Progesterone supplementation extends uterine receptivity for blastocyst implantation in mice

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Abstract

We previously showed that blastocyst can initiate implantation beyond the normal 'window' of uterine receptivity on day 5 of pregnancy and pseudopregnancy (PSP) in mice. In this study, we investigated whether uterine receptivity for blastocyst implantation can be further extended on day 6 of PSP and the role of progesterone (P_4) on this event. Embryo transfers, experimentally induced decidualization, *in situ* hybridization and [3H]thymidine incorporation were performed. Blastocysts initiate attachment reaction within 48 h when transferred on day 5, but not on day 6 of PSP. Likewise, decidualization reaction occurred on days 4 and 5 of PSP, but completely failed on day 6. However, P_4 supplementation partially retains uterine receptivity for blastocyst implantation and decidualization on day 6 of PSP. In addition, certain indicators of uterine receptivity, such as cell proliferation profile and expression patterns of implantation-related genes were similarly observed on days 4 and 5 of PSP, but not on day 6. Consistent with embryo transfer and decidualization, exogenous administration of P_4 partially restores these indicators on day 6 of PSP. We concluded that critical physiological changes occur between days 4 and 5 of PSP, leading to uterine non-receptivity on day 6, but P_4 is able to extend the uterine receptivity through day 6.

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Introduction

Uterine milieu for blastocyst implantation is classified into prereceptive, receptive, and non-receptive phases. Uterine receptivity is defined as the window of limited time, when the uterine environment is conducive to support blastocyst growth, attachment reaction, and subsequent events of implantation (Psychoyos 1973, Paria *et al.* 1993, Dey 1996). Extensive embryo transfer experiments were performed on various days of pseudopregnancy (PSP), and the first sign of attachment reaction in the implantation process, the increased vascular permeability, was examined 24 h after embryo transfer to understand uterine receptivity in mice. These experiments suggested that blastocyst can implant only for a limited period on day 4 of pregnancy or PSP. The establishment of uterine receptivity for blastocyst implantation in rodents depends on an appropriate combination of P_4 and estrogen, but the molecular events by which estrogen converts the P_4 -primed uterus

to the receptive state, activates the blastocyst, and initiates the implantation process remain ill-defined.

We have previously shown that the 'window' of uterine receptivity for blastocyst implantation in mice remains open for an extended period at lower estrogen (E_2) levels (3 ng) but rapidly closes at higher levels (Ma *et al.* 2003). Rapid closure of uterine receptivity at high E_2 levels is accompanied by aberrant expression of implantation-related genes. These results demonstrate that optimal levels of ovarian steroids are critical in regulating molecular mechanisms for uterine receptivity in mice. This study utilized delayed implantation model where recipient mice for embryo transfer were ovariectomized and treated with exogenous hormones to monitor implantation rates under various experimental conditions. Thus, one question remains to be answered is whether the 'window' of uterine receptivity can be extended in mice with or without hormonal manipulation.

Cytosolic phospholipase A₂ (cPLA₂) is a fatty acid liberating enzyme which sits upstream of cyclooxygenases in prostaglandin (PG) biosynthetic pathway (Murakami *et al.* 2000). Consistent with critical roles of PGs in implantation and decidualization (Lim *et al.* 1997), cPLA₂-deficient female mice also exhibit uterine defects (Song *et al.* 2002). During analysis of implantation phenotypes in these mice, we found that blastocysts which failed to initiate the attachment reaction during the expected time show implantation one day later on day 6 of pregnancy (Song *et al.* 2002). This suggested that blastocysts require longer time to initiate the implantation process under the condition of sub-optimal uterine receptivity such as uteri deficient of cPLA₂ with lower levels of PGs at the time of implantation. Further investigation with extensive embryo transfers using normal recipients on day 5 of PSP demonstrated that blastocyst can initiate the implantation process in conventionally referred non-receptive uterus (Song *et al.* 2002).

These findings led us to revisit the definition of 'window' of implantation with extensive embryo transfer on extended days of PSP and investigate the role of progesterone in retaining uterine receptivity in mice. We show here that blastocyst can implant on day 5, but not on day 6 of PSP and that P₄ supplementation partially extends uterine receptivity for blastocyst implantation on day 6 of PSP possibly by conditioning uterine environment for implantation via modulation of uterine cell proliferation and expression of some implantation-related genes.

Materials and Methods

Mice

Adult female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or PSP respectively. The morning of finding a vaginal plug was designated day 1 of pregnancy. Implantation sites (IS) on days 5, 6, or 7 were visualized by i.v. injection (0.1 ml/mouse) of 1% Chicago Blue dye solution in

saline (Paria *et al.* 1993). Mice were housed in the semi-barrier animal care facility according to NIH and institutional guidelines for laboratory animals.

Blastocyst transfer to pseudopregnant mice

Day 4 blastocysts were transferred to the uteri of recipients in the morning (1000 h) of various days of PSP. Recipient mice were killed at 0900 h on designated days of PSP. P₄ (Sigma) was supplemented to several groups of days 6 and 7 pseudopregnant mice by daily injection from day 5 to the day when the mice were killed. Time of injection is indicated in Table 1. The number of IS was recorded by i.v. injection (0.1 ml/mouse) of Chicago Blue dye solution (1% in saline) 48 h after embryo transfer. Mice were killed 5 min later and IS were demarcated as discrete blue bands along the uteri.

Experimentally induced decidualization in pseudopregnant mice

To induce artificial decidualization, sesame oil was infused intraluminally in one uterine horn on days 4–6 of PSP. The contra-lateral horn served as control. Mice were killed 4 days later and uterine weights of the infused and non-infused (control) horns were recorded. Fold increases in uterine weights were used as an index of decidualization. P₄ was supplemented to one group of day 6 pseudopregnant mice by daily injection (0900 h) from day 5 to the day when the mice were killed.

Uterine cell type-specific proliferation in pseudopregnant mice

To examine whether uterine cell type-specific proliferation is associated with the uterine receptivity, the mice on days 4–6 of PSP received an injection (i.p.) of [methyl-³H]thymidine (25 µCi/0.1 ml; PerkinElmer, Boston, MA, USA) in the morning (0800 h) and were killed 2 h later. Nuclear uptake of [³H]thymidine was detected in uterine sections by autoradiography (Lim *et al.* 1997).

Table 1 Blastocyst implantation beyond normal 'window' of uterine receptivity in mice.

Days of transfer	No. of recipients	No. of embryos transferred	No. of recipients with IS (%)	No. of IS (%)
Day 4	5	60	5 (100)	31 (52)
Day 5	7	92	6 (86)	44 (48)
Day 6	8	94	0 (0)	0 (0)
(I) Day 6 (P ₄ : 0900 h)	15	221	9 (60)	50 (23)
(II) Day 6 (P ₄ : 1400 h)	6	69	0 (0)	0 (0)
(III) Day 7 (P ₄ : 0900 h)	7	90	0 (0)	0 (0)
(IV) Day 7 (P ₄ + E ₂ : 0900 h)	7	98	0 (0)	0 (0)

Day 4 blastocysts were transferred into uteri of recipients on various days of pseudopregnancy. P₄ supplemented recipients received an injection of P₄ (2 mg/mouse) on day 5 morning (0900 h: I, III, IV) or afternoon (1400 h: II) and continuous daily P₄ on the morning from day 6 until killed to examine IS. IV recipients were injected with E₂ (25 ng/mouse) along with P₄ on day 7 (0900 h) before embryo transfer. All recipients were killed 48 h after embryo transfer to examine IS by the blue dye method. Data for embryo transfer performed with day 4 and day 5 pseudopregnant recipients, used as control for other embryo transfers in this table, were previously published (Song *et al.* 2002).

In situ hybridization

In situ hybridization was performed as previously described (Das *et al.* 1994, Lim *et al.* 1997, Song *et al.* 2002). Small pieces of tissues were flash-frozen in liquid Histo-Freeze (Fisher Scientific, St Louis, MO, USA). Frozen sections (12 µm) were mounted onto poly-L-lysine-coated slides, fixed in cold 4% paraformaldehyde solution in PBS, acetylated, and hybridized at 45 °C for 4 h in hybridization buffer containing the ³⁵S-labeled antisense cRNA probes. After hybridization, the sections were incubated with RNase A (RNase A, 20 µg/ml) at 37 °C for 20 min. RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion (Eastman Kodak, Rochester, NY, USA). Sections hybridized with sense probes served as negative controls. Sense and antisense ³⁵S-labeled RNA probes were generated using the appropriate polymerases.

Results

Blastocysts can initiate attachment reaction in the non-receptive uterus beyond normal 'window' of uterine receptivity

Uterine environment at day 5 of PSP was considered non-receptive (Paria *et al.* 1993). When blastocysts are transferred to these uteri, IS are generally not visible if examined by 24 h. Recently, we have shown that blastocyst can still implant in the non-receptive uterus on day 5 of pregnancy and PSP beyond normal 'window' of uterine receptivity in mice, but they require longer time to initiate the attachment reaction (Song *et al.* 2002). This led us to hypothesize that blastocyst may initiate the implantation on extended days of PSP in conventionally non-receptive uterus. To test this hypothesis, we performed embryo transfer experiments to recipients on various days of PSP and examined IS 48 h later rather than 24 h by blue dye injection. As previously shown (Song *et al.* 2002), 48% of blastocysts implanted in the day 5 pseudopregnant uteri of recipients by 48 h after transfer. This was comparable to implantation rate of embryo transfers performed on day 4 of PSP (52%; Table 1). These clearly demonstrated that blastocysts can initiate the implantation process on day 5 of PSP in non-receptive uterus in mice. However, whether blastocyst can implant on later days of PSP in conventional non-receptive uterus still remain unanswered. To examine this, we performed embryo transfers to recipients on days 6 and 7 of PSP. In contrast, to comparable implantation rate of day 5 embryo transfer, blastocysts transferred to recipients on days 6 and 7 of PSP completely failed to implant even 48 h after embryo transfers (Table 1). These results indicate that day 5 pseudopregnant uterus is able to support attachment reaction but requires longer time (48 vs 24 h) to manifest the first sign of attachment reaction, the increased vascular permeability. However, this 'window' to initiate attachment reaction is shut off after day 5.

Progesterone supplementation retains uterine receptivity for blastocyst implantation on day 6 of PSP

The observation from embryo transfer experiments suggested that the uterine environment with respect to uterine receptivity for blastocyst implantation significantly changes between days 5 and 6 of PSP. Since it is well established that optimal levels of ovarian steroids are key regulators of uterine receptivity, we hypothesized that levels of progesterone could be a key factor to maintain uterine receptivity for blastocyst implantation. In fact, it was reported that P₄ levels are maintained during days 3–5 of PSP and the levels significantly decrease by day 6 (Wakuda *et al.* 1999). Thus, we investigated whether exogenous ovarian steroids could further extend the receptive state for blastocyst implantation. We injected P₄ (2 mg/mouse) daily (0900 h) from day 5 of PSP to the day when the recipients were killed and performed embryo transfers on day 6. Recipients were killed at 48 h after embryo transfer. Interestingly, 9 of 15 day 6 pseudopregnant recipients (60%) with daily P₄ supplementation showed IS when examined at 48 h after embryo transfer (Table 1, I). This suggests that decreased level of P₄ is a key factor for the uterus to become non-receptive (day 6). The number (23%) of IS visualized by the blue dye method in recipients on day 6 of PSP is lower than those of days 4 and 5 of PSP (52 and 48%) respectively, suggesting that P₄ supplementation is not able to fully restore uterine receptivity on day 6. To examine if timing of P₄ availability is important, we supplemented P₄ in the afternoon (1400 h) rather than in the morning (0900 h) on day 5 to the recipient. Surprisingly, all the blastocysts transferred to those recipients failed to initiate the attachment reaction (Table 1, II). This result suggests that timely P₄ supplementation is critical to maintain uterine environments for providing a more accessible condition for blastocyst implantation. Hormonal supplementation on day 7 of PSP did not help to improve implantation rate in day 7 PSP recipients (Table 1, III–IV). Collectively, the results suggest that critical changes in uterine physiology occur in the uterus between days 5 and 6 of PSP.

Uterus responds to decidualization stimuli beyond normal 'window' of uterine receptivity

Above results show that blastocysts are capable of initiating attachment reaction beyond normal 'window' of receptivity under appropriate P₄ level as judged by increased vascular permeability at the site of implantation. The attachment reaction is immediately followed by decidual response by stromal cells underlying the luminal epithelial cells contacting the blastocyst. As decidual response is a critical component of successful implantation, we tested whether uteri of pseudopregnant recipients on different days respond to an artificial stimulus to induce decidualization beyond the normal

'window' of uterine receptivity. We gave an intraluminal oil infusion to recipients on days 4, 5, or 6 of PSP and examined the extent of decidual response 4 days later. Artificially induced decidualization occurred in most of the uteri of days 4 and 5 of PSP (5/6 and 6/8), but not in the uteri of day 6 of PSP (0/7; Fig. 1). To investigate whether P_4 supplementation sustain the uterine responsiveness to a decidualogenic stimulus on day 6 as in the case of the attachment reaction, we gave P_4 (2 mg/mouse) injections on days 5 and 6 of PSP (0900 h) in this experimental model. As shown in Fig. 1, P_4 injection improved decidual responses in day 6 pseudopregnant mice (5/17) although fold increases in uterine weight (7.4 ± 0.9) and percentage of mice with decidualization responses (29%) were lower than those of days 4 and 5 of PSP (12.1 ± 2.0 and 12.8 ± 1.2 fold increases in uterine weight, and 83 and 75% of mice respectively). These results collectively show that initial attachment reaction and subsequent decidual response to the extent comparable to day 4 pseudopregnant uterus can occur on day 5 of PSP, but not thereafter. In addition, P_4 supplementation partially improves the uterine condition on day 6 of PSP to support attachment reaction and decidualization.

Cell proliferation profile is altered in the uterus in non-receptive phase

In mice, the establishment of the receptive uterus for supporting implantation is regulated by coordinated effects of P_4 and E_2 on differential cell proliferation and

Figure 1 Experimentally induced decidualization in mice on days 4–6 of pseudopregnancy (PSP). Female mice received intraluminal oil infusion on days 4–6 of PSP. A group of day 6 pseudopregnant mice (day 6 (P_4)) was given with P_4 (2 mg/mouse) by daily injection from day 5 (0900 h) to the day when killed. Mice were killed 4 days after oil infusion and uterine weights were recorded. Fold increases denote comparison of weights between infused and non-infused uterine horns. Results of days 4 and 5 of PSP, used as control for decidualization with day 6 of PSP in this experiment, were previously published (Song *et al.* 2002). The numbers above bars indicate the numbers of responding mice/total mice. Results are mean $\pm$ S.E.M.

gene expression (Huet *et al.* 1989). We suspected that failure of implantation and decidualization on day 6 of PSP is due to uterine delinquency arising from abnormal cell proliferation and/or differentiation. Thus, we compared uterine cell proliferation profiles between days 4 and 6 of PSP by [3 H]thymidine incorporation (Fig. 2). As previously observed (Huet *et al.* 1989), proliferation was restricted to stromal cells. However, no difference in proliferation pattern was noted between days 4 and 5 of PSP. However, [3 H]thymidine incorporation was aberrantly noted in luminal epithelial cells with very low to undetectable levels in the stroma (Fig. 2) in day 6 pseudopregnant mice. In addition, the number of proliferating cells on day 6 of PSP was quite lower than those on days 4 and 5 of PSP. Exogenous supplement of P_4 (2 mg/mouse) on day 5 of PSP (0900 h) maintained normal uterine cell proliferation profiles in some uteri of day 6 pseudopregnant mice.

Figure 2 Nuclear incorporation of [3 H]thymidine in proliferating uterine cells on days 4–6 of pseudopregnancy (PSP). Mice on days 4–6 of PSP received an injection (i.p.) of [3 H]thymidine in the morning (0800 h) and were killed 2 h later. A group of day 6 pseudopregnant mice (day 6 (P_4)) was given with a single injection of P_4 (2 mg/mouse) on day 5 (0900 h). Note nuclear [3 H]thymidine uptake in epithelial cells on day 6 of PSP, but in stromal cells of uteri of day 6 pseudopregnant mice injected with P_4 on day 5. Patterns of [3 H]thymidine incorporation in various days of PSP were shown under darkfield at 40 $\times$ (left panel) and 200 $\times$ (right panel) respectively. These experiments were repeated thrice with similar results.

The result that uteri on day 6 PSP no longer maintain cell proliferation profile of typical P₄ dominance suggests that the uterus on this day possibly undergoes critical change in hormonal milieu leading to uterine refractoriness to implantation.

Differential expression of implantation-related genes during changing uterine receptivity

Plasma levels of P₄ during pregnancy or PSP is one principal factor to provide the receptive environment by regulating certain local mediators. Thus, we examined expression of several implantation-related genes, such as COX-1 (*Ptgs1*), Amphiregulin (*Areg*), *Hoxa10* (*Hoxa10*), Flk-1 (*Kdr*), and LIF (*Lif*) using *in situ* hybridization on days 4–6 of PSP to investigate whether change in hormonal milieu on day 6 of PSP is accompanied by aberrant expression patterns of these genes (Figs 3 and 4). All of these genes are expressed in a cell type-specific manner during implantation (Chakraborty *et al.* 1995, Das *et al.* 1995a, Benson *et al.* 1996, Song *et al.* 2000). *Ptgs1*, as reported previously, was expressed mainly in luminal epithelium with modest expression in glandular epithelium on day 4. Interestingly, the accumulation of *Ptgs1* mRNA in the luminal epithelium gradually decreased on day 5 and was no longer detected on day 6, while its expression in the glandular epithelium was intensified on day 6. *Areg* was expressed in the entire luminal epithelium on day 4, but gradually decreased and became almost

undetectable on day 6. However, *Hoxa10* and *Kdr* maintained similar expression patterns in the stroma throughout this period. Since *Hoxa10* and *Areg* are both P₄-regulated genes, it is interesting to note that only the epithelial expression of *Areg*, but not the stromal expression of *Hoxa10*, decreases to undetectable levels on day 6 when blood levels of P₄ sharply decrease (Wakuda *et al.* 1999). Surprisingly, while exogenous P₄ supplementation partly extends the uterine receptivity for the attachment reaction and decidualization, the expression patterns of *Ptgs1* and *Areg* on day 6 were not influenced by this treatment.

In contrast to implantation-related genes examined above, P₄ supplementation had an effect on the expression of *Lif* (Fig. 4). *Lif*, expressed in uterine glands prior to implantation on day 4 of pregnancy (Bhatt *et al.* 1991, Song *et al.* 2000), maintained expression in glands on days 4 and 5 of PSP. However, the expression rapidly decreased and became undetectable on day 6 of PSP (Fig. 4). Although it was previously shown that *Lif* expression is mainly regulated by estrogen (Bhatt *et al.* 1991, Shen & Leder 1992), P₄ supplementation in some day 6 pseudopregnant mice maintained its expression in uterine glands (arrows in the right panel of bottom). Collectively, these results suggest that uterine environment on day 6 of PSP undergoes certain molecular changes that reduce the efficiency of implantation and that P₄ is a key factor, even if not a sole one, to retain uterine environment for blastocyst implantation.

Figure 3 Expression of implantation-related genes in uteri of days 4–6 of pseudopregnant mice. *In situ* hybridization of *Areg*, *Ptgs1*, *Hoxa10*, *Kdr* mRNAs in uterine sections on days 4–6 of PSP are shown under darkfield at 40 $\times$. A group of day 6 pseudopregnant mice (day 6 (P₄)) was given with a single injection of P₄ (2 mg/mouse) on day 5 (0900 h). le, Luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium. These experiments were repeated twice with similar results.

Figure 4 Differential expression of *Lif* in uteri of days 4–6 of pseudopregnant mice. *In situ* hybridization of *Lif* mRNAs is represented under darkfield at 40 $\times$. Day 6 (P_4) represents the uterus of day 6 pseudopregnant mice with a single injection of P_4 (2 mg/mouse) on day 5. Arrows indicate the uterine glands that *Lif* expression is still retained on day 6 of pseudopregnancy. le, Luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium. These experiments were repeated thrice with similar results.

Discussion

Synchronization of embryo development to the blastocyst stage with uterine receptivity is a prerequisite for successful implantation. Indeed, implantation failure due to inappropriate uterine receptivity is one of the major causes of infertility in humans, particularly in the case of embryo transfers following *in vitro* fertilization (Nikas *et al.* 1999). However, molecular mechanisms by which the uterus achieves receptive state for blastocyst implantation still remain elusive. The highlight of the present investigation is that blastocysts can initiate attachment reaction on days 5 and 6 (with P_4 injection) of PSP, which is beyond the conventional 'window' of uterine receptivity in mice. In addition, decidualization, another aspect of the implantation process, can occur similarly. The finding that day 6 pseudopregnant uteri support attachment reaction only under exogenous P_4 supplementation suggests that declining levels of P_4 is one critical factor restricting uterine receptivity.

The molecular and cellular events leading to uterine receptivity for blastocyst implantation in mice depend on ovarian steroids (Dey 1996). Various local factors including growth factors, cytokines, transcription factors, and PGs are implicated in molecular events of implantation under the influence of P_4 and E_2 (Das *et al.* 1994, 1995a, Benson *et al.* 1996, Lim *et al.* 1997b, Song *et al.* 2000). Our study shows that implantation-related genes of the epithelial compartments are decreased on day 6 of PSP, suggesting that epithelial cells are not conditioned for attachment reaction on this day. P_4 supplementation only partially improves gene

expression and it is coincident with partial rescue of implantation rate on day 6 of PSP.

Among implantation-specific genes, *Lif* is considered critical for implantation since $LIF^{-/-}$ mice show implantation and decidualization failures (Stewart *et al.* 1992, Song *et al.* 2000). Thus, it is interesting to see that *Lif* is expressed in uterine glands as long as uterus is receptive for blastocyst implantation (days 4 and 5; Fig. 4). *Lif* expression is very low to undetectable on day 6 of PSP when blastocyst fails to initiate implantation. These results suggest that *Lif* is a critical factor contributing to the receptive state of uterus for blastocyst implantation. One explanation of partial rescue by P_4 is that other negative factors for receptivity increase in the uterus as time passes. One example is the endocannabinoid signaling, which was previously identified as an important regulator of uterine receptivity for implantation (Das *et al.* 1995b, Paria *et al.* 1996, Schmid *et al.* 1997). The level of anandamide, an endogenous cannabinoid, gradually increases with changing uterine sensitivity from receptive (on day 4) to non-receptive phase (on day 6; Paria *et al.* 2001). In addition, its level in $LIF^{-/-}$ mice was higher than that of wild-type mice on day 4 of pregnancy (Paria *et al.* 2001). It will be interesting to investigate whether P_4 supplementation to non-receptive uterus affects levels of anandamide or not.

Our present study shows that blastocysts can initiate implantation beyond the normal 'window' of uterine receptivity when P_4 level is appropriate. However, there is evidence that temporally deferred implantation beyond normal 'window' of uterine receptivity in wild-type mice may accompany various defects in embryo developments leading to embryonic demise before birth (Song *et al.* 2002, Wang & Dey 2006). One-day delay of implantation process in $cPLA_2^{-/-}$ mice indeed results in defective postimplantation developments (Song *et al.* 2002). These results are consistent with increased risk of early pregnancy loss in embryo implantation with sub-optimal uterine receptivity in humans (Wilcox *et al.* 1999). While our work provides solid evidence that P_4 is one crucial factor regulating uterine receptivity, other factors involved in optimizing uterine environment for implantation needs further identification.

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