Alkaline phosphatases contribute to uterine receptivity, implantation, decidualization, and defense against bacterial endotoxin in hamsters

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Abstract

Alkaline phosphatase (AP) activity has been demonstrated in the uterus of several species, but its importance in the uterus, in general and during pregnancy, is yet to be revealed. In this study, we focused on identifying AP isozyme types and their hormonal regulation, cell type, and event-specific expression and possible functions in the hamster uterus during the cycle and early pregnancy. Our RT-PCR and *in situ* hybridization studies demonstrated that among the known *Akp2*, *Akp3*, *Akp5*, and *Akp6* murine AP isozyme genes, hamster uteri express only *Akp2* and *Akp6*; both genes are co-expressed in luminal epithelial cells. Studies in cyclic and ovariectomized hamsters established that while progesterone (P₄) is the major uterine *Akp2* inducer, both P₄ and estrogen are strong *Akp6* regulators. Studies in preimplantation uteri showed induction of both genes and the activity of their encoded isozymes in luminal epithelial cells during uterine receptivity. However, at the beginning of implantation, *Akp2* showed reduced expression in luminal epithelial cells surrounding the implanted embryo. By contrast, expression of *Akp6* and its isozyme was maintained in luminal epithelial cells adjacent to, but not away from, the implanted embryo. Following implantation, stromal transformation to decidua was associated with induced expressions of only *Akp2* and its isozyme. We next demonstrated that uterine APs dephosphorylate and detoxify endotoxin lipopolysaccharide at their sites of production and activity. Taken together, our findings suggest that uterine APs contribute to uterine receptivity, implantation, and decidualization in addition to their role in protection of the uterus and pregnancy against bacterial infection.

Reproduction (2013) **146** 419–432

Introduction

The uterus enters into the receptive state following mating to support blastocyst attachment, which then induces the decidualization program in rodents. Defects in uterine receptivity, implantation, and decidualization are known causes of compromised fertility in females (van Mourik *et al.* 2009), but details of the uterine molecular reprogramming involved in these processes have not yet been established. The uterus is also susceptible to bacterial infection and intrauterine infection is a leading cause of pelvic inflammatory disease, endometritis, infertility, subfertility, early pregnancy loss, fetal defects, and preterm birth (Adamson & Baker 2003, Goldenberg *et al.* 2008, Aisemberg *et al.* 2010, Keelan 2011, Sweet 2012). However, the molecules that the uterus uses to neutralize the toxicity

of bacterial toxins (endotoxins) in general and during pregnancy remain unidentified.

Alkaline phosphatase (AP, EC 3.1.3.1) is an ancient enzyme that was thought to have insignificant physiological roles as it hydrolyzes phosphate esters at high alkaline pH (Millan 1990). However, this perception has changed, and a new chapter of AP physiology has emerged with the following findings: i) this enzyme can act at very close to neutral pH (Millan 2006); ii) genetic ablation of AP isozymes in mice revealed distinct phenotypes such as skeletal defects in *Akp2*-null mice (Waymire *et al.* 1995), altered fatty acid transport in the gut in *Akp3*-null mice (Narisawa *et al.* 2003, Nakano *et al.* 2007), delayed parturition, and reduced litter size in *Akp5*-null mice (Dehghani *et al.* 2000); and iii) AP may contribute to host defense against pathogen-induced inflammation (Poelstra *et al.* 1997a, 1997b,

Koyama *et al.* 2002, Goldberg *et al.* 2008, Malo *et al.* 2010, Ramasamy *et al.* 2011). AP isozymes are membrane-bound molecules that are divided into two groups, tissue-nonspecific AP (TNAP) and tissue-specific APs (TSAPs). TNAP, which is also commonly known as the kidney/bone/liver isozyme, is encoded by the *Akp2* (a.k.a. *Alpl*) gene in mice. TSAPs in mice include duodenum-specific intestinal AP (dIAP) that is encoded by the *Akp3* gene, global IAP (gIAP) that is encoded by the *Akp6* gene, and embryonic AP (EAP) that is encoded by the *Akp5* gene (Millan 2006, Narisawa *et al.* 2007). AP activity studies in the mouse and rat uteri during early pregnancy have demonstrated a correlation of AP activity with decidua formation as its activity is strong in decidual stromal cells following implantation (Finn & Hinchliffe 1964, Manning *et al.* 1969, Murdoch *et al.* 1978, Pollard *et al.* 1990, Bucci & Murphy 1995). However, its physiological role in the uterus of any species prior to and during pregnancy has not been assigned. To our knowledge, neither the uterine AP gene(s) nor the AP activity pattern with respect to uterine changes during the cycle and early pregnancy has been reported in the hamster, unlike mice and rats in which maternal ovarian estrogen secretion is required for initiation of implantation, but similar to guinea pigs, rabbits, pigs, horses, monkeys, and humans in which AP supports blastocyst implantation only in the progesterone (P_4)-primed uterus (Reese *et al.* 2008). Thus, an attempt was made to test a hypothesis that AP isozymes expressed in the hamster uterus, showing cyclic, hormonal, and pregnancy-related changes, are involved in regulation of the processes of implantation, decidualization, and detoxification of endotoxin.

Materials and methods

Animals

Adult virgin male and female golden hamsters (*Mesocricetus auratus*; 8–10 weeks-old) were purchased from Charles River Laboratory (Wilmington, MA, USA) and housed in a 14 h light:10 h darkness cycle in the Laboratory Animal Facility of the Vanderbilt University Medical Center with *ad libitum* access to water and food according to the Institutional Guidelines on the Care and Use of Laboratory Animals. All experimental animal procedures were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

Uterine tissue collection during the estrous cycle

The day of vaginal discharge in hamsters is considered as the estrous day (Zhang & Paria 2006). Cyclic uterine tissues were collected (0800–0900 h) at estrus, metestrus, diestrus, and proestrus. Tissues were instantly frozen in pre-chilled Friendly Freeze'it (Curtin Matheson Scientific, Houston, TX, USA) and stored at -80°C .

Uterine tissue collection during early pregnancy of hamsters

Female hamsters were mated with fertile males on the evening of proestrus. Vaginal secretions were checked the next morning for the presence of sperm, which indicated day 1 of pregnancy (Zhang & Paria 2006). Whole hamster uteri from days 1 to 3 of pregnancy were collected at 0830–0900 h. Although whole hamster uteri were also collected in the morning of day 4 at 0900 h, implantation sites were collected at 1800 h on day 4 and 0900 h on day 5 after an i.v. injection of 1% Chicago blue B dye solution (Sigma; 0.25 ml 1% dye in saline) (Zhang & Paria 2006). On days 6–8 of pregnancy, implantation sites of hamsters showed distinct uterine swelling and were collected without blue dye injection. A part of the liver and small intestine were also obtained. All tissues were immediately frozen and stored at -80°C .

Induction of decidualomata by intrauterine silk suture

A short section of silk suture was placed inside one uterine horn of pregnant hamsters in the morning (0600 h) of day 4 of pregnancy for the purpose of disturbing normal embryo implantation and induction of decidualomata. The contralateral horns of these animals were not disturbed for normal embryo implantation and decidua formation. Animals were killed on day 6 of pregnancy and inspected for the presence of intermittent implantation sites in the undisturbed uterine horn and the decidualomata in the suture-containing horn. The embryo-induced decidua and suture-induced decidualomata were collected and stored at -80°C .

Hamster blastocyst collection

Pregnant hamsters were killed at 0100 h on day 4 to recover blastocysts from their uteri. Hamster blastocysts were washed thoroughly in hamster embryo culture medium-2 to eliminate uterine cell contamination (Wang *et al.* 2002). Twenty to 25 blastocysts were grouped in a sterile 1.5 microcentrifuge tube, frozen, and stored at -80°C .

Uterine tissue collection from ovariectomized P_4 - and estradiol-17 β -treated hamsters

Female hamsters were ovariectomized regardless of their stage of the cycle and rested for at least 10–15 days to eliminate circulating steroids (Zhang & Paria 2006). Animals then received a single injection (s.c.) of sesame seed oil as the vehicle (0.2 ml/hamster), P_4 (2 mg/0.2 ml/hamster), estradiol-17 β (E_2) (1 μg /0.2 ml/hamster), or P_4 plus E_2 . Control hamsters injected with vehicle were killed 6, 12, and 24 h later. All steroid-treated hamsters were also killed 6, 12, and 24 h after steroid injection. Uteri were immediately frozen and stored at -80°C .

Total RNA extraction

Total RNA from the uterus, liver, and small intestine was extracted using TRIZOL reagent (Invitrogen Life Technologies) according to the manufacturer's instruction. The isolated RNAs

were treated with DNase 1 for 30 min at 37 °C followed by phenol–chloroform extraction. To precipitate RNA, an ammonium acetate solution was added to the aqueous RNA solution to a concentration of 2.5 M. Then 2.5 vol. of pre-chilled ethanol was added and the solution was chilled for at least 2 h at –20 °C. The precipitated RNA was separated by centrifugation, washed with 75% ethanol, and dissolved in RNase-free water (Wang *et al.* 2002). Total RNA from blastocysts was extracted as described previously (Wang *et al.* 2002).

RT-PCR

DNase-treated total RNAs were subjected to cDNA synthesis by RT using oligo(dT) primers according to the manufacturer's instruction (Invitrogen). PCR was performed using sense and antisense primers from mouse *Akp2*, *Akp3*, *Akp5*, *Akp6*, and a housekeeping gene *Rpl7*. Primer sequences were as follows: *Akp2* (sense: 5'-GTG GAT ACA CCC CCC GGG GC-3'; antisense: 5'-GGT CAA GGT TGG CCC CAA TGC A-3'), *Akp3* (sense: 5'-GCT GGA ACC CCA GAC CCC GAG-3'; antisense: 5'-GGC CCT CTC GAT GGC TAA GTC G-3'), *Akp5* (sense: 5'-CGC ACC AGT GAG CAG GAC ACG-3'; antisense: 5'-GCC CGG GCT CAC TGC ACT GC-3'), *Akp6* (sense: 5'-AGA CAG GTC CCA GAC AGC G-3'; antisense: 5' CCA CCG AGG ATC ACA TCA A-3'); and *Rpl7* (sense: 5'-TGA ATG GAG TAA TCC CAA AG-3'; antisense: 5'-CAA GAG ACC GAG CAA TCA AG-3'). The *Akp2*, *Akp3*, and *Akp5* primer pairs were used previously by Hahnel *et al.* (1990), and *Rpl7* primer pairs were used by Wang *et al.* (2002). Primers for *Akp6* were designed from GenBank accession number NM_001081082. PCRs were performed under the following conditions: initial denaturation at 94 °C for 5 min, 40 cycles consisting of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. The reactions were carried out in a final volume of 20 µl. PCR-generated products were resolved electrophoretically (1.2% agarose gel) along with 100-bp ladder, stained with ethidium bromide, and photographed. Only *Rpl7* positive samples were used for *Akp2*, *Akp3*, *Akp5* and *Akp6* expression studies. The PCR products were cloned into pCR-II-TOPO cloning vector using a TOPO TA Cloning kit, version K2 (Invitrogen). Nucleotide sequencing of these clones were performed to verify the identity and orientation of each clone. The GenBank accession numbers for the hamster *Akp2* and *Akp6* are JQ_928734 and JQ_966128 respectively.

Real-time PCR

DNase-treated total RNAs (1 µg) were reverse transcribed as described earlier in the Materials and Methods section for RT-PCR. One microliter of the first strand was amplified in 25 µl total volume in an iCycler (Bio-Rad Laboratories, Inc.) using iQ SYBER Green Supermix (cat. # 170-8880; Bio-Rad). The following PCR protocol was used: 95 °C for 3 min followed by 45 cycles of 95 °C for 10 s and 55 °C for 30 s. All reactions were run in triplicates. The quantification was performed by the iQ 5 Standard Edition Optical System Version 2.0. Data from real-time PCR analysis were normalized to hypoxanthine phosphoribosyltransferase 1 (*Hprt* (*Hprt1*)) expression for analysis and results transformed to $2^{-\Delta\Delta C_t}$ with the oil-treated

control groups as the reference for clarity of presentation. Hamster-specific *Akp2* and *Akp6* primer sequences were as follows: *Akp2* (GenBank accession number JQ_928734; sense: 5'-GGC TAC AAG GTG GTG GAT GG-3'; antisense: 5'-GCA AAG ACT GCC ACA TCT TCC-3'), *Akp6* (GenBank accession number JQ_966128; sense: 5'-ACA GCC ACC GCC TAT CTC T-3'; antisense: 5'-GCT TGG CAC GAT ACA TCA CT-3'). Primers used to detect the *Hprt* gene (GenBank accession number NM_013556; sense: 5'-GCT TGG CAC GATA CAT CAC T-3'; antisense: 5'-CCC TGA AGT ACT CAT TAT AGT CAA GGG CAT-3') were previously used by us (Wang *et al.* 2011).

RNA probe preparation

Plasmids bearing hamster *Akp2* and *Akp6* cDNAs were extracted, purified, and linearized (*Akp2*: *SpeI*/*T7* for antisense, *EcoRV*/*SP6* for sense; *Akp6*: *EcoRV*/*SP6* for antisense, *SpeI*/*T7* for sense). For *in situ* hybridization, ³⁵S-labeled antisense and sense cRNA probes were generated using appropriate RNA polymerases (Wang *et al.* 2002).

In situ hybridization

In situ hybridization was performed as described previously by our group (Wang *et al.* 2002). Briefly, frozen uterine sections were fixed in cold 4% paraformaldehyde solution in PBS for 15 min on ice. Following pre-hybridization, sections were hybridized to ³⁵S-labeled antisense probes at 45 °C for 4 h. Sections hybridized with ³⁵S-labeled sense probes were used as negative control. After hybridization and washing, sections were incubated with RNase A at 37 °C for 20 min. RNase A-resistant hybrids were detected autoradiography using Kodak NTB-2 liquid emulsion (Eastman Kodak Co.). The slides were then stained with hematoxylin and eosin (Wang *et al.* 2002).

Histochemical detection of AP activity

Uterine cryosections (12 µm) were fixed in cold 4% paraformaldehyde solution in PBS and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate solution (Sigma) for 1–2 min at 37 °C. The histochemical reaction of AP was monitored under a stereomicroscope. Sections were rinsed in PBS and mounted in GVA solution. As a negative control, some slides with mounted uterine sections were microwaved (highest power level) for 5–10 min in PBS prior to incubation with substrate solution (Jones *et al.* 1974). To determine levamisole-sensitive and -insensitive forms of uterine AP isozymes, some slides were incubated with levamisole (TNAP inhibitor: 20 mg/ml) for 4 h at room temperature prior to addition of substrate solution. Sections were not counterstained in order to avoid obscuring phosphatase activity.

Cell-specific localization of gIAP by immunohistochemistry

Uterine cryosections were fixed in 10% buffered formalin and then stained with rabbit polyclonal gIAP antibody at a dilution of 1:250 in PBS as described previously

(Narisawa *et al.* 2007). The specificity of the staining was confirmed with antibody replaced with equal amounts of non-immune rabbit IgG. Sections were post-stained with hematoxylin and photographed.

Lipopolysaccharide-dephosphorylation assay by uterine homogenates and recombinant TNAP and gIAP proteins

Lipopolysaccharide (LPS)-dephosphorylating activity present in homogenates of day 1 uteri and day 7 implantation sites was assayed according to the protocol described by Goldberg *et al.* (2008). Briefly, tissue lysates equivalent to 2 µg protein were added into a 100 µl reaction buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM MgCl₂, and 20 µM ZnCl₂) with/without LPS (0.25 mg/ml; *Escherichia coli* (serotype 055:B5) from Sigma (cat. # L2880)) followed by incubation at 37 °C for 3 h. As negative controls, heat-inactivated (95 °C for 1 h) tissue lysates were added to the reaction mixture. To confirm uterine AP isozyme is involved in LPS dephosphorylation, uterine lysates were incubated with levamisole (5 mM) on ice for 1 h prior to their addition to the reaction buffer. To detect Pi released from LPS, malachite green solution (1:4) was added for 10 min and activity was then determined from spectrophotometric absorbance readings (650 nm wave length) taking into account the background readings. Each assay was performed in triplicate.

Using the above-described method, levamisole sensitivity toward the LPS-dephosphorylating activity of recombinant TNAP and gIAP proteins was also determined (Narisawa *et al.* 2007). LPS hydrolysis by TNAP or gIAP in control groups was considered as 100%.

Histochemical detection of LPS dephosphorylation at cellular sites of AP production and activity

To identify LPS dephosphorylation sites in the uterus, cryostat cut uterine sections from cyclic and early pregnant (days 1–8) uteri were fixed in formalin-Macrodex for 10 min and incubated with Tris/maleic acid buffer (pH 7.6) containing MgSO₄ and Pb(NO₃)₂ with or without LPS (3.2 mg/ml) for 2 h at room temperature. To ascertain uterine TNAP and gIAP involved in LPS dephosphorylation, sections were incubated with levamisole (5 mM) at 37 °C for 4 h prior to addition of reaction buffer with LPS. Slides were next washed with water, incubated with Na₂S (2%) for 30 s, washed with water, post-stained with hematoxylin, dehydrated with ascending strengths of alcohol, washed in xylene, and mounted with Distrene, Plasticiser, Xylene (DPX; Bentala *et al.* 2002). Uterine sites of dark brown lead sulfide deposits were examined under bright field.

Statistical analysis

Statistical analysis was performed on all LPS dephosphorylation sites and real-time PCR data using the Student's *t*-test or one-way ANOVA followed by Tukey's test. Statistical significance was declared when *P* value ≤ 0.05. Data are presented as means ± S.D. or ± S.E.M.

Results

Akp2 and Akp6 isozyme genes in the hamster uterus

PCR was used to detect transcripts of *Akp2*, *Akp3*, *Akp5*, and *Akp6* in day 1 uterus and day 6 implantation sites. Among the *Akp2*, *Akp3*, *Akp5*, and *Akp6* genes, the uterus of the hamster expressed the TNAP isozyme gene *Akp2* and the gIAP isozyme gene *Akp6*. *Akp2* and *Akp6* mRNAs were expressed in both the day 1 uterus and day 6 implantation sites (Fig. 1). The transcript of the *Akp3* gene that encodes dIAP was not expressed in any hamster tissues used in this study but was expressed in the mouse intestine (mouse data not shown). The transcript of the *Akp5* gene that encodes EAP was only detected in the hamster blastocyst.

Akp2 and Akp6 mRNA expression and total AP activity showed cyclic variations in the uterine luminal epithelium during the estrous cycle

To test the cellular source of the *Akp2* and *Akp6* genes in the non-pregnant uterus, *in situ* hybridization study was performed on uteri obtained from four stages of the estrous cycle. Endometrial expression of both genes was primarily observed in cells of the luminal epithelium (LE) and showed cyclic variations. The diestrous and proestrous uterine sections showed strong *Akp2* mRNA expression in the LE, and thereafter, the *Akp2* expression from the uterine LE was gradually reduced from the proestrous day to the metestrous day (Fig. 2A). Uterine expression of *Akp6* mRNA was also strong in the LE of the diestrous, proestrous, and estrous days with reduced expression in the metestrous day (Fig. 2B). Expression of *Akp2* and *Akp6* mRNAs above the background level was not

Figure 1 Expression of AP isozyme genes in the hamster uterine tissues. RT-PCR-amplified products of *Akp2*, *Akp3*, *Akp5*, and *Akp6* in day 1 uteri, day 6 implantation site (D6 IS), blastocysts, liver, and intestine of hamsters. Water and DNaseI-treated RNAs (D6 IS for *Akp2*; blastocysts for *Akp5*; and intestine for *Akp6*) without RT (–RT) were used as negative controls. *Rpl7* was used as a constitutive gene control. These experiments were performed three times with three independent samples with similar results.

Figure 2 Cyclic changes of the uterine AP isozyme gene expression and total AP activity. Uterine cross sections were processed to demonstrate cyclic variations of *Akp2* (A) and *Akp6* (B) mRNA expression and total AP activity (C) during the estrous cycle. GE, glandular epithelium; LE, luminal epithelium; S, stroma. (A) *Akp2* mRNA expression by *in situ* hybridization. Photographs were captured under dark field at 40 $\times$ magnification ($n=3$ /day of the estrous cycle). (B) *Akp6* mRNA expression by *in situ* hybridization. Photographs were captured under dark field at 40 $\times$ magnification ($n=3$ /day of the estrous cycle). (C) Total AP activity by histochemistry using BCIP/NBT solution. Photographs were captured under bright field at 200 $\times$ magnification ($n=3$ /day of the estrous cycle). Specificity of AP staining in sections from diestrus uteri was demonstrated by destroying endogenous enzyme activity through microwave heating.

observed in cells of the glandular epithelium, stroma, and myometrial layers in any day of the estrous cycle.

In agreement with luminal epithelial expression of mRNAs of *Akp2* and *Akp6*, AP activity was also primarily observed in cells of the uterine LE (Fig. 2C). A few AP-positive cells were seen in the stromal compartment, but their identities were not determined in this study. AP activity appeared to be primarily localized in the apical cell surface layer of the LE. AP activity in the uterine LE was strong in uterine sections from the diestrus and proestrus days, and thereafter, its activity from the uterine LE gradually reduced from the proestrus day to metestrus day (Fig. 2C). These cyclic variations in the pattern of luminal epithelial AP activity were positively correlated with epithelial expression patterns of *Akp2* and *Akp6* mRNAs in cyclic uteri. The observed alterations in AP isozyme gene expression and total AP activity in the uterine LE during the estrous cycle are indicative of modulation by steroid hormones.

Induction of luminal epithelial *Akp2* by P_4 and *Akp6* by both P_4 and E_2 in ovariectomized hamsters

Using the ovariectomized hamster model, we next examined whether *Akp2* and *Akp6* expression in the ovariectomized uterus is regulated by steroid hormones in the same manner as observed in the cyclic uterus. No detectable *in situ* hybridization signal for *Akp2* or *Akp6* mRNAs was observed in any uterine cell types of ovariectomized animals treated with oil. Compared with oil-injected controls, the E_2 -treated ovariectomized hamster uterus failed to show any change in *Akp2* mRNA expression by 6 h, but exhibited a slight increase, albeit with a lower intensity, in luminal epithelial *Akp2* expression at 12 h followed by a decline to the control level at 24 h (Fig. 3A). By contrast, we noted a gradual increase in the expression of *Akp6* mRNA in luminal epithelial cells from 6 to 24 h after E_2 treatment when compared with oil-injected controls (Fig. 3B). When ovariectomized hamsters were treated with P_4 alone, increased *Akp2* and *Akp6* mRNA expression in uterine luminal epithelial cells was noted by 6 h. Thereafter, mRNA expression of both these genes in the LE remained elevated until 24 h post-injection (Fig. 3A and B). The combined E_2/P_4 treatment showed a synergistic effect on *Akp6*, but not on *Akp2*, mRNA expression. Cells of the uterine gland, stroma, and myometrium showed no specific autoradiographic signals for *Akp2* and *Akp6* in either P_4 - or E_2 -treated ovariectomized hamsters (Fig. 3A and B). The *in situ* hybridization results at the 12-h time point were independently confirmed by real-time PCR. The levels of *Akp2* (Fig. 3C) and *Akp6* (Fig. 3D) mRNAs after hormone treatment corroborated with the patterns of expression of *Akp2* and *Akp6* as observed by *in situ* hybridization.

Figure 3 Steroid hormonal regulation of uterine AP isozyme gene expression in ovariectomized hamsters. Vehicle- and E₂- and/or P₄-treated animals ($n=3$ /treatment group) were killed at 6, 12, and 24 h after injection. Uterine cross sections from ovariectomized hamsters treated with vehicle (sesame seed oil), E₂, P₄, or E₂ plus P₄ were processed to determine hormonal regulation of *Akp2* (A) and *Akp6* (B) mRNA expression by *in situ* hybridization. LE, luminal epithelium; S, stroma. Photographs were captured under dark field at 100 $\times$ magnification. Total RNAs were extracted from vehicle- and hormone-treated groups at the 12-h time point and subjected to real-time PCR for *Akp2* (C) and *Akp6* (D). Different letters on top of bars show a significant difference ($P<0.05$) among these groups. Data are shown as mean $\pm$ s.d.

While the uterine LE remains a source of both TNAP and gIAP prior to and during implantation, decidual cells following implantation are the only source of TNAP

Because hamster uterine *Akp2* and *Akp6* expressions are regulated by steroid hormones, we then examined localization and expression patterns of *Akp2* and *Akp6* mRNAs by *in situ* hybridization and gIAP protein (the product of *Akp6*) by immunohistochemistry in the uterus during the preimplantation, implantation, and decidualization periods (days 1–8 of pregnancy) (Fig. 4A, B, and C). Cell-specific localization of the *Akp2* product TNAP was not performed as commercially available TNAP antibodies failed to recognize hamster TNAP protein. During the preimplantation period (days 1–4 morning), the day 1 uterus showed moderate expression of *Akp2* and *Akp6* mRNAs only in cells of the LE. Both the *Akp2* (Fig. 4A) and *Akp6* (Fig. 4B) mRNA signals were then reduced in the day 2 uterus. However, *Akp2* and *Akp6* mRNA expression in the uterine LE became stronger on days 3 and 4 of pregnancy than on days 1 and 2 of pregnancy. Uterine myometrial and stromal cells showed little or no expression of *Akp2* and *Akp6* mRNAs from days 1 to 4 of pregnancy. Immunoreactivity of gIAP was detected in uterine LE cells, and gIAP protein expression patterns were very similar to *Akp6* mRNA expression patterns in preimplantation uteri. The expression of gIAP protein was lower on day 2 than on day 1 of pregnancy but showed an increase on days 3 and 4 of pregnancy (Fig. 4C). We next conducted studies to determine whether their expression patterns at the uterine implantation site show any cell specificity in

response to implantation. On day 5 of pregnancy, stronger *Akp2* mRNA expression was observed in cells of the entire LE away from the implantation site when compared with its expression in luminal epithelial cells surrounding the implanted embryo (Fig. 4A). By contrast, *Akp6* mRNA showed strong expression in the LE cells surrounding the implanted embryo as well as in cells of the LE immediately above the implantation chamber toward the mesometrial side and reduced expression in the LE cells further away from the implantation chamber (Fig. 4B). This unusual *Akp6* mRNA expression in day 5 implantation sites led us to examine its expression pattern in implantation sites obtained from day 4 of pregnancy at 1800 h when the initial blastocyst–uterine attachment reaction occurs in hamsters (Reese *et al.* 2008). A similar pattern of *Akp6* mRNA expression that was observed at the day 5 implantation site was also noted on the early day 4 implantation site (Fig. 4B). The cellular expression pattern of gIAP protein at days 4 and 5 implantation sites followed the similar expression pattern of *Akp6* mRNA. Immunoreactive gIAP protein was primarily detected in the LE cells adjacent to the implanted blastocyst on the evening of day 4 and morning of day 5 (Fig. 4C).

As the early events of implantation induce transformation of uterine stromal cells into decidual cells, decidual cells surrounding the implantation chamber began to show *Akp2*, but not *Akp6*, mRNA expression at low levels on day 5 of pregnancy. However, *Akp2* gradually showed stronger expression in the entire decidual zone surrounding the implanted embryo with

Figure 4 Event-specific change in the uterine cell-specific localization of AP isozyme genes and proteins during early pregnancy. Cross or longitudinal sections from day 1 to day 4 uteri and day 4 to day 8 implantation sites were processed to demonstrate cell-specific expression of *Akp2* (A) and *Akp6* (B) mRNAs and glAP protein (C). BL, blastocyst; EM, embryo; LE, luminal epithelium; PDZ, primary decidual zone; SDZ, secondary decidual zone. (A) *Akp2* mRNA expression by *in situ* hybridization. Photographs were captured under dark field ($n=3$ /day of pregnancy). Sections hybridized with *Akp2* sense probes served as negative controls. (B) *Akp6* mRNA expression by *in situ* hybridization. Photographs were captured under dark field ($n=3$ /day of pregnancy). Longitudinal sections from day 4 morning (0900 h) uteri and day 4 evening (1800 h) implantation sites were used for hybridization. Inset shows higher magnification (200 $\times$) picture. Sections hybridized with *Akp6* sense probes served as negative controls. (C) Immunohistochemical detection of glAP. Photographs were captured under bright field at 400 $\times$ magnification ($n=3$ or 4/day of pregnancy). The specificity of immunostaining was confirmed by replacing primary antibody with non-immune rabbit IgG. Arrow indicates apical immunolocalization of glAP.

the progression of the implantation/decidualization process from days 6 to 8 of pregnancy (Fig. 4A). Embryos at the days 7 and 8 implantation sites showed low levels of *Akp2*, but not *Akp6*, mRNA expression (Fig. 4A and B). Remaining luminal epithelial cells at the mesometrial side of the implantation site showed low levels of both *Akp2* and *Akp6* mRNA expression. These data suggested that while mRNAs for both the *Akp2* and *Akp6* isozyme genes and glAP protein are expressed in uterine luminal epithelial cells, the *Akp2* isozyme gene is specific for uterine decidual cells following implantation.

Histochemical detection of uterine total AP activity and its isozyme-specific contributions during the preimplantation, implantation, and decidualization phases

Having established that cellular *Akp2* and *Akp6* mRNAs showed event-specific expression in uterine cells prior to, during, and following implantation, we next investigated the total biochemical activity of uterine AP enzymes in the uterus during the preimplantation, implantation, and decidualization periods to correlate total AP activity patterns with the observed expression patterns of *Akp2* and *Akp6* mRNAs. In addition, because of uterine

expression of both the *Akp2* and *Akp6* gene products, histochemical examination of uterine AP activity was performed in the presence or absence of levamisole to differentiate levamisole-sensitive and levamisole-insensitive uterine total AP activity. Levamisole is a well-known and widely used uncompetitive inhibitor of the *Akp2* gene product TNAP (Kozlenkov *et al.* 2004), but its sensitivity toward the activity of glAP is unknown. As no specific inhibitor of glAP has been reported, we assumed at this stage that the levamisole-insensitive AP activity in the uterine LE would be contributed by a product of an AP isozyme gene other than *Akp2*.

The results in Fig. 5A and B showed that the total activity of uterine APs from days 1 to 4 of pregnancy correlates well with the combined expression patterns of both *Akp2* and *Akp6* mRNAs on these days. The results in Fig. 5B showed that levamisole failed to block complete activity of AP in uterine epithelial cells during the preimplantation period. The pattern of the levamisole-insensitive AP activity in the preimplantation uterus correlates well with the *Akp6* mRNA and glAP protein expression pattern on these days. Thus, we predicted that the remaining levamisole-insensitive uterine AP activity in the preimplantation uterus is due to the presence of glAP protein.

Figure 5 Total and levamisole-sensitive and -insensitive AP activities in the peri-implantation hamster uterus. Cross sections from days 1 to 4 uteri and days 4 to 8 implantation sites were processed to demonstrate cell-specific localization of total AP (A) and levamisole-insensitive AP (B) activities. Photographs were captured under bright field ($n=3$ or 4/day of pregnancy). BL, blastocyst; EM, embryo; LE, luminal epithelium; PDZ, primary decidual zone; S, stroma; SDZ, secondary decidual zone. (A) Total AP activity in peri-implantation uteri (days 1–8) as determined by histochemistry. Specificity of AP staining in sections from day 1 uteri was demonstrated by destroying endogenous enzyme activity through microwave heating. (B) Residual AP (levamisole-insensitive) activity after levamisole treatment in the peri-implantation (days 1–6) uterine sections.

We next investigated the total AP activity pattern as well as levamisole sensitivity of APs at the initial period of implantation and during decidualization. The LE cells adjacent to and away from days 4 to 5 implantation sites showed strong AP activity (Fig. 5A and B). However, while levamisole treatment on sections from days 4 to 5 implantation sites eliminated the AP activity from the LE cells away from the implantation site, a large proportion of the total AP activity still persisted in LE cells surrounding the implanted blastocyst (Fig. 5B). This epithelial levamisole-insensitive AP activity pattern surrounding the implanted blastocyst corresponds to the expression pattern of *Akp6* mRNAs and glAP protein. Together, these findings clearly show unique epithelial

glAP protein expression and activity at the implantation site. Uterine stromal cells surrounding the implanted embryo begin to show AP activity early on day 5 of pregnancy. The entire decidual zone surrounding the implanted embryo showed strong AP activity from days 6 to 8 of pregnancy. In decidual cells, however, the AP activity pattern overlapped with only the *Akp2* mRNA expression pattern on these cells. Furthermore, when uterine sections from day 6 implantation sites were pretreated with levamisole, the AP activity was considerably inhibited in luminal epithelial cells and completely abolished from cells of the day 6 decidua (Fig. 5B). These data suggested that while AP isozyme activities in uterine luminal epithelial cells are contributed by the TNAP and glAP isozymes, AP activity in decidual cells following implantation is contributed by only the TNAP isozyme.

Decidual Akp2 and TNAP activity is not influenced by the implanted blastocyst

In an attempt to examine whether expression of *Akp2* mRNA and TNAP activity in decidual tissues following implantation is solely a function of the implanted blastocyst, *Akp2* mRNA and TNAP activity was checked in sections obtained from day 6 decidual tissues induced by the embryo (decidua) or suture (deciduomata). *Akp2* mRNA (Fig. 6A) and TNAP activity (Fig. 6B) were noted in sections from both the decidua and deciduomata. However, the intensity of *Akp2* mRNA and AP activity was stronger in the decidua than in the deciduomata. AP activity in cells of both the decidua and deciduomata was levamisole sensitive (Fig. 6B). These results suggest that decidual expression of the *Akp2* gene and TNAP activity is not exclusively regulated by the embryo. An additional noticeable finding in this study was that the remaining epithelial layer at the implantation site and suture-induced decidual area and intact epithelial cells at the mesometrial side were AP positive and levamisole insensitive.

Uterine AP possessed the property of LPS detoxification by dephosphorylation

To explore the role of hamster uterine AP in LPS dephosphorylation, biochemical AP activity was measured in homogenates from the day 1 uterus and day 7 implantation sites using LPS as a substrate. Tissue homogenates in the presence of LPS showed a significant increase in inorganic phosphate (Pi) release compared with the control group in which LPS was not added. When enzyme activity of uterine homogenates was inactivated at 95 °C, no considerable change in Pi release was observed after LPS addition when compared with the control group without LPS (Fig. 7A and B). In order to define the uterine-specific APs that are responsible for the LPS dephosphorylation, levamisole

Figure 6 Expression of *Akp2* mRNA (A) and total AP activity (B) in the day 6 embryo-induced deciduum and suture-induced deciduomata. EM, embryo. (A) Expression of *Akp2* mRNA by *in situ* hybridization. Photographs were captured under dark field ($n=3$). (B) Histochemical staining of AP activity in the presence and absence of levamisole (Lev). Photographs were captured under bright field ($n=4$). Inset shows higher magnification (200 $\times$) of levamisole-insensitive AP activity in residual epithelial cells.

(inhibitor of TNAP) was added into homogenates prior to and during incubation with LPS. While levamisole entirely blocked the Pi release from LPS by homogenates of day 7 implantation sites (Fig. 7B), it was only partially effective in inhibiting Pi release from LPS by day 1 uterine homogenates (Fig. 7A). The day 1 uterine LPS-dephosphorylating effect that was not blocked by levamisole treatment indicated that this fraction of the remaining uterine AP activity is insensitive to levamisole. At this point, it is unclear whether the residual day 1 uterine LPS dephosphorylation activity is due to the presence of gIAP as it is not known whether gIAP activity is sensitive or insensitive to levamisole. Thus, an LPS hydrolysis assay was performed using recombinant TNAP and gIAP proteins in the presence or absence of levamisole (Fig. 7C). The majority of the observed TNAP activity (99.95%) was inhibited by levamisole while gIAP activity was partially (32.08%) affected by levamisole. Together, our data suggested that TNAP is the primary AP responsible for LPS dephosphorylation at the day 7 implantation sites of the hamster. However, the day 1 uterine AP responsible for LPS dephosphorylation was partially contributed by TNAP as well as gIAP.

LPS dephosphorylation was detected at uterine cellular sites of the AP isozyme mRNA expression and activity

After establishing LPS dephosphorylation biochemically by uterine APs, we examined histochemically the sites of LPS dephosphorylation in the cyclic, preimplantation, and post-implantation uterus. Staining of sections from cyclic uteri, days 1 and 3 of preimplantation uteri, and implantation sites from days 5 to 7 of post-implantation uteri for AP activity at pH 7.6 using LPS as a substrate yielded a pattern corresponding with the pattern and cell-specific localization of AP activity as demonstrated using the substrate BCIP at pH 9.5 (Fig. 8). Control sections incubated in the buffer without LPS exhibited no staining. Uterine sections from four stages of the cycle (Fig. 8A), and days 1 and 3 of pregnancy (Fig. 8B), displayed lead sulfide precipitates in the apical surface of the LE cells in the presence of LPS. Parallel to AP activity observed during the cycle using BCIP (Fig. 2), the LE cells of the diestrous uterus showed stronger LPS dephosphorylation than the uterine LE cells from the rest of the cycle. Similarly, parallel to AP activity seen in the preimplantation uteri using BCIP (Fig. 5), day 3 uterine LE cells showed stronger LPS dephosphorylation than day 1 uterine LE cells. While uterine LE cells surrounding as well as away from the day 5 implantation sites showed dark brown staining, only cells of the decidual of day 7 implantation site exhibited brown staining in the presence of LPS. The results presented in the lower panel of Fig. 8B showed that levamisole failed to block complete activity of AP in uterine epithelial cells in sections from days 1 to 3 of pregnancy. Similarly, the residual AP activity was also observed in the LE cells surrounding and immediate to the day 5 implantation sites in the presence of levamisole. The remaining levamisole-insensitive AP activity in these sections may be indicative of LPS dephosphorylation by gIAP. However, levamisole treatment on sections from the day 7 implantation site completely abolished LPS dephosphorylation from the decidual cells, suggesting occurrence of LPS dephosphorylation by TNAP in these cells.

Discussion

The experiments described in this study established at the molecular and cellular levels that cells of the hamster uterine LE express two AP isozyme genes *Akp2* and *Akp6* that encode TNAP and gIAP respectively. These findings support a previous study that emphasized the existence of levamisole-sensitive TNAP and levamisole-insensitive IAP activities in the uterus of the ovariectomized hamster (Grusheikaia & Loktionov 1980). Our findings also agree with their observations that the intestinal type of AP isozyme is primarily expressed in uterine epithelial cells. However, while their study using only histochemical techniques claimed TNAP isozyme expression in stromal cells, our combined studies involving cell-specific localization of *Akp2* mRNAs and TNAP activity

clearly showed TNAP isozyme expression in uterine epithelial, but not in stromal, cells. Expression of uterine *Akp6* seems to be specific in the hamster as studies in the mouse uterus showed expression of only *Akp2* (Pollard *et al.* 1990).

Our results on *Akp2* and *Akp6* mRNA expressions and total AP activity in the uterus of cyclic hamsters showed cyclic variations suggesting regulation of epithelial AP isozymes by steroid hormones. The diestrous uterus in hamsters is influenced by increased plasma levels of P_4 on the metestrous day. However, ovulation and sexual receptivity in this species are characteristics of the estrous stage under the surge of circulating plasma P_4 and estrogen that occurs in the afternoon of the proestrous day (Lukaszewska & Greenwald 1970, Baranczuk & Greenwald 1973). Thus, the increase in the luminal epithelial *Akp2* message on the diestrous and proestrous days when compared with the estrous and metestrous day is coincident with the influence of P_4 on the uterus. However, the strong expression of *Akp6* message on the estrous day in addition to diestrous and proestrous day when compared with the metestrous day is suggestive of regulation by both P_4 and estrogen. The pattern of uterine luminal epithelial total AP activity was positively correlated with epithelial *Akp2* and *Akp6* gene expression patterns in cyclic uteri. The predicted contributions of P_4 and/or estrogen in the regulation of uterine *Akp2* and *Akp6* mRNA expressions during the estrous cycle were next ascertained by hormone replacement experiments in the ovariectomized hamsters. The regulation of *Akp2* mRNA expression in the uterus by P_4 is confirmed, but no synergistic influence of combined treatment of E_2 and P_4 on *Akp2* was noticed. Induction of *Akp2* mRNA and TNAP activity by P_4 has been demonstrated previously in human breast cancer cells (Di *et al.* 1991). As an earlier report in mice suggested regulation of the uterine TNAP activity by E_2 (Manning *et al.* 1969), control of *Akp2* gene expression by P_4 in hamsters seems species specific. As expected from results of the estrous cycle, expression of *Akp6* mRNA in the uterus of ovariectomized hamsters showed regulation by both P_4 and E_2 and the combined P_4/E_2 treatment showed a synergistic effect. This information partly agrees with a previous study in hamsters that

Figure 7 LPS dephosphorylation by day 1 uteri and day 7 implantation sites. LPS-dephosphorylating activity by homogenates from day 1 uteri (A), day 7 implantation sites (B), and recombinant TNAP and gIAP isozymes (C). (A) Biological LPS-dephosphorylating activities present in day 1 uterine homogenates. Levamisole (Lev) failed to totally inhibit LPS-dephosphorylating activities of day 1 uterine homogenates. Results were expressed as mean absorbance value $\pm$ s.d. ($n=5$). Data were analyzed using one-way ANOVA followed by Turkey's test (*, $P<0.05$). (B) Biological LPS-dephosphorylating activities present in homogenates of day 7 implantation sites. Lev inhibited the major fraction of LPS-dephosphorylating activities of day 7 uterine homogenates. Results were expressed as mean absorbance value $\pm$ s.d. ($n=5$). Data were analyzed using one-way ANOVA followed by Turkey's test (*, $P<0.05$). (C) Effect of Lev on the LPS dephosphorylation by recombinant TNAP and gIAP. LPS hydrolysis by TNAP and gIAP in control (Con) groups was considered as 100%. Data were mean $\pm$ s.e.m. of three different determinations (Student's *t*-test, *, $P<0.05$).

Figure 8 LPS dephosphorylation at uterine sites of AP production during the cycle and peri-implantation period. Histochemical localization of AP activity in sections from cyclic (A) and peri-implantation uteri (day 1, day 3 and implantation sites of days 5 and 7) (B) using LPS as a substrate. All sections were counterstained with hematoxylin. A dark brown staining indicated lead sulfide precipitates. Photographs were captured under bright field ($n=4$). Control sections without LPS were completely negative. Residual stainings in levamisole (Lev)-treated sections from day 1, day 3, and day 5 uteri indicated Lev-insensitive glAP activity. BL, blastocyst; EM, embryo; LE, luminal epithelium; S, stroma.

showed increased activity of the intestinal type of AP isozyme in the uterus of ovariectomized hamster treated with only benzoestrol (Grusheikaia & Loktionov 1980).

Previous studies have demonstrated that AP activity is associated with the differentiative state, but not the proliferative state, of epithelial cells (Wood *et al.* 2003, Marvin-Guy *et al.* 2008). Consistent with this view, we observed the lowest AP activity in day 2 epithelial cells when these cells were proliferating and strong AP activity in days 3 and 4 uterine epithelial cells when these cells were differentiating to support implantation (Zhang & Paria 2006). We suggest that in the hamster as opposed to the mouse, in which AP activity in the preimplantation uterus gradually decreased from day 1 onward (Manning *et al.* 1969, Murdoch *et al.* 1978, Bucci & Murphy 1995), uterine epithelial AP activity or mRNA expression patterns of *Akp2* and *Akp6* uphold the promise of useful markers for the receptive uterus. However, it is unknown what role TNAP and glAP might play in uterine LE cells during the receptive state. Although a growing body of evidence supports their involvement in pathophysiological conditions like obstructive jaundice, rickets, hypophosphatasia, and intestinal inflammatory diseases (Narisawa *et al.* 1997, Goldberg *et al.* 2008, Millan *et al.* 2008, Ramasamy *et al.* 2011), neither the specific biological substrate(s) for AP isozymes nor the interrelationship of these isozymes is known. Luminal epithelial cells show apical plasma membrane alterations such as polarity changes and flattening of microvilli with reorganization of apical molecules during epithelial cell surface preparation for blastocyst attachment in a variety of species including the hamster, mouse, rabbit, camels, and human (Bucci & Murphy 1995, tin-Ley 2000, Bagot *et al.* 2001). These changes in the surface of epithelial cells may be associated with phosphorylation/dephosphorylation of proteins and phospholipids in their membrane lipid bilayer. Thus, our findings suggest that hamsters may utilize the AP activity in epithelial cells to support the phosphorylation status of the cell surface molecules that are helpful at the time of the uterine

receptivity and blastocyst attachment. Regarding this possibility, it is known that several plasma membrane enzymes such as metalloproteinases and lipid-hydrolyzing enzymes are involved in alteration of cellular plasma membrane properties during the embryo–uterine attachment reaction (Giudice 1999) and sperm–egg fusion for fertilization (Boldt *et al.* 1988) respectively. However, we are not in a position to predict which AP isozyme (TNAP or glAP) contributes the most to the establishment of uterine receptivity. The co-expression of both TNAP and glAP in the luminal epithelial cells of the preimplantation uterus indicates that these two isozymes may play a compensatory role in the uterine LE. However, the differential expression of *Akp2* and *Akp6* at the early implantation site of this species indicates that the uterus may favor the product of *Akp6* over *Akp2* to bring changes in epithelial surface morphology and biochemistry that are needed at the blastocyst attachment site.

Studies in the post-implantation sites from days 5 to 8 of pregnancy and suture-induced decidual cells in hamsters suggest that any local stimulus that has the ability to induce transformation of stromal cells to decidual cells is adequate to induce decidual TNAP expression. These findings support previous studies in mice that have considered TNAP as an indicator of stromal cell decidualization as strong TNAP activity is observed in cells of both the decidua and deciduomata (Manning *et al.* 1969). On the basis of these results, we suggest that decidual *Akp2* expression may only be associated with inflammatory aspects of normal implantation as stromal cell decidualization as a result of implantation or an other external stimulus has been considered as an inflammatory reaction (Bilinski *et al.* 1998). There is no information about the AP activity in the human decidua following implantation, although the presence of AP activity is reported in uterine pre-decidual cells during the secretory phase of the menstrual cycle (Wilson 1969). AP, along with Bmp2 and IL11R α , is a widely accepted decidual marker. Bmp2 and IL11R signaling at the implantation sites in mice

have been demonstrated to be required for decidual development at the implantation site (Bilinski *et al.* 1998, Lee *et al.* 2007, Ramathal *et al.* 2010). Bmp2 and IL11R α are also potent *Akp2* mRNA and TNAP activity-inducing agents (Suga *et al.* 2001, Kim *et al.* 2004). Thus, it is possible that AP mediates the actions of Bmp2 and IL11 signaling in decidua development. A direct role of AP in decidua formation and function is not yet identified in any species including mice as *Akp2*-null mice die prior to weaning due to skeletal defects and seizures (Waymire *et al.* 1995, Narisawa *et al.* 1997).

Several studies have identified that LPS is a substrate for AP (Bentala *et al.* 2002, Koyama *et al.* 2002, Beumer *et al.* 2003). LPS is a constituent of Gram-negative bacterial cell wall and elicits strong inflammatory responses in tissues. If uterine AP represents a true protective enzyme against LPS, it should be able to detoxify LPS by dephosphorylation, and uterine cells that express AP would be the sites of LPS dephosphorylation. In this study, we found that homogenates of day 1 uterus and day 7 implantation sites when incubated with LPS showed significant amount of Pi release, suggesting uterine AP activity in both days of pregnancy. However, while levamisole fully restrained the LPS dephosphorylation activity of day 7 implantation sites, it only reduced about 50% of LPS dephosphorylation activity of day 1 uterine homogenates. These observations suggest that while the day 1 uterus possesses both the levamisole-sensitive and -nonsensitive AP activities, the day 7 implantation sites only contain levamisole-sensitive TNAP activity. This biochemical assay, however, did not indicate the site of LPS dephosphorylation in uterine cells. Results from our histochemical studies in cyclic and pregnant uteri using LPS as a substrate for AP activity at the physiological pH level showed the same pattern as the AP activity using the conventional substrate BCIP. Based on these findings, we inferred that uterine epithelial and decidual cells are the initial responding cells to endotoxin, and uterine AP isozymes have the potential to detoxify LPS at their site of expression.

In conclusion, we report that two AP isozymes TNAP and gIAP are expressed in the hamster uterus and they might be involved in the process of uterine receptivity, implantation, and decidualization. In addition, they may function as endotoxin detoxification molecules under normal physiological conditions as well as during uterine infection by pathogens. In this regard, it is worth noting that i) *in vivo* studies have demonstrated dephosphorylation of LPS by i.v. administration of exogenous AP (Chen *et al.* 2011) and ii) IAP treatment is beneficial to human ulcerative colitis and a mouse model of chronic colitis (Ramasamy *et al.* 2011). Thus, our studies raise the possibility that exogenous treatment with any AP that has strong catalytic activity could be used as a candidate drug for preventing uterine infection

in general or during pregnancy and for lowering the risk of infection-induced pregnancy loss/defects.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by National Institutes of Health grants HD044741 and ARRA Administrative Supplements to HD044741. Dr H Ni is a recipient of visiting Scholar scholarship from China Scholarship Council to study abroad.

Acknowledgements

The authors thank Dr Klaas Poelstra (University of Groningen, The Netherlands) for kindly sharing the protocol for histochemical detection of LPS dephosphorylation by endogenous AP. The authors wish to express their appreciation to the members of the Collaborative Team of Interdisciplinary Research on Blastocyst Implantation (NICHHD) for their encouragement during the course of this study.

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Received 12 April 2013

First decision 7 May 2013

Revised manuscript received 31 July 2013

Accepted 8 August 2013