[9] lung fibroblasts,[6] and decidual cells,[20] LIF was up-regulated by IL-1α or IL-1β.

The aim of this study was to investigate the regulatory effects of IL-1β and IL-1ra on the LIF gene expression in the peri-implantation embryos and the uterine endometrial cells in mouse.

MATERIALS AND METHODS

1. Collection of the oocytes and embryos

ICR mice (Department of Biology, Hanyang University) were bred under the condition controlled environment with a 14 hours light: 10 hours dark cycle. The female mice (8 to 10 weeks old) were superovulated by intraperitoneal injection of 5 IU pregnant mare's serum gonadotrophin (PMSG, Sigma) followed by the injection of 5 IU human chorionic gonadotrophin (hCG, Sigma) 48 hours later. Females were paired overnight with ICR males (10 to 12 weeks old). Mating was confirmed with the presence of a vaginal plug.

The oocytes at Metaphase I (germinal vesicle, GV oocytes) were collected by ovarian follicle puncture and the oocytes at Metaphase II (ovulated oocytes) were collected from the ampullae of the oviducts at 18 hour after the hCG injection.

The embryos of 1-, 2-, 4-, 8-cell, morula, and blastocyst stages were collected from either

the oviducts or the uteri by flush with a fine Pasteur pipette at 20, 48, 56, 65, 72, and 96 hours after the hCG injection, respectively.

2. Culture of the embryos *in vitro*

Late 2-cell embryos were recovered from the oviducts by flush with a fine Pasteur pipette at 48 hour after the hCG injection and allowing the embryos to be dispersed into the KSOM medium.

Late 2-cell embryos were cultured in the medium drops with/without interleukin-1β (IL-1β, 500 pg/ml, R&D System) or interleukin-1 receptor antagonist (IL-1ra, 60 ng/ml, R&D System) under mineral oil (Sigma) in a plastic culture dish (60×15 mm, Corning) under the condition at 37℃, 100% humidified incubator (Cellstar, QJW 100), supplied with 5% CO_2 in the air.

The embryos of 4-, 8-cell, morula, and blastocyst stage were collected at 8, 17, 24, and 48 hours after the culture of the late 2-cell embryos, respectively.

3. Preparation of the uterine endometrial cells

To obtain the fresh uteri of pregnant mouse, female mice (8 to 10 weeks old) were synchronized by intraperitoneal injection of 5 IU PMSG followed 5 IU hCG 48 hours later. After mating confirmed with the presence of a vaginal plug, their uteri were removed at 24, 48, 72, 96, and 108 hours after the hCG injection. To collect the uterine endometrial cells, the uteri were treated in PBS containing collagenase (10 mg/ml) and incubated under the condition as above for 1 hour. After incubation, an equal volume of medium containing 10% fetal bovine serum (FBS) was added to the tube and the tube was shaken and transferred the supernatant to new 15 ml cornical tube to centrifugation. Thereafter the supernatant was removed and 5 ml fresh medium (Ham's F-10 supplemented with 10% FBS) was added to the tube, and the pellet was resuspended.

4. Culture of the uterine endometrial cells

Ham's F-10 (Gibco BRL) medium was used to culture of the endometrial cells. The process of resuspension with fresh medium (10% FBS) was followed by seeding on the culture dish coated with 0.1% gelatin (Sigma). When the viable endometrial cells attached on the culture dish, the culture medium supplemented 10% FBS was removed and the serum-free Ham's F-10 was added to the culture dish for 1 hour to wash out the remained serum. After washing step, the attached endometrial cells were cultured in fresh serum-free medium containg with/without IL-1β (500 pg/ml, R&D System) or IL-1ra (60 ng/ml, R&D System) for 48 hours.

5. Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was carried out with total volume 40 μl of DEPC-treated distilled water 21.7 μl, extracted RNA, 4 μl 10× PCR buffer, 4 μl 10 mM dNTP mixtures, 2.5 pmol oligo (dT) adaptor primer, 8 μl $MgCl_2$, 20 units RNase inhibitor, and 5 units AMV (Avian myeloblastosis virus) reverse transcriptase XL (Takara). RT reaction was carried out in the 4800 PCR thermal cycler (Takara) by using program with the following parameter: 42℃, 60 min; 99℃, 5 min. After the reaction was complete, samples were either directly used for PCR or stored at -20℃.

Polymerase chain reaction was conducted each 3 embryos or 50 cells. The reactions were carried out in 40 μl of 4 μl 10× PCR buffer, 4 μl 2.5 mM dNTP mixtures, 4 μl 25 mM $MgCl_2$, 1.5 units of Taq polymerase (Takara), 10 pmol each of the appropriate 3' and 5' primers (Bioneer), 23.7 μl DEPC-treated distilled water, and 4 μl of the RT product. After mixing all components in a 0.5 ml tube (Gene Amp thin-walled tube), the reaction mixture was covered with 70 μl mineral oil. PCR cycles were composed 1 cycle of 94℃ for 3 min and 40 cycles of 30 sec at 94℃, 30 sec at 65℃,

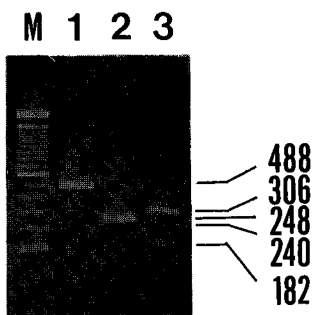

Figure 1. Agarose gel electrophoresis of amplified LIF transcript subjected to MspI and MboII digestion. M, 100 bp ladder; Lane 1, amplified and undigested sample; the major band is 488 bp in length. Lane 2, amplified and digested sample with MspI; there are two bands, 240 and 248 bp. Lane 3, amplified and digested sample with MboII; there are two bands, 182 and 306 bp.

30 sec at 72℃. The reaction was terminated at 72℃ for 10 min and quenched at 4℃. The PCR products were run on 2.5% agarose gel (Gibco BRL), stained with ethidium bromide (Et-Br), and photographed under UV light.

6. Restriction enzyme digestion of LIF PCR product

After completion of RT-PCR, PCR products were confirmed with restriction enzyme digestion. Enzyme digestion was carried out in 21 μl of 18 μl PCR products, 2 μl L buffer, and 1 μl (5 units) MspI or MboII (Takara) at 37℃ for 1 hour followed by 72℃ for 10 min as terminating the reaction. Electrophoresis was performed on 2.5% agarose gel and then agarose gel was photographed under UV light and verified.

RESULTS

1. Identification of the PCR products by restriction enzyme digestion

The identity of the diagnostic fragment was confirmed by restriction enzyme analysis. From the mRNA sequence, the diagnostic fragment should carry an MspI and MboII site, such that restriction enzyme digestion should give rise to

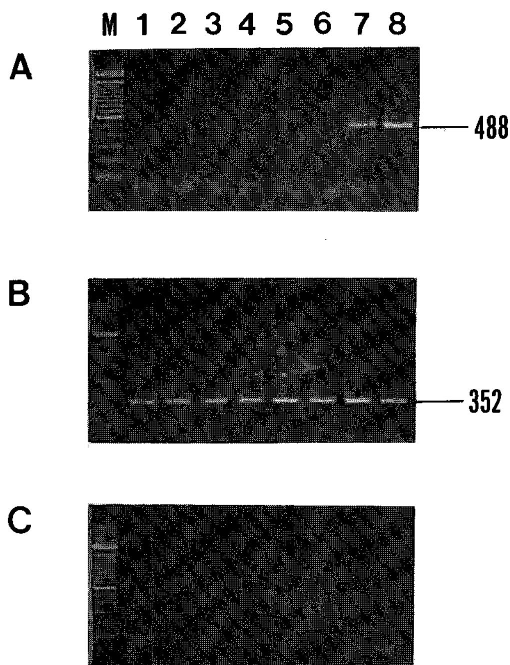

Figure 2. Expression pattern of LIF mRNA in the oocytes and the embryos *in vivo*. **A:** LIF. **B;** Hprt, positive control. **C;** negative control. M, 100 bp ladder; Lane 1, GV oocyte; Lane 2, ovulated oocyte; Lane 3, 1-cell; Lane 4, 2-cell; Lane 5, 4-cell; Lane 6, 8-cell; Lane 7, morula; Lane 8, blastocyst.

each two fragments. In the case of using the MspI, the fragments digested one 240 bp and the other 248 bp, and using the MboII, the fragments digested one 182 bp and the other 306 bp in the length (Figure 1). Two fragments (240 bp and 248 bp) were hard to distinguish as similar band in length, in the use of MspI.

2. Expression pattern of LIF gene in the embryos *in vivo* and *in vitro*

Using the RT-PCR, LIF mRNA was detected in the embryos from morula to blastocyst both *in vivo* and *in vitro*. This transcript was not detected from GV oocyte to 8-cell stage embryos *in vivo*, and not detected from 2-cell to 8-cell stage embryos *in vitro*. The amount of LIF mRNA was slightly increased at blastocyst stage compared with morula stage *in vivo* (Figure 2A) and *in vitro* (Figure 3A). RT-PCR was also performed for Hprt gene as a internal

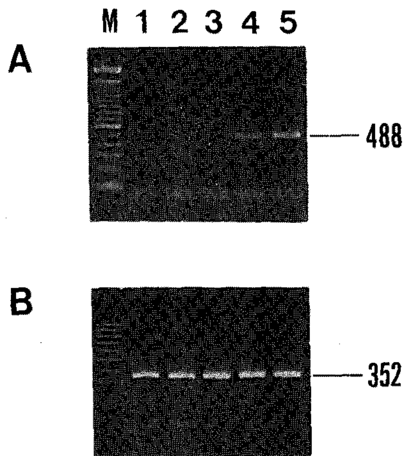

Figure 3. Expression pattern of LIF mRNA in the embryos cultured *in vitro*. **A:** LIF. **B;** Hprt, positive control. M, 100 bp ladder; Lane 1, 2-cell; Lane 2, 4-cell; Lane 3, 8-cell; Lane 4, morula; Lane 5, blastocyst.

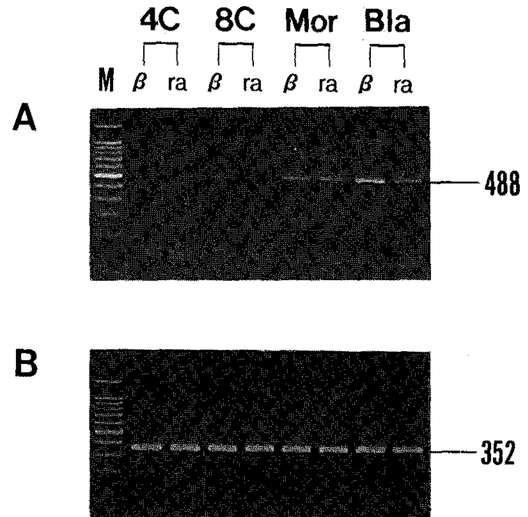

Figure 4. Expression pattern of LIF mRNA in the embryos (4-cell, 8-cell, morula, and blastocyst) cultured *in vitro* with IL-1β or IL-1ra. **A:** LIF. **B;** Hprt, positive control. M, 100 bp ladder; 4C, 4-cell; 8C, 8-cell; Mor, morula; Bla, blastocyst; β, IL-1β; ra IL-1ra treated embryos.

control for this procedure (Figure 2B, 3B), and performed for RNA from the embryos that had not been reversely transcribed as a negative control for a possible genomic DNA contamination (Figure 2C).

3. Expression pattern of LIF gene in the embryos treated with IL-1β or IL-1ra

LIF mRNA was detected from 8-cell stage to blastocyst stage in the embryos treated with IL-1β. But LIF mRNA was detected in the embryos from morula to blastocyst treated with IL-1ra. In IL-1β treatment group, the amount of LIF mRNA was increased slightly in the blastocyst stage in comparison with 8-cell and morula stage embryos. In contrast with treatment of IL-1β, the amount of LIF mRNA was decreased slightly in blastocyst stage embryos treated with IL-1ra (Figure 4A). RT-PCR was also performed for Hprt gene as a internal control for this procedure (Figure 4B).

4. Expression pattern of LIF gene in the uterine endometrial cells

LIF mRNA was detected in the endometrial cells of the day 1 (GD 1) and day 4 (GD 4) of pregnant mouse uterus *in vivo* (Figure 5A) and *in vitro* cultured in the medium supplemented 10% FBS for 24 hour (Figure 5B). The amount of LIF mRNA was abundant in GD 4 endometrial cells than GD 1 *in vivo* and *in vitro*. RT-PCR was also performed for Hprt gene as a internal control for this procedure (Figure 5C & 5D).

5. Expression pattern of LIF gene in the uterine endometrial cells treated with IL-1β or IL-1ra

LIF mRNA was detected from day 1 to 5 of the endometrial cells of pregnancy treated with IL-1β and was detected at only day 4 of the endometrial cells treated with IL-1ra (Figure 6A). RT-PCR was also performed for Hprt gene as a internal control for this procedure (Figure 6B).

DISCUSSION

It has been known that LIF expression is up-regulated by IL-1 in human synovial fibroblasts,[9]

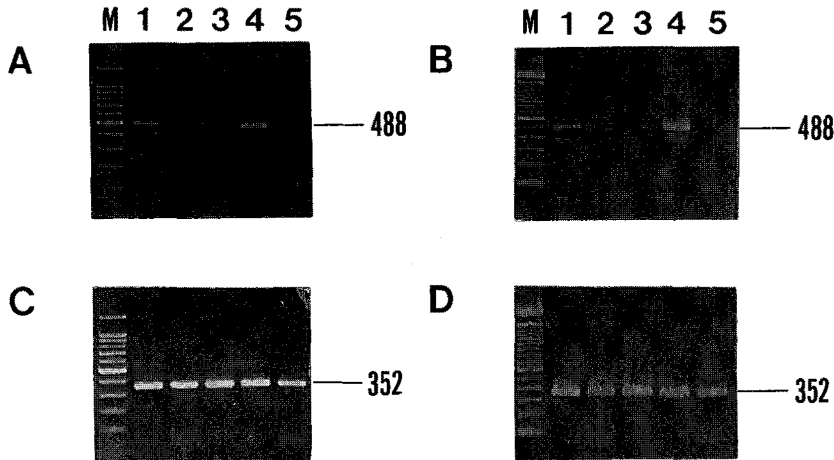

Figure 5. Expression pattern of LIF mRNA in the uterine endometrial cells of pregnant mouse. **A;** LIF (*in vivo*). **B;** LIF (*in vitro* cultured in the medium supplemented 10% FBS for 24 hour). **C;** Hprt (*in vivo*). **D;** Hprt (*in vitro*). C and D, positive control. M, 100 bp ladder; Lane 1, GD 1; Lane 2, GD 2; Lane 3, GD 3; Lane 4, GD 4; Lane 5, GD 5 uterine endometrial cells of pregnant mouse. GD, gestation day.

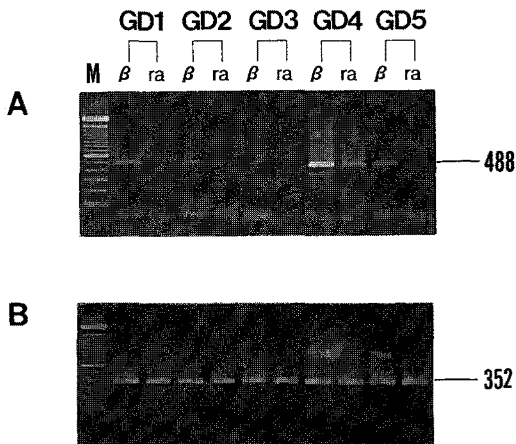

Figure 6. Expression pattern of LIF mRNA in the *in vitro* cultured uterine endometrial cells of pregnant mouse. **A;** LIF (cultured with IL-1β or IL-1ra). **B;** Hprt, positive control. M, 100 bp ladder; GD, gestation day; β, IL-1β; ra, IL-1ra treated endometrial cells.

lung fibroblasts,[6] and decidual cells[20] in a dose-dependent manner.

So far the maternal expression of LIF was one of the proven factor guarantying the implantation in mouse.[24] In this study, LIF mRNA expression was examined in the embryos and endometrial cells of pregnant mouse uterus treated with/without IL-1β or IL-1ra, to investigate whether LIF mRNA expression is also regulated by IL-1 in the embryos and endometrial cells during implantation process.

LIF mRNA was detected from morula to blastocyst *in vivo* (Figure 2A) and *in vitro* cultured in the medium alone (Figure 3A). LIF mRNA was detected from *in vitro* developed 8-cell to blastocyst treated with IL-1β. It is interesting that the early onset of LIF mRNA expression in 8-cell embryos by IL-1β. On the other hand, the LIF mRNA was decreased in morula and blastocyst treated with IL-1ra (Figure 4A). It suggests that temporal expression of LIF mRNA is up- and down-regulated by IL-1β and IL-1ra, respectively in the embryos.

In the endometrial cells treated with IL-1β, LIF mRNA was detected throughout the day 1 to 5 of pregnancy. On the other hand, LIF mRNA expression was decreased in the endometrial cells treated with IL-1ra (Figure 6A). Being assumed that the endometrial cell culture remained in their original status of in the uterus in the absence of exogenous IL-1, LIF induction in day 2 and 3 endometrial cell culture clearly documents the potentiation of LIF ex-

pression by IL-1. Moreover IL-1ra abolished day 1 expression of LIF and decreased day 4 expression of LIF. However, LIF expression was not completely blocked by IL-1ra in the day 4 endometrial cell culture (Figure 6A). It suggested that IL-1ra antagonized IL-1 signaling that leading to up-regulation of LIF expression.

In conclusion, it was found that the expression of LIF mRNA is controlled by IL-1 in both the embryos and uterine endometrial cells of pregnancy, similar with human synovial fibroblasts, lung fibroblasts and decidual cells. The regulation of LIF mRNA expression by IL-1 suggests that the crosstalk between LIF and IL-1 is essential for the successful implantation.

Acknowledgment

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