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 \textit{in situ} hybridization studies demonstrated that among the known \textit{Akp2}, \textit{Akp3}, \textit{Akp5}, and \textit{Akp6} murine AP isozyme genes, hamster uteri express only \textit{Akp2} and \textit{Akp6}; both genes are co-expressed in luminal epithelial cells. Studies in cyclic and ovariectomized hamsters established that while progesterone (P\textsubscript{4}) is the major uterine \textit{Akp2} inducer, both P\textsubscript{4} and estrogen are strong \textit{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, \textit{Akp2} showed reduced expression in luminal epithelial cells surrounding the implanted embryo. By contrast, expression of \textit{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 \textit{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.

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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 \textit{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 \textit{et al}. 2008, Aisemberg \textit{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 \textit{Akp2}-null mice (Waymire \textit{et al}. 1995), altered fatty acid transport in the gut in \textit{Akp3}-null mice (Narisawa \textit{et al}. 2003, Nakano \textit{et al}. 2007), delayed parturition, and reduced litter size in \textit{Akp5}-null mice (Dehghani \textit{et al}. 2000); and iii) AP may contribute to host defense against pathogen-induced inflammation (Poelstra \textit{et al}. 1997a, 1997b, 1998, 1999, 2002, 2007; Poelstra \& Millan 1997, 2002).
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. Alph) gene in mice. TSAPs in mice include duodenum-specific intestinal AP (dILAP) 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 & Hinchliff 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 (P4)-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 deciduomata 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 deciduomata. The contralateral horns of these animals were not disturbed for normal embryo implantation and deciduomata 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 deciduomata in the suture-containing horn. The embryo-induced decidum and suture-induced deciduomata 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 P4- and estradiol-17β-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), P4 (2 mg/0.2 ml/hamster), estradiol-17β (E2) (1 μg/0.2 ml/hamster), or P4 plus E2. 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′-GGT CAA GGT TGG CCC CAA TGC A-3′; antisense: 5′-GGT GCT GGA ACC CCA GAC CCC GAG-3′; Akp3 (sense: 5′-GGT GCT GGA ACC CCA GAC CCC GAG-3′; antisense: 5′-GGC CCT CTC GAT GGC TAA GTC G-3′; Akp5 (sense: 5′-GCC ACC CTG GAG CAC 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 GAA TCC CAA AG-3′; antisense: 5′-CAG ACC GAC CCA GAA CAG-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_070181082. 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-III-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−ΔΔCt 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′-GGT CAC TAC AAG CAA TCA CTG A-3′; antisense: 5′-GCA AAG ACT GCC ACA TCT CCC-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 GAG 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: Spel/T7 for antisense, EcoRV/Sph for sense; Akp6: EcoRV/Sph for antisense, Spel/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 involvement 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 wavelength) 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 dEAP 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
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 diestrous and proestrous days, and thereafter, its activity from the uterine LE gradually reduced from the proestrous day to metestrous 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 P₄ and Akp6 by both P₄ and E₂ 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 E₂-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 E₂ treatment when compared with oil-injected controls (Fig. 3B). When ovariectomized hamsters were treated with P₄ 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 E₂/P₄ 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 P₄- or E₂-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.
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
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 gIAP 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 isoyme-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 gIAP is unknown. As no specific inhibitor of gIAP 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 gIAP 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 gIAP protein.
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 gIAP protein. Together, these findings clearly show unique epithelial

gIAP 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 gIAP 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 (decidum) or suture (deciduomata). Akp2 mRNA (Fig. 6A) and TNAP activity (Fig. 6B) were noted in sections from both the decidum and deciduomata. However, the intensity of Akp2 mRNA and AP activity was stronger in the decidum than in the deciduomata. AP activity in cells of both the decidum 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
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 decidua 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 isoform is primarily expressed in uterine epithelial cells. However, while their study using only histochemical techniques claimed TNAP isoform expression in stromal cells, our combined studies involving cell-specific localization of Akp2 mRNAs and TNAP activity

(ihibitor 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.
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 P4 on the metestrous day. However, ovulation and sexual receptivity in this species are characteristics of the estrous stage under the surge of circulating plasma P4 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 P4 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 P4 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 P4 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 P4 is confirmed, but no synergistic influence of combined treatment of E2 and P4 on Akp2 was noticed. Induction of Akp2 mRNA and TNAP activity by P4 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 E2 (Manning et al. 1969), control of Akp2 gene expression by P4 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 P4 and E2 and the combined P4/E2 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 ±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 ±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 ± S.E.M. of three different determinations (Student's t-test, *, P<0.05).
showed increased activity of the intestinal type of AP isozyme in the uterus of ovareiectomized hamster treated with only benzestrol (Grushiekaia & 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 in days 3 and 4 uterine epithelial cells when these cells were proliferating and strong AP activity was observed in day 2 epithelial 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 gIAP) contributes the most to the establishment of uterine receptivity. The co-expression of both TNAP and gIAP 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 predecidual cells during the secretory phase of the menstrual cycle (Wilson 1969). AP, along with Bmp2 and IL11Rz, is a widely accepted decidual marker. Bmp2 and IL11R signaling at the implantation sites in mice.

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 gIAP activity. BL, blastocyst; EM, embryo; LE, luminal epithelium; S, stroma.
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 IL11Rα 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 levamisolose-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.

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Role of uterine alkaline phosphatase


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