—Mini Review—

Immune System Cooperatively Supports Endocrine System-Primed Embryo Implantation

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Abstract: *Attachment to endometrial epithelium is an essential process for human embryos. Although it is widely accepted that this process is largely regulated by the endocrine system, the precise molecular mechanism(s) remains unclear. Recent evidence suggests that immune cells actively contribute to the establishment of embryo implantation. In accordance with this, we found that peripheral blood immune cells positively affect the differentiation of maternal endometrium to facilitate embryo implantation during early pregnancy. From these findings, we propose a novel concept that circulating immune cells are important regulators of embryo implantation. Lately, implantation failure in patients treated with in vitro fertilization and embryo transfer has received increasing attention. Based on our hypothesis, we have successfully developed a new therapy for implantation failure using autologous peripheral blood immune cells. These findings suggest that supportive mechanisms via the immune system facilitate embryo implantation and will be useful in the field of assisted reproductive technology.*

Key words: *Embryo implantation, Immune system, Maternal recognition, PBMC, Zona pellucida*

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Regulation of Embryo Implantation by Endocrine System

Embryo implantation in the uterus is an essential phenomenon for mammals. In general, it has been widely accepted that the endocrine system regulates endometrial differentiation to prepare for embryo implantation. Through progesterone stimulation, estrogen-primed endometrium is further differentiated into secretory endometrium, which is suitable for embryo implantation. After ovulation, progesterone is secreted from the corpus luteum that is a newly formed endocrine organ derived from the ovulated follicle.

In humans, luteinizing hormone (LH) is known to be a main regulator of corpus luteum function. During the luteal (secretory) phase, several molecules that mediate direct interaction between the embryo and maternal endometrium have been reported to be expressed on the cell surface of the luminal epithelial cells [1, 2]. About 4–5 days after fertilization, the human embryo enters the uterine cavity. Currently, it is considered that the embryo attaches to the luminal epithelium after cross-talking with the maternal endometrium.

After attachment to endometrial epithelial cells, the trophectoderm of the human embryo is activated and increases its invasive property. Then, the human embryo invades the endometrial stromal tissues, through epithelial cells that normally construct a tightly connected layer, and becomes buried within endometrial stromal tissue [3]. Several molecules such as activated leukocyte adhesion molecule (ALCAM)/CD166

and trophinin have been proposed as activators of the trophectoderm of the human embryo [4, 5].

Although the precise mechanisms remain unknown, after becoming buried within endometrial stromal tissue, lucunar spaces are formed within the trophectderm layer and maternal blood streams into this space and then returns to the systemic circulation. At this stage, human chorionic gonadotropin (HCG) that is abundantly produced by trophectoderm can be transmitted to the ovary. This hormone stimulates the corpus luteum to produce progesterone through the HCG receptor that is shared with LH, maintaining embryo implantation. During the human menstrual cycle, corpus luteum function persists for only 14 days. However, when pregnancy occurs, HCG stimulates corpus luteum of the menstrual cycle to transform into the corpus luteum of pregnancy in order to maintain embryo implantation. Accordingly, corpus luteum of pregnancy is an essential organ for embryo implantation and HCG is considered a main regulator of the corpus luteum of pregnancy. However, there is considerable evidence suggesting other mechanisms are involved. For example, in patients with ectopic pregnancy or natural abortion, despite an elevated HCG level in blood, progesterone production decreases and spontaneous abortion proceeds [6]. In addition, it was reported that exogenous HCG cannot maintain progesterone or relaxin production and corpus luteum regression proceeds in non-pregnant women [7]. However, there is no soluble factor other than HCG that has been identified to date [8] and the precise regulatory mechanisms remain unknown [9].

Regulation of Embryo Implantation by the Immune System

When we look at the implantation site again, maternal blood that enters lucunar spaces within the trophectoderm layer contains peripheral blood mononuclear cells (PBMC), that is lymphocytes and monocytes. These cells return to maternal systemic circulation to reach the ovary along with HCG. Therefore, we hypothesized that not only HCG, but also PBMC transmit information about the presence of an implanting embryo to maintain the function of corpus luteum. In other words, we propose that not only soluble factors, but also circulating cells contribute to the promotion of progesterone production by the corpus luteum. Considering that the presence of developing embryo in the female genital tract is one of the most important events for the mother, it is reasonable to speculate that the immune system, an excellent information transmitting system, plays some role in this process [10].

To prove this hypothesis, we examined the effects of PBMC derived from women in early pregnancy on progesterone production in human luteal cell culture. Interestingly, progesterone production was significantly promoted by PBMC derived from pregnant women. In addition, the production of Th-2 cytokines such as interleukin 4 and 10, was also elevated in this culture. Furthermore, these cytokines increased progesterone production as much as HCG, indicating that circulating immune cells can transmit information from the embryo to the ovary, contributing to the maintenance of progesterone production and embryo implantation in the uterus [11].

Based on these findings, we extended our hypothesis to the further concept that circulating immune cells transmit information about the presence of a developing embryo to the various organs throughout the whole body and induce adequate functional change or differentiation in these organs to facilitate embryo implantation [12].

Based on this speculation, we then examined the effects of immune cells on endometrial differentiation and embryo implantation in the uterus. In mice, it is well known that estrogen stimulation is necessary to start embryo implantation. In the delayed implantation model in which progesterone alone is supplemented after ovariectomy on pseudopregnant day 3, intrauterine transferred blastocyts remain floating in the uterine cavity. However, when estrogen is administered, the expression of leukemia inhibitory factor (LIF) is induced in the endometrium and embryo implantation restarts. In this model, when we administered spleen cells derived from pregnancy day 4 mice to the delayed implantation mice, LIF expression and subsequent embryo implantation were induced without estrogen stimulation. These findings indicate that immune cells can induce endometrial differentiation that is the indispensable process for embryo implantation [13].

It is also well known that when blastocysts are transferred into the uterine cavity of a pseudopregnant mouse, successful implantation can only be achieved within 3–5 days after ovulation when the endometrium has been adequately differentiated. This period is called the implantation window [14]. Notably, when spleen cells derived from mice on pregnancy day 4 were intravenously administered to pseudopregnant mice, embryo implantation was induced in recipient mice on pseudopregnancy day 1 or 2 when the embryo normally cannot implant [15]. However, splenocytes

derived from mice on day 4 of pseudopregnancy did not show any significant effect on the implantation window, suggesting that the presence of pre-implanted embryos in the genital tract is an important influence on splenocyte function in mice donating spleen cells.

Mechanisms of Immune Cell Recognition of the Developing Embryo and Induction of Endometrial Differentiation

Since the function of spleen cells of pregnant mice is already altered on day 4 of pregnancy, it is reasonable to speculate that the immune system can recognize the developing embryo in the Fallopian tube. In agreement with this, it was reported that endometrial differentiation could be induced in pregnant mice even when the utero-tubal transition sites were ligated and entrance to the uterine cavity of the developing embryos was inhibited [16]. This finding indicates that developing murine embryos in the Fallopian tube could affect endometrial differentiation in the absence of direct interaction with the endometrium. It also suggests that there are certain systemic mediators between the mother and the embryo in the Fallopian tube. In addition, direct injection of splenocytes derived from pregnant mice (day 4) into the endometrium induced embryo implantation on day 2 of pseudopregnancy, and this cellular effect was higher with a T-lymphocyte-rich preparation than with a monocyte-rich preparation [13]. Furthermore, intraendometrial administration of CD4(+/–)CD8(–) cells from thymocytes enhanced embryo implantation in mice receiving embryo transfers on day 2 of pseudopregnancy [17]. Recently, it was shown that residual dendritic cells were activated by the embryo and migrate to local lymph nodes, regulating the immune cell reaction [18]. Altogether, we speculate that the residual immune cells in the Fallopian tube, such as intraepithelial lymphocytes and dendritic cells, receive information on the presence of developing embryos and migrate through the afferent pathway to the lymphoid organs and amplify this information. Thereafter, other effector immune cells are mobilized into systemic blood circulation to travel to target organs such as the endometrium, the ovary and so on and then induce tissue differentiation and/or functional changes to support classical regulatory mechanisms by the endocrine system [12].

If the above speculation is true, we further assume that the embryo produces certain soluble factor(s) in the Fallopian tube before hatching, because it is physically impossible for immune cells to directly interact with the developing embryos that are surrounded by the zona pellucida (ZP). To correctly support embryo implantation, the maternal immune system must distinguish the developing non-self embryo that belongs to the same species from organisms belonging to another species. Consequently, soluble signals from the embryo should be embryo- and species-specific. As a candidate for these factors, we have pay attention to the ZP since it consists of species-specific glycoproteins [19, 20]. ZP is normally protected from immunological attack during oogenesis in the ovarian follicle. In addition, ZP-specific glycoproteins have been proposed to play important roles in the species- and oocyte-specific binding of sperm. In this regard, the ZP contains an abundant store of oocyte- and species-specific glycoproteins. Notably, the ZP is degraded by developing embryos starting just after fertilization until hatching. Therefore, it is theoretically reasonable to consider that ZP-degradation products can activate the maternal immune system from the very early stages [21].

Just before hatching, the human embryo produces an embryo-specific hormone, HCG. Previously, we found that PBMC derived from women in early pregnancy enhanced trophectoderm invasion of murine embryos [22]. These PBMC were also demonstrated to promote invasion of BeWo cells, a human trophoblastic cell line derived from choriocarcinoma, by producing chemoattractive factors [23]. Strictly speaking, BeWo cells differ from the activated trophectderm of invading human embryo just after endometrial attachment. However, these findings obtained using BeWo cells suggest that circulating mononuclear immune cells are involved in the initial step of human embryo invasion. More importantly, when PBMC derived from non-pregnant women were incubated with HCG, HCG-treated PBMC more highly promoted murine embryo and BeWo cell invasion by increasing production of chemoattractive factors [22, 23]. These findings suggest that HCG alters PBMC function to facilitate embryo invasion.

Several decades ago, crude HCG purified from urine was reported to suppress immune reactions [24]. However, it was later shown that highly purified HCG had no effect on lymphocyte function [25, 26]. Therefore, the effects of HCG on immune cells have remained controversial for a long time. Recently, we found that recombinant-HCG enhanced IL-8 production by human monocytes at a high concentration. However, the cell surface expression of LH/HCG receptor was not detected on monocytes [27]. Therefore, it is speculated that there is a different pathway, other than LH/HCG-receptor system, which can respond to a high HCG con-

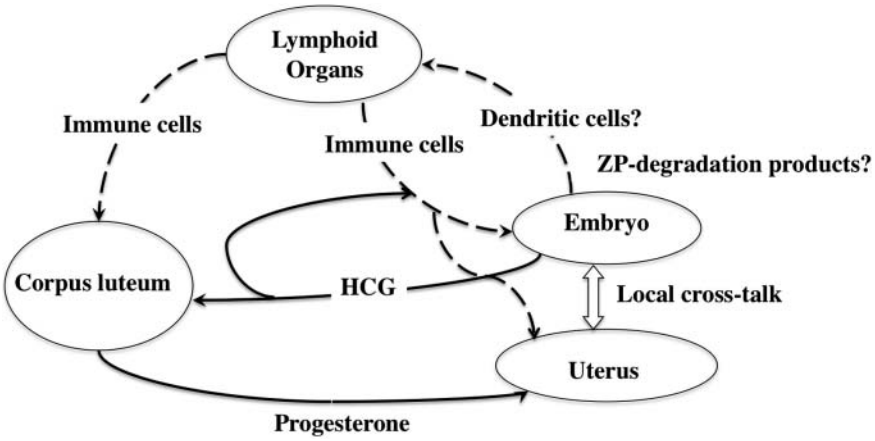

Fig. 1. Proposed supporting mechanisms of the immune system involved in endocrine system-primed embryo implantation. The residual dendritic cells in the Fallopian tube receive signals (degraded products of ZP glycoprotein) from developing embryos and migrate through the afferent pathway to the lymphoid organs and amplify this information. Thereafter, other effector immune cells are mobilized into systemic blood circulation in order to travel to target organs such as the endometrium, ovary etc., where they induce tissue differentiation and/or functional changes in order to cooperatively support classical regulatory mechanisms of the endocrine system. HCG also stimulates immune cells to produce chemokines that induce embryo invasion.

centration. HCG is an evolutionally-new hormone in primates that shares a receptor with LH. The most important difference between LH and HCG is the presence of abundant sugar chains at the C-terminal of the HCG β-chain. Actually, HCG-induced IL-8 production is inhibited by an exogenous excess of sugars, suggesting that HCG affects PBMC function through sugar chain receptors, which is a primitive mechanism in the immune system [27].

Although the precise mechanisms involved in the stepwise invasion processes of human embryos remain unknown, it is well known that the initial change around the implantation site is an increase in vascular permeability [28, 29], and recruitment of a specific population of immune cells at this site [30]. Accordingly, it is speculated that locally high concentrations of HCG stimulate recruited or resident immune cells to produce chemoattractants which in turn induces embryo invasion. In agreement with this thesis, a recent study demonstrated that human invading trophoblasts at the implantation site produce hyperglycosylated HCG and the more glycosylated HCG up-regulated trophoblast invasion [31].

Taken together, it can be proposed that degraded products of ZP glycoprotein and HCG are potential candidates as embryo- and species-specific signals for maternal recognition by the immune system (Fig. 1).

Clinical Application of Additional Supporting Mechanisms of the Immune System in Infertility Treatment

An implantation window is also supposed to exist in humans. To confirm its presence, we developed an attachment assay using human choriocarcinoma-derived BeWo cell mass and a human endometrial epithelial cell culture. In this assay, high attachment rates were observed in endometrial culture derived from the mid-luteal phase, suggesting that human endometrial receptivity increases during the implantation period. Importantly, when co-cultured with PBMC, attachment rates were significantly increased, showing that PBMC derived from non-pregnant women contain a population of cells that promote endometrial cell receptivity *in vitro* [32]. In support of this finding, we previously observed that thymocytes derived from immature non-pregnant female mice significantly promoted embryo implantation rates on day 2 of pseudopregnancy. These thymocytes also induced LIF expression in the uterus in the delayed implantation model mice, indicating that even without pregnancy, certain murine immune cell populations can induce endometrial differentiation and promote embryo

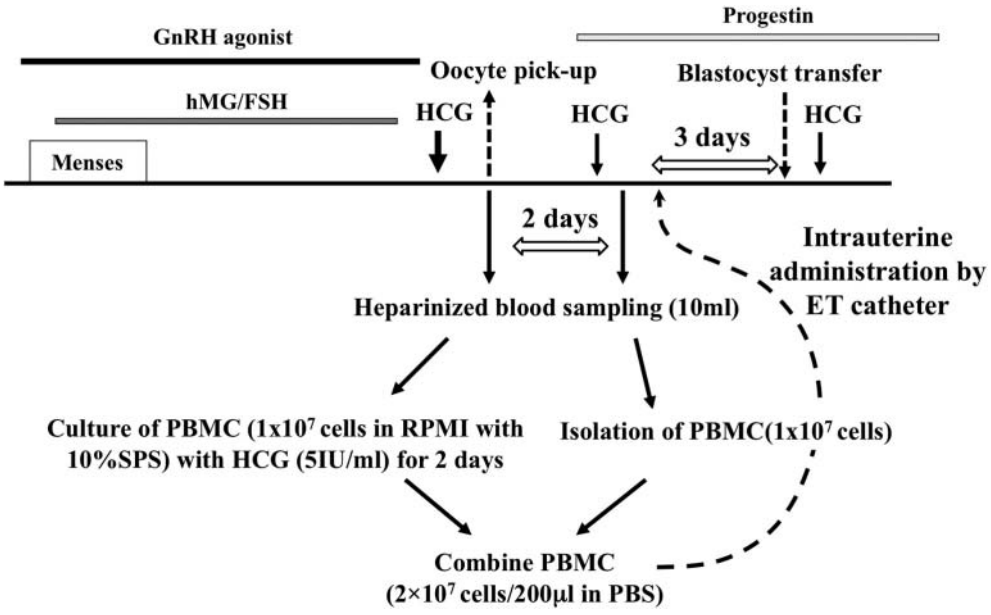

Fig. 2. Protocol of PBMC therapy for infertile patients receiving IVF-ET. PBMC are isolated from the patients and incubated with HCG in order to activate PBMC for 2 days. Thereafter, activated PBMC and freshly isolated PBMC are combined and are administered into the uterine cavity to induce adequate endometrial differentiation. After 3 days, blastocysts are transferred into the uterine cavity.

Table 1. Estimated mechanisms for induction of embryo implantation by autologous PBMC

1. PBMC induce endometrial differentiation suitable for embryo attachment.
2. PBMC evoke favorable inflammatory reactions in the uterine cavity.
3. PBMC secrete proteases which may effectively change the function or structure of surface molecules expressed on the endometrial luminal epithelial cells.
4. PBMC move from the uterine cavity toward the endometrial stromal tissue, creating the leading pathway for subsequent embryo attachment and invasion.

implantation *in vivo* [17].

Based on our findings, we have developed a novel therapy using autologous PBMC. In this therapy, PBMC are isolated from the patients and incubated with HCG in order to activate PBMC for 2 days. Thereafter, activated PBMC are administered into the uterine cavity to induce adequate endometrial differentiation. Three days later, blastocysts are transferred into the uterine cavity (Fig. 2). We applied this treatment to patients with 4 or more repeated failures in IVF therapy and found that PBMC treatment effectively improved the pregnancy and implantation rates [33]. Although PBMC are autologous cells from the patient, PBMC by themselves are expected to evoke favorable inflammatory reactions in the uterine cavity *in vivo*. It is also noted that PBMC can secrete proteases that may effectively change the function or structure of surface molecules expressed on the endometrial luminal epithelial cells. Furthermore, locally administered PBMC is considered to move from the uterine cavity toward the endometrial stromal tissue, creating the leading pathway for the subsequent embryo attachment and invasion (Table 1).

Conclusion

In this manuscript, we have described our new hypothesis that immune cells receive information about the presence of a developing embryo and transmit this information through blood circulation to various organs throughout the whole body, inducing functional changes or differentiation in these organs which facilitate embryo implantation in cooperation with the endocrine system.

Based on this theory, when infertile patients insufficiently respond to endocrine stimulation, we can clinically utilize these alternative mechanisms in infertility therapy. Since the procedure does not require special instruments, this protocol can be safely applied to the conventional infertility therapy cycle including intrauterine insemination. By improving the culture condition for PBMC, more effective changes in PBMC function can be expected in the future. We hope that this unique approach will also contribute to improvements in the breeding of domestic animals.

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