Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids

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ABSTRACT

Cyclo-oxygenase (COX) is a rate-limiting enzyme that converts arachidonic acid to prostaglandins (PGs) and exists in two isoforms, COX-1 and COX-2. In the rodent, increased uterine vascular permeability at sites of blastocyst apposition is one of the earliest prerequisite events in the implantation process. This event is preceded by generalized uterine edema and luminal closure, and coincides with the initial attachment reaction between the trophectoderm and luminal epithelium. Vasoactive PGs are implicated in these processes. Here we demonstrate that COX genes are differentially regulated in the peri-implantation mouse uterus. During the preimplantation period (days 1–4), the COX-1 gene was expressed in the uterine epithelium mainly on day 4 until the initiation of attachment reaction in the evening after which the expression was downregulated. This COX-1 expression coincides with the generalized uterine edema required for luminal closure. In contrast, the COX-2 gene was expressed in the luminal epithelium and subepithelial stromal cells at the anti-mesometrial pole exclusively surrounding the blastocyst at the time of attachment reaction on day 4 and persisted through the morning of day 5. This uterine gene was not expressed at the sites of blastocyst apposition during progesterone (P_4)-treated delayed implantation, but was readily induced in the uterus surrounding the activated blastocysts after termination of the delay by estradiol-17β (E_2). The results suggest that PG synthesis catalyzed by COX-2 is important for localized increased uterine vascular permeability and attachment reaction. The COX-1 gene that was downregulated from the time of attachment reaction on day 4 was again expressed in the mesometrial and anti-mesometrial secondary decidual beds on days 7 and 8. These results suggest that PGs generated by COX-1 are involved in decidualization and/or continued localized endometrial vascular permeability observed during this period. In contrast, the COX-2 gene, expressed at the anti-mesometrial pole on days 4 and 5, switched its expression to the mesometrial pole from day 6 onward. These results suggest that PGs produced at this site by COX-2 are involved in angiogenesis for the establishment of placenta. In the ovariectomized mice, the COX-1 gene was induced in the epithelium by a combined treatment with P_4 and E_2. However, P_4 and/or E_2 treatments failed to influence the uterine COX-2 gene. Overall, the results suggest that the uterine COX-1 gene is influenced by ovarian steroids, while the COX-2 gene is regulated by the implanting blastocyst during early pregnancy.

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INTRODUCTION

The synchronized development of the embryo to the blastocyst stage, escape of the blastocyst from its zona pellucida, and attainment of the uterus to the receptive state are all essential to the implantation process (Psychoyos 1973). The establishment of a receptive uterus for supporting implantation is primarily regulated by co-ordinated effects of progesterone (P_4) and estrogen (Psychoyos 1973,

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Paria *et al.* 1993*a*). In the rodent, the first conspicuous sign for the initiation of implantation is an increased endometrial vascular permeability at the site of the blastocyst. This is detected as discrete blue bands along the uterus after an intravenous injection of a blue dye solution (Psychoyos 1973), and coincides with the initial attachment reaction between the luminal epithelium and trophectoderm (Enders & Schlafke 1967). This reaction is analogous to proinflammatory reaction and considered to be one of the earliest prerequisite events in implantation (Psychoyos 1973). In the mouse, the attachment reaction occurs at 2200–2300 h on day 4 of pregnancy, and is preceded by uterine luminal closure which results in intimate apposition of the trophectoderm with the luminal epithelium (Enders & Schlafke 1967, Psychoyos 1973, Enders 1976). The attachment reaction is followed by stromal decidualization and apoptosis of the luminal epithelium at the implantation site (Parr *et al.* 1987). This results in subsequent adherence and penetration by trophoblast cells through the underlying basement membrane (Schlafke & Enders 1975). In the mouse, ovariectomy in the morning of day 4 of pregnancy prior to preimplantation ovarian estrogen secretion results in blastocyst dormancy and failure in attachment reaction, a condition termed delayed implantation. Delayed implantation can be maintained by continued P_4 treatment, and terminated by an injection of estrogen with blastocyst activation and initiation of attachment reaction (Yoshinaga & Adams 1966, Huet & Dey 1987). The luminal closure and apposition occur during delayed implantation, but the attachment reaction and the subsequent processes do not occur unless estrogen is provided (Psychoyos 1973, Nilsson 1974, Enders 1976).

The attachment reaction results from an intimate 'cross-talk' between the trophectoderm of the active blastocyst and luminal epithelium of the receptive uterus. However, the molecular basis of this 'cross-talk' remains an enigma. Vasoactive prostaglandins (PGs) are implicated as important mediators of localized endometrial vascular permeability at the time of attachment reaction (Lau *et al.* 1973, Kennedy 1977, Johnson & Dey 1980, Malathy *et al.* 1986, Gupta *et al.* 1989). Furthermore, PGs are considered to be important for the decidual cell reaction (Kennedy 1985, Tawfik *et al.* 1987). Cyclo-oxygenase (COX) is the first rate-limiting enzyme in the biosynthesis of PGs from arachidonic acid (reviewed in DeWitt 1991). COX exists in two isoforms, COX-1 and COX-2, and these isoforms are the products of two different genes (Fletcher *et al.* 1992, Kraemer *et al.* 1992). The murine COX-1 is a 600–602 amino acid, glycosylated, membrane associated hemeprotein that is encoded by a 2·8 kb mRNA transcribed from a 22 kb gene (Kraemer *et al.* 1992). COX-1 is considered a constitutive enzyme, present in virtually all cell types, and is considered to be involved in PG synthesis necessary for normal cellular processes. Murine COX-2 is a 604 amino acid protein encoded by a 4·7kb mRNA transcribed from an 8 kb gene (Fletcher *et al.* 1992). COX-2 exhibits about 60% amino acid identity with COX-1, and is induced by proinflammatory stimuli, growth factors, cytokines and mitogens in a variety of cells (Lin *et al.* 1989, Kujubu *et al.* 1991, Lee *et al.* 1992, DeWitt & Meade 1993, Ristimaki *et al.* 1994).

COX is present in the rodent uterus and embryo, and synthesizes PGs during early pregnancy (Pakrasi & Dey 1983, Malathy *et al.* 1986, Parr *et al.* 1988). A recent study described differential distribution of immunoreactive COX-1 and COX-2 proteins in the mouse uterus, and regulation of COX-2 in uterine cells by cytokines in culture (Jacobs *et al.* 1994). However, information regarding the regulation and expression of these genes in the mammalian uterus or embryo during early pregnancy or under the influence of steroid hormones is still limited. We therefore examined the developmental and hormonal regulation of the COX-1 and COX-2 genes in the peri-implantation mouse uterus. We report herein that the COX-1 and COX-2 genes are discoordinately expressed and regulated in the mouse uterus during the peri-implantation period or under steroid hormonal stimulation.

MATERIALS AND METHODS

Animals and tissue preparation

CD-1 mice (Charles River Laboratories, Raleigh, NC, USA) were housed in the animal care facility at the University of Kansas Medical Center according to NIH and institutional guidelines on the care and use of laboratory animals. Adult female mice (20–25 g, 48–60 days old) were mated with fertile males of the same strain. The morning of finding a vaginal plug was designated day 1 of pregnancy. Mice were killed between 0830 and 0900 h on days 1–8 of pregnancy. To collect uteri at the onset of attachment reaction, mice were killed between 2200 and 2300 h on day 4 of pregnancy. Whole uteri were collected on days 1–7 of pregnancy, while the deciduum and myometrium were surgically separated on day 8. No attempt was made to separate the embryos from the decidua. Early implantation sites on day 4 (2200–2300 h) and day 5 (0830–0900 h) were visualized by intravenous injections

(0·1 ml/mouse) of a Chicago Blue B dye solution (1% in saline). Mice were killed 5 min later (Paria *et al.* 1993*a*). Uteri were processed for Northern and *in situ* hybridization, as well as immunohistochemistry. Pseudopregnant mice produced by mating with vasectomized males were also killed at these times to examine whether embryonic influences were operative.

To induce and maintain delayed implantation, mice were ovariectomized on the morning (0830–0900 h) of day 4 of pregnancy and received daily injections of P_4 (Sigma Chemical Co., St Louis, MO, USA; 2 mg/mouse) from days 5 to 7 (Yoshinaga & Adams 1966, Paria *et al.* 1993*a*,*b*). To terminate the delay and induce implantation, the P_4-primed delayed mice were given an injection of estradiol-17β (E_2; Sigma Chemical Co.; 25 ng/mouse) on the third day of delay (day 7). Dexamethasone (Dex) has been shown to inhibit implantation in the rat (Johnson & Dey 1980). Therefore, to examine whether it inhibits implantation in the mouse, Dex (10 μg/mouse) was injected on day 6, and on day 7 2 h prior to an injection of E_2. Mice were killed 24 h after E_2 injection. Implantation sites were visualized by blue dye injection. Tissues were isolated for *in situ* hybridization.

To determine the effects of estrogen and P_4, mice were ovariectomized without regard to the stage of the estrous cycle and rested for 2 weeks. They were then treated with an injection of E_2 (100 ng/mouse), an injection of P_4 (1 mg/mouse), or a combination of the same doses of P_4 and E_2. All steroids were dissolved in sesame oil and injected s.c. (0·1 ml/mouse). The control animals received vehicle only (0·1 ml/mouse). Mice were killed at various times after the hormone injections and their uteri collected for RNA extraction and *in situ* hybridization.

To determine whether uterine expression of COX genes is influenced by cytokines, ovariectomized mice were injected intraperitoneally with bacterial lipopolysaccharide (LPS; Sigma Chemical Co., 100 μg/mouse in saline) or Dex (10 μg/mouse) alone or in combination before or 24 h after treatment with E_2 (75 ng/mouse). Mice were killed at various times after treatments. Total RNA was isolated for Northern blot hybridization.

Hybridization probes

A mouse cDNA fragment from the 5′ coding region of COX-1 (Asn_{82}–Gln_{360}) (DeWitt *et al.* 1990) or COX-2 (Met_1–Gln_{270} including 55 nt at 5′ untranslated region) (DeWitt & Meade 1993) was subcloned into a pGEM vector at the PstI site or EcoRI/PstI sites respectively. The mouse amphiregulin (AR) cDNA clone (pBSMAR) encompassing the entire coding sequence in pBluescript SKII+ vector was used (Das *et al.* 1995). A rat cDNA fragment of heparin-binding epidermal growth factor-like growth factor (HB-EGF) was subcloned into a pGEM vector (Abraham *et al.* 1993, Das *et al.* 1994). For Northern hybridization, antisense ^{32}P-labeled cRNA probes were generated, while for *in situ* hybridization, sense and antisense ^{35}S-labeled cRNA probes were generated using the appropriate polymerases. Probes had specific activities of about 2×10^9 d.p.m./μg.

Northern blot hybridization

Total RNA was extracted from whole uteri by a modified guanidine thiocyanate procedure (Han *et al.* 1987, Das *et al.* 1992, 1994). Total RNA (2·0 or 6 μg) was denatured, separated by formaldehyde–agarose gel electrophoresis, and transferred to nylon membranes. RNA was cross-linked to the membranes by u.v. irradiation (Spectrolinker, XL-1500; Spectronics Corp., Westbury, NY, USA) and the blots were prehybridized, hybridized, and washed as described previously (Das *et al.* 1992, 1994). After hybridization, the blots were washed under stringent conditions, and the hybrids detected by autoradiography (Das *et al.* 1994). The stripping of the hybridized probe for subsequent rehybridization was achieved as described previously (Das *et al.* 1992, Wang *et al.* 1994). Each blot was hybridized with the COX-1 and COX-2 probes, sequentially. The blots containing uterine RNA samples from steroid-treated mice were hybridized in sequence with the COX-1, COX-2, HB-EGF and AR. The HB-EGF and AR cRNA probes were used as positive controls for E_2 and/or P_4 action (Wang *et al.* 1994, Das *et al.* 1995). In all experiments, duplicate gels were stained with acridine orange to document the integrity of RNA samples and to confirm that equal amounts of RNA had been loaded onto each lane. Hybrids were detected by autoradiography. Exposure times are indicated in the Figure legends.

In situ hybridization

In situ hybridization followed the protocol described previously (Das *et al.* 1994). Uteri were excised, cleaned of fat tissues, cut into 4–6 mm pieces, and flash frozen in freon. Frozen sections (10 μm) from days 1–4 or days 5–8 of pregnancy were mounted onto poly-L-lysine-coated slides, air-dried and stored desiccated at − 80 °C until use. Sections were brought to room temperature, fixed

in cold 4% paraformaldehyde solution in PBS, acetylated and hybridized at 45 °C for 4 h in 50% formamide buffer containing ^{35}S-labeled antisense cRNA probes specific to COX-1 or COX-2. After hybridization and washing, the slides were incubated with RNase A (20 μg/ml) at 37°C for 15 min, and RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion. Parallel sections hybridized with the sense probes served as negative controls. Slides were post-stained with hematoxylin and eosin.

Antibodies to COX-1 and COX-2

Antipeptide antibodies to COX-1 or COX-2 were kindly provided by Dr J L Pace of our Institute. These antibodies were produced using the synthetic peptide encompassing the unique sequences in the N-terminal region of mouse COX-1 (DeWitt *et al.* 1990) and the C-terminal region of mouse COX-2 (Kujubu *et al.* 1991) using a protocol published previously (Kujubu *et al.* 1993) with some modifications. Briefly, rabbits were immunized with the appropriate peptides coupled to a carrier protein using glutaraldehyde. The peptide, ADPGVPSPV, corresponding to amino acids 1–9 of the mature COX-1 (protein lacking N-terminal signal sequence), was coupled to keyhole limpet hemocyanin and used to produce a rabbit antiserum to COX-1. The peptide, NASASHSRLDDINPT, corresponding to amino acids 563–577 of the mature COX-2 protein, was coupled to thyroglobulin and used to produce a rabbit antiserum to COX-2. Specific antipeptide antibodies were affinity purified over Affi-gel 15 columns to which had been coupled the appropriate peptide/BSA conjugate.

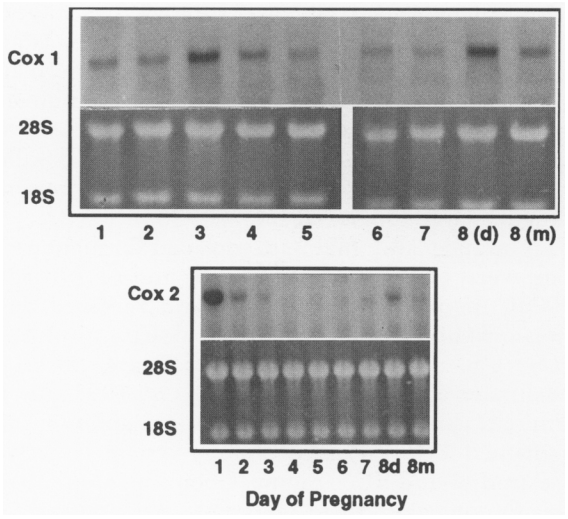
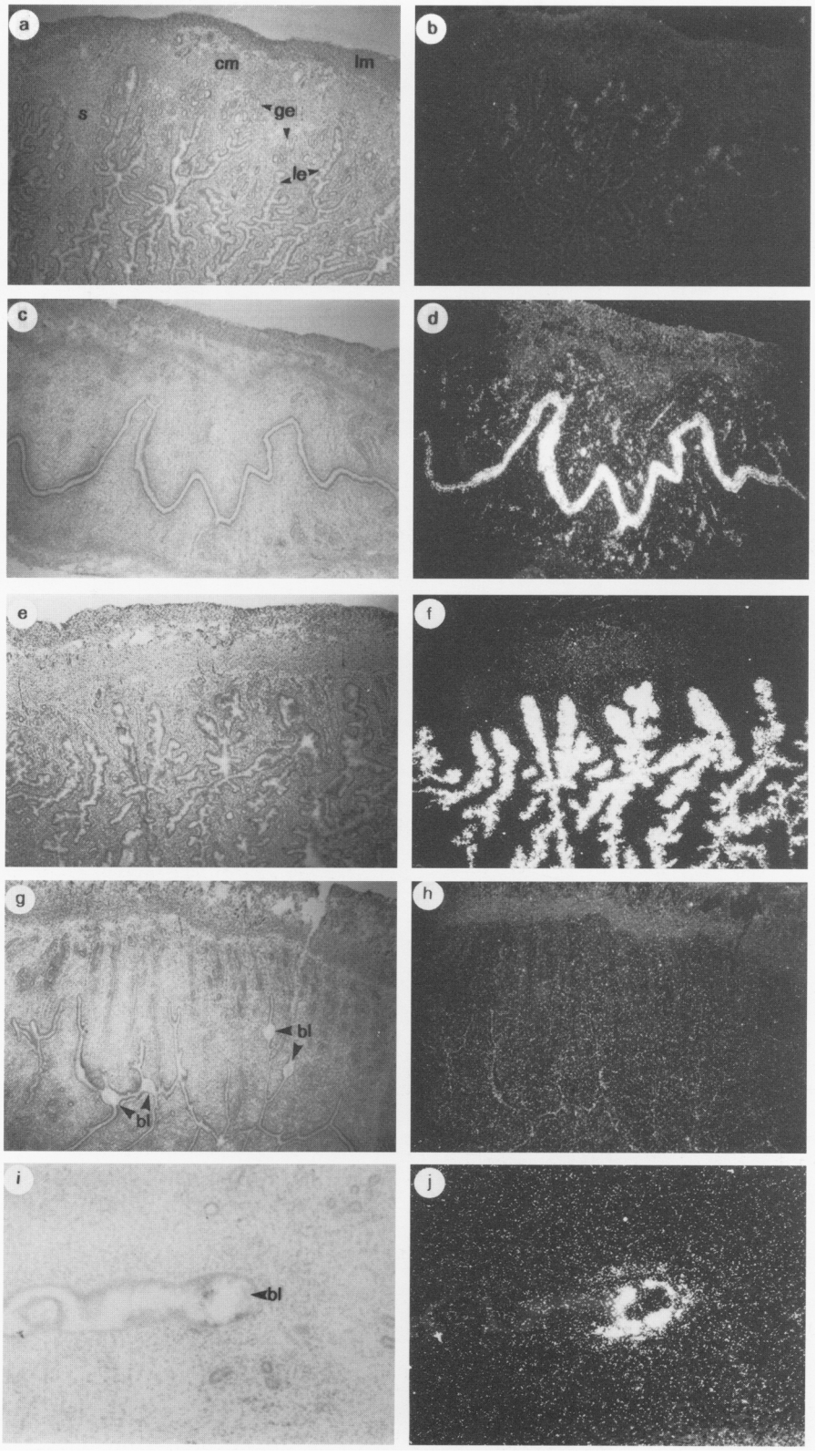


FIGURE 1. Northern blot analysis of COX-1 and COX-2 mRNAs in the mouse uterus during the peri-implantation period. The steady-state levels of uterine COX-1 and COX-2 mRNAs were detected in whole uterine total RNA samples obtained on days 1–7 or the separated deciduum (d) and myometrium (m) on day 8 of pregnancy. Total RNAs (2 μg) were separated by formaldehyde–agarose gel electrophoresis, transferred to nylon membranes, u.v. cross-linked and hybridized to ^{32}P-labeled cRNA probes specific for mouse COX-1 and COX-2 mRNAs. The upper panel shows the COX-1 transcript (2·8 kb), while the lower panel shows that of COX-2 (4·7 kb). RNA samples in duplicate gels were stained with acridine orange to ensure equal loading. The mobilities of 28S and 18S rRNAs are indicated. These experiments were repeated twice with two separate sets of animals in each group and similar results were obtained.

Immunohistochemistry

Immunohistochemical localization of COX-1 and COX-2 was performed following the protocol described previously (Das *et al.* 1994). In brief, frozen sections of uteri (10 μm) were mounted onto poly-L-lysine-coated slides, fixed in Bouin's solution for 20 min, washed in PBS and incubated in blocking solution (10% normal goat serum) for 10 min, followed by incubation with antibodies to COX-1 and COX-2 for 24 h at 4 °C in a moist chamber. Immunostaining was performed using

FIGURE 2. *In situ* hybridization of COX-1 and COX-2 mRNAs in the preimplantation (days 1–4) mouse uterus. Frozen sections (10 μm) were mounted onto poly-L-lysine-coated slides and fixed in 4% cold paraformaldehyde in PBS for 10 min. Sections were hybridized with a ^{35}S-labeled COX-1 or COX-2 antisense cRNA probe. Uterine sections from days 1–4 were mounted onto the same slide. RNase A resistant hybrids were detected after 8 days of autoradiography using Kodak NTB-2 liquid emulsion. COX-1 mRNA distribution in day-1 and day-4 uterine sections is shown in (a and c) brightfield and (b and d) darkfield photomicrographs respectively. COX-2 mRNA distribution in day-1, day-4 morning (0900 h) and day-4 evening (2300 h) sections is shown in (e, g and i) brightfield and (f, h and j) darkfield photomicrographs respectively. White grains under darkfield indicate the sites of mRNA accumulation. All photomicrographs are shown with a magnification of 22 ×, except (i) and (j), which are shown at 55 ×. le, luminal epithelium; ge, glandular epithelium; s, stroma; cm, circular muscle; lm, longitudinal muscle; bl, blastocyst.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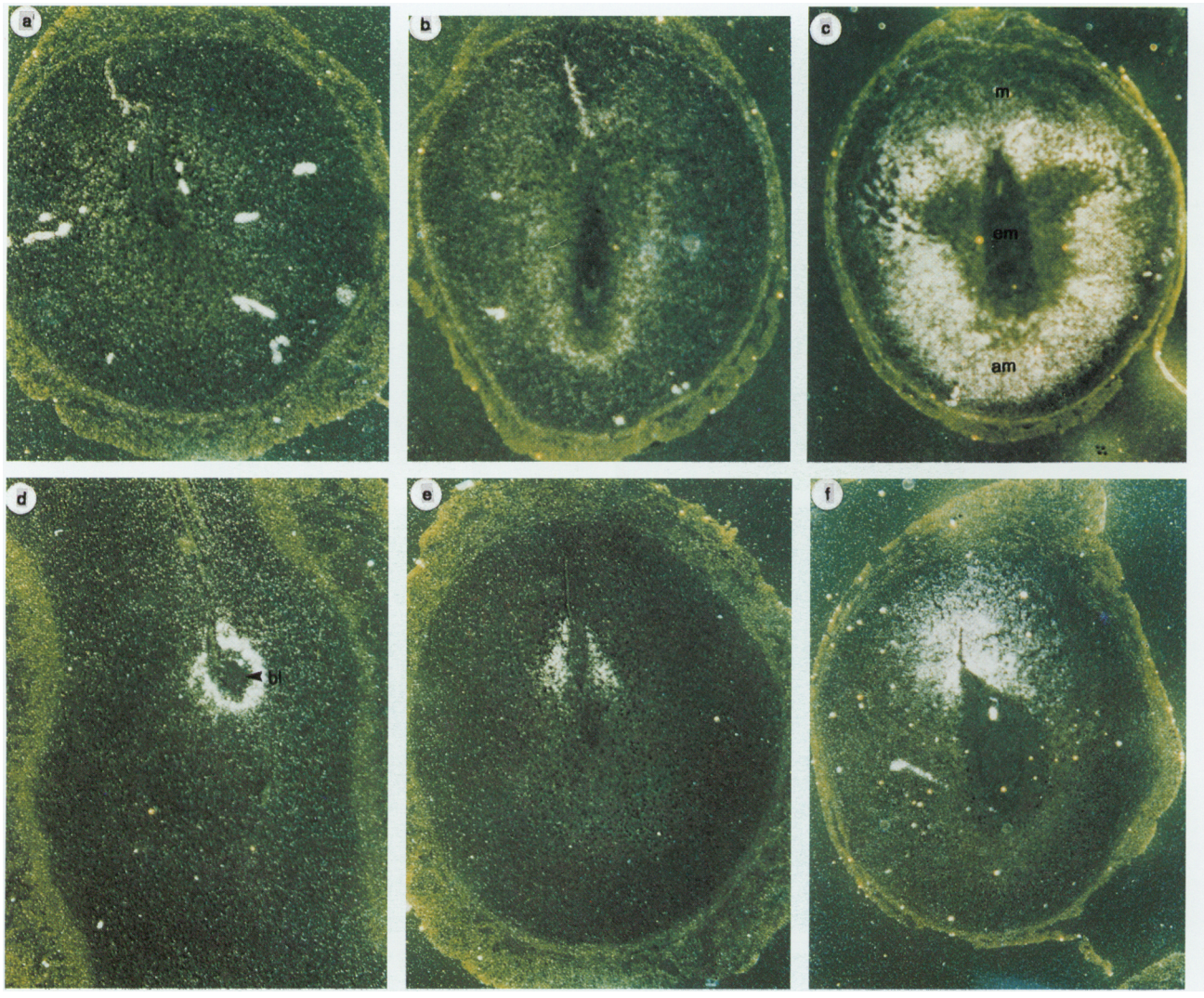


FIGURE 3. *In situ* hybridization of COX-1 and COX-2 mRNAs in the postimplantation (days 5–8) mouse uterus. Sectioning and hybridization were as described in the legend to Fig. 2. COX-1 mRNA is shown in the upper panels (a, day 6; b, day 7; c, day 8), while COX-2 mRNA is shown in the lower panels (d, day 5; e, day 6; f, day 8). All photomicrographs were taken under a darkfield microscope and are shown at a magnification of 28 ×, except panels (c) and (f), which are shown at 17·5 ×. White grains indicate the sites of mRNA accumulation. bl, blastocyst; m, mesometrial pole; am, antimesometrial pole; em, embryo.

a Zymed-Histostain-SP kit (Zymed Laboratories, San Francisco, CA, USA) containing a biotinylated secondary antibody, a horseradish peroxidase–streptavidin conjugate and a substrate–chromogen mixture. Endogenous peroxidase activity was blocked by 0·23% periodic acid in PBS for 30 s after incubating with the secondary antibody. Specificity of the staining was checked by neutralizing the antibodies with tenfold molar excess of the peptides used to generate the antibodies. Red deposits indicate the sites of positive immunostaining. Slides were counterstained lightly with hematoxylin.

RESULTS

Northern blot analysis of COX mRNAs in the peri-implantation uterus

The steady-state levels of COX-1 and COX-2 mRNAs in the uterus on days 1–8 of pregnancy were analyzed by Northern blot hybridization,

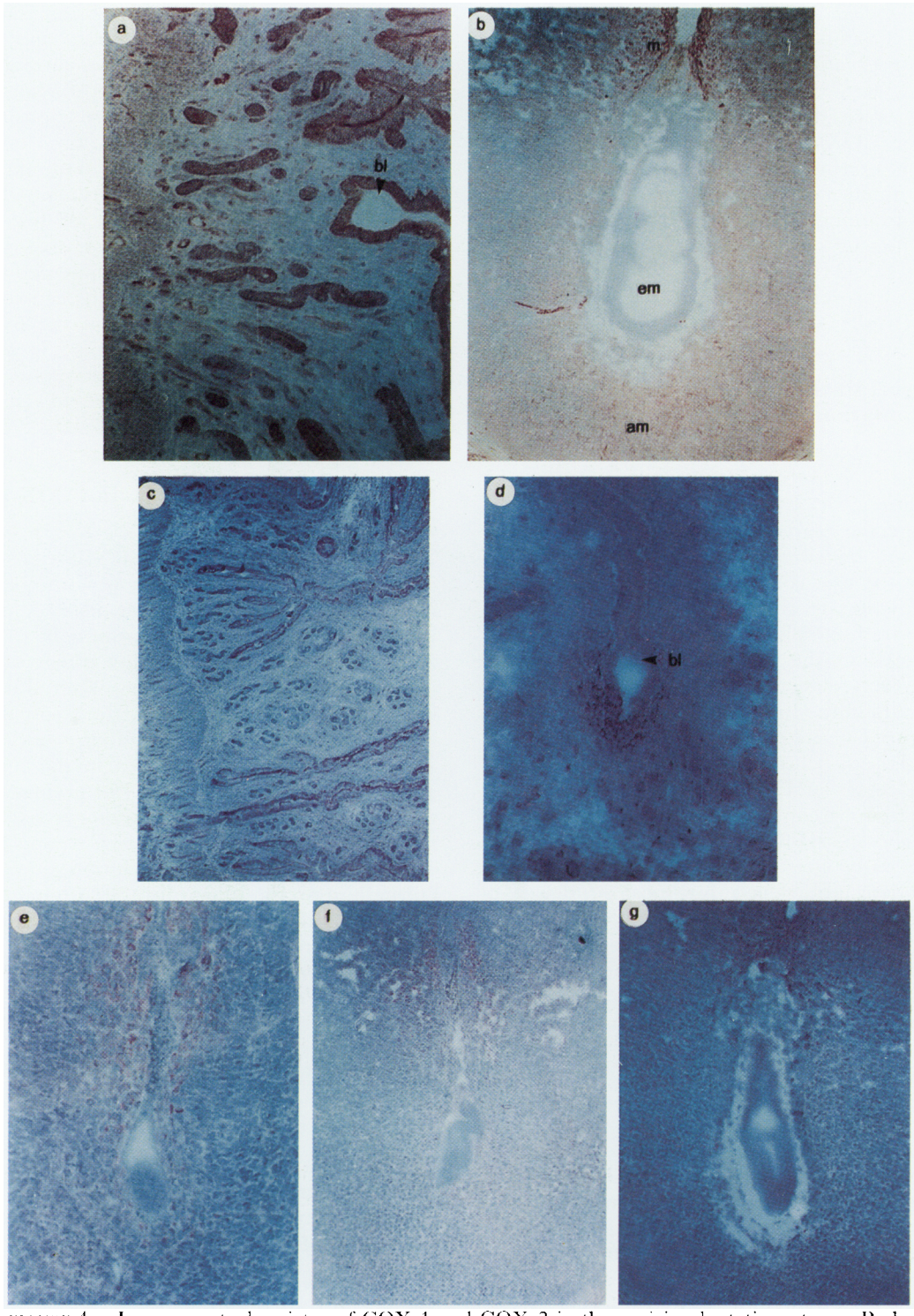


FIGURE 4. Immunocytochemistry of COX-1 and COX-2 in the peri-implantation uterus. Red deposits indicate the sites of positive immunostaining. Photomicrographs of COX-1 immunostaining on (a) day 4 and (b) day 8 are shown in the upper panel. Photomicrographs of COX-2 immunostaining on (c) day 1 and (d–g) days 5–8 are shown in the middle and lower panels respectively. Magnification is 73 × for days 1 and 4–6, and 29 × for days 7 and 8. bl, blastocyst; em, embryo; m, mesometrial pole; am, antimesometrial pole.

using ^{32}P-labeled antisense cRNA probes specific for mouse COX-1 and COX-2. Consistent with previous observations in other tissues (Fletcher *et al.* 1992, Kraemer *et al.* 1992), 2·8 and 4·7kb transcripts were detected in uterine total RNA samples for COX-1 and COX-2 mRNAs respectively. The uterine levels of COX-1 mRNA did not show much variation on the various days of pregnancy examined, except for modest changes in the day-3 uterus or day-8 decidua (Fig. 1). The levels of COX-2 mRNA were high on day 1 of pregnancy, but remained at relatively low levels thereafter (Fig. 1).

In situ hybridization of COX mRNAs in the peri-implantation uterus

The above results suggested that if the expression of COX genes is important for implantation and/or decidualization, it would be through localized cell-type specific expression in the uterus. Thus, we examined the distribution of COX-1 and COX-2 mRNAs in the uterus by *in situ* hybridization. The results demonstrate that on day 1 of pregnancy, modest autoradiographic signals for COX-1 mRNA were detected in the glandular epithelium, while high levels were detected for COX-2 mRNA in the luminal epithelium (Fig. 2a, b, e and f). However, no signal for COX-2 mRNA could be detected in the glandular epithelium (Fig. 2e and f). On days 2 and 3, the levels of COX-1 mRNA did not show much variation as compared with the day-1 uterus (data not shown). However, COX-2 mRNA levels declined rapidly after day 1, reaching and maintaining undetectable levels for most of day 4 of pregnancy (Fig. 2g and h). In contrast, the accumulation of COX-1 mRNA was remarkably high in the luminal and glandular epithelium on day 4 (Fig. 2c and d). With the initiation of the attachment reaction (blue reaction) on the evening of day 4 (2200–2300 h), an interesting pattern of expression was noted for these genes. COX-1 mRNA levels declined dramatically and remained low through day 6 of pregnancy; only some glands and blood vessel endothelium in the decidual bed exhibited signals for this mRNA (Fig. 3a). On day 7 of pregnancy, a modest accumulation of COX-1 mRNA began to appear in the secondary decidual cells, which dramatically increased in these cells at both the mesometrial and antimesometrial poles on day 8 (Fig. 3b and c). On the other hand, COX-2 mRNA, which exhibited no accumulation in the uterus for most of day 4 of pregnancy, was again expressed in specific cell types at the time of attachment reaction, i.e. the autoradiographic signals were detected in the luminal epithelium and adjacent subepithelial stromal cells surrounding the blastocyst at the antimesometrial pole at 2200–2300 h on day 4 (Fig. 2i and j). No autoradiographic signals could be detected in the epithelial or stromal cells inbetween sites of blastocyst apposition, or in the pseudopregnant uteri at any time on day 4 or thereafter (data not shown). On day 5, the distribution of COX-2 mRNA remained similarly localized around the blastocysts at the antimesometrial pole (Fig. 3d). In contrast, on days 6–8, the COX-2 mRNA expression disappeared from the antimesometrial pole, but was instead localized in the decidualizing stromal cells at the mesometrial pole of the implantation site (Fig. 3e and f). No specific autoradiographic signals were detected when uterine sections were hybridized with the sense probes (data not shown).

Immunostaining of COX-1 and COX-2 in the peri-implantation uterus

To examine whether COX mRNAs were translated in the mouse uterus consistent with their mRNA expression pattern, the cellular distribution of these isoforms was examined by immunohistochemistry. Red deposits indicated the sites of positive immunostaining (Fig. 4). The patterns of accumulation were consistent with those of the *in situ* hybridization results. The highlights of these results are that COX-1 was localized in the luminal and glandular epithelium on day 4 of pregnancy, and in the deciduum at both the mesometrial and antimesometrial poles on day 8 (Fig. 4a and b). On the other hand, COX-2 was localized in the luminal epithelium on day 1, and in the luminal epithelium and subepithelial stromal cells surrounding the blastocyst on the morning of day 5 of pregnancy (Fig. 4c and d). Consistent with the mRNA localization, on days 6–8 of pregnancy, COX-2 was localized in the decidual cells at the mesometrial pole (Fig. 4e–g). No positive immunostaining was obtained when sections were incubated in the presence of antibodies preneutralized with an excess of specific antigens (data not shown).

In situ hybridization of COX-2 mRNA in the delayed implanting uterus before and after initiation of implantation

To examine further whether the expression of COX-2 mRNA requires the presence of an activated blastocyst, *in situ* hybridization was performed on uterine sections obtained from P_4-treated delayed implanting mice, or after the initiation of blastocyst activation and implantation by an E_2 injection (Fig. 5). No hybridization signals were detected in

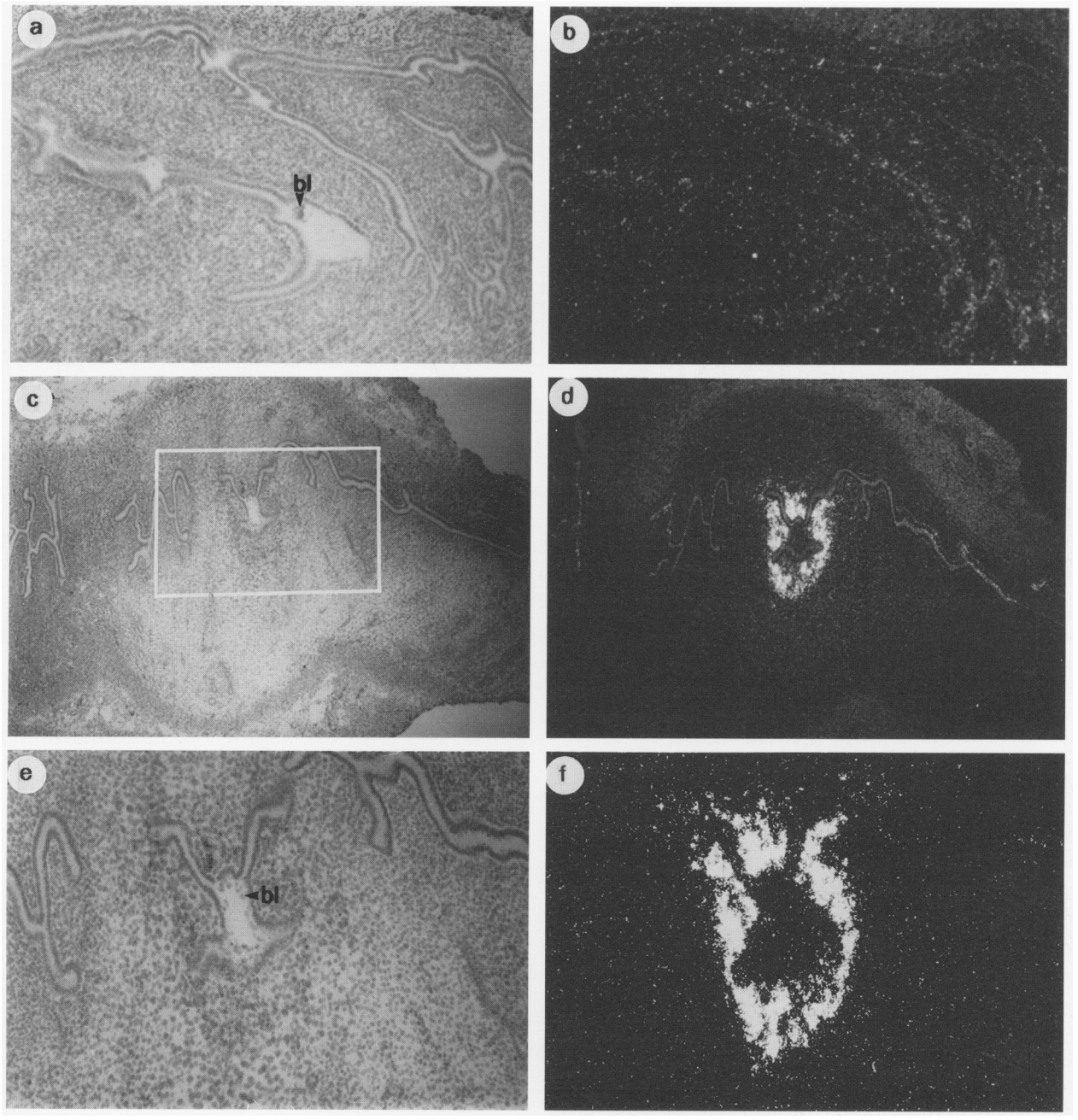


FIGURE 5. *In situ* hybridization of COX-2 mRNA in the delayed implanting uterus before and after the initiation of implantation. COX-2 mRNA distribution in uterine sections from P_4-treated delayed implanting or P_4+E_2-treated mice is shown at 30 × in (a and c) brightfield and (b and d) darkfield photomicrographs respectively. (e) and (f) are higher magnification (76 ×) of (c) and (d). bl, blastocyst.

the luminal epithelial or stromal cells surrounding the dormant blastocysts that had been in close apposition with luminal epithelial cells for 3 days during P_4 treatment (Fig. 5a and b). In contrast, distinct expression of COX-2 mRNA was evident in the subepithelial stromal cells surrounding the activated blastocyst (Fig. 5c–f). Dex, which is known to inhibit the expression of the COX-2 gene

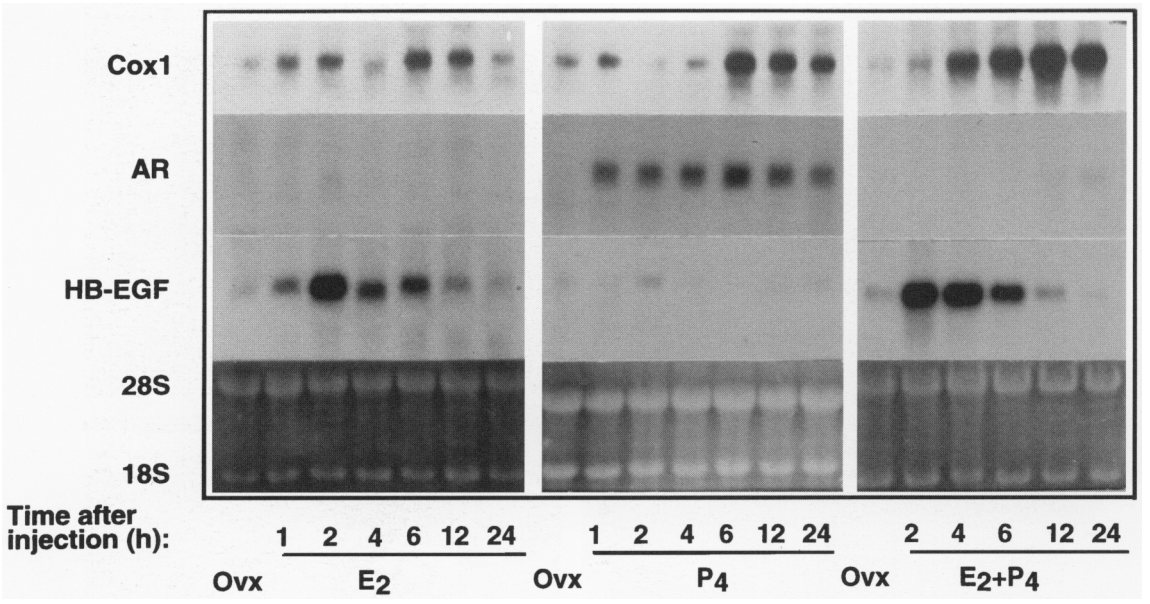


FIGURE 6. Northern blot analysis of COX-1, COX-2, AR and HB-EGF mRNAs in steroid-treated adult ovariectomized mice. Mice were given a single injection of E_2, P_4, or a co-injection of E_2 with P_4. Total RNAs were isolated from uteri collected at the indicated times (h) after steroid treatments. Uterine total RNA from ovariectomized mice isolated 6 h after oil injection served as controls (Ovx). Treatment with E_2 and/or P_4 did not induce uterine COX-2 mRNA at any time-point examined (data not shown). The transcript of AR is 1·4 kb, while that of HB-EGF is 2·4 kb. The mobilities of 28S and 18S rRNAs are indicated. These experiments were repeated twice with two separate sets of animals in each group and similar results were obtained.

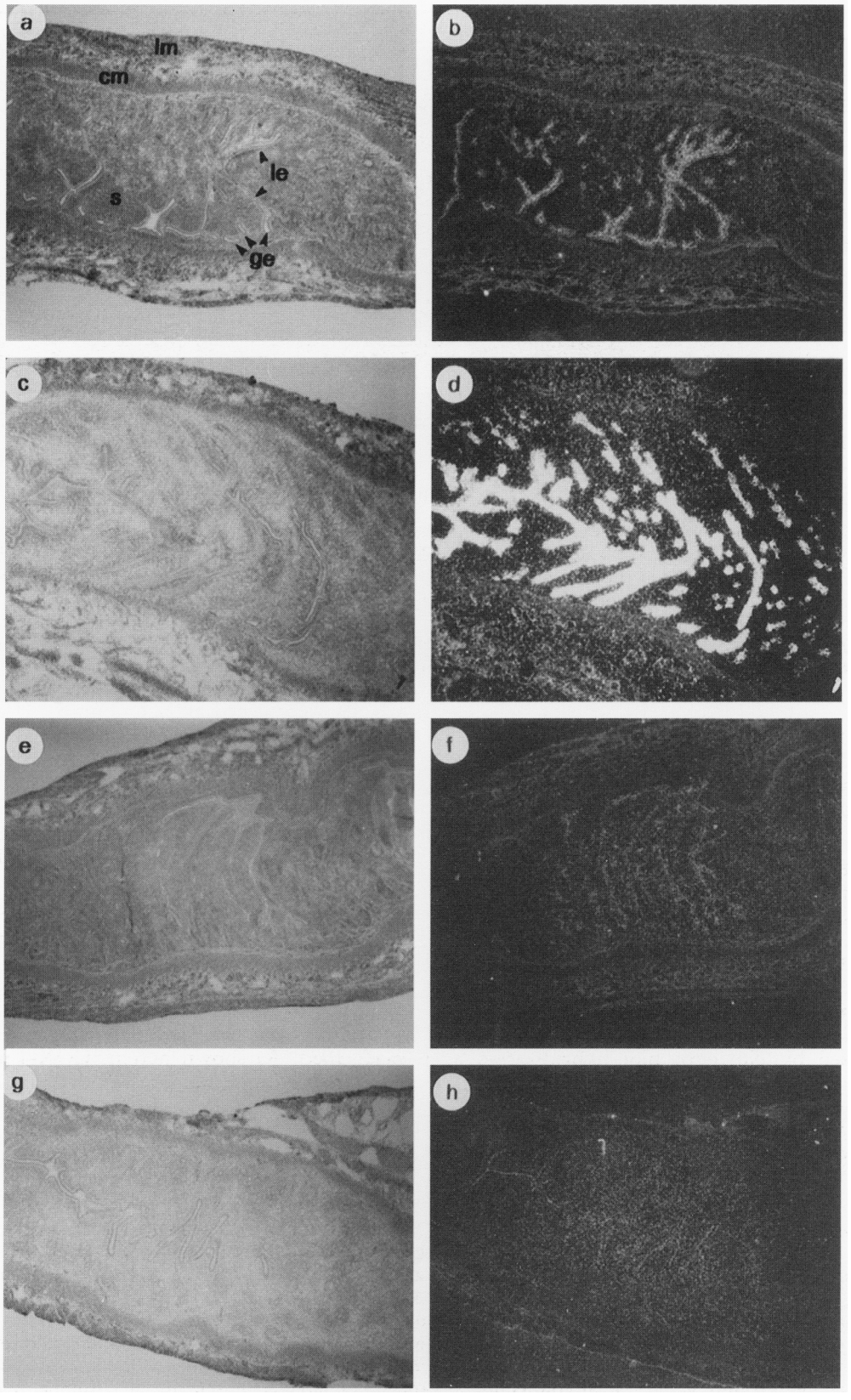
in other systems (DeWitt & Meade 1993), failed to inhibit implantation of the activated embryos or downregulate the expression of COX-2 at the implantation sites (data not shown).

Analysis of COX-1 and COX-2 mRNAs in the adult ovariectomized mouse uterus treated with E_2 and/or P_4

To determine whether ovarian steroids influenced the induction of the COX-1 or COX-2 gene, ovariectomized mice were treated with E_2 and/or P_4, or the vehicle (oil). Total uterine RNA samples were analyzed by Northern blot hybridization at various times after treatment (Fig. 6). The results indicate that treatment with E_2 and/or P_4 did not influence the expression of COX-2 mRNA at any time-point (data not shown). In contrast, E_2 or P_4 alone modestly increased the levels of COX-1 mRNA as compared with the control (oil). However, the onset of accumulation of this mRNA was more rapid (1 h) after treatment with E_2 than with P_4 (6 h). The most dramatic increase was achieved by a coinjection of E_2 with P_4. The induction of AR or HB-EGF mRNA, used as controls for the steroid treatments, corresponded to previously observed patterns (Wang *et al.* 1994, Das *et al.* 1995).

To determine whether these steroid treatments induced the expression of these genes in specific uterine cell types, *in situ* hybridization was performed. As shown herein, COX-1 mRNA persisted in the uterine epithelial cells of ovariectomized mice treated with the vehicle only (oil), albeit at low levels (Fig. 7a and b). However, consistent with the results of Northern blot

FIGURE 7. *In situ* hybridization of COX-1 and COX-2 mRNAs in steroid-treated adult ovariectomized mice. Mice were given a single injection of E_2, P_4, or a co-injection of E_2 with P_4 and killed 12 h later. COX-1 mRNA distribution in uterine sections from ovariectomized, oil-treated and E_2+P_4-treated mice are shown in (a and c) brightfield and (b and d) darkfield photomicrographs (33 ×) respectively. COX-2 mRNA distribution in similarly treated uterine sections is shown in (e and g) brightfield and (f and h) darkfield photomicrographs (33 ×) respectively. An injection of E_2 or P_4 alone induced a modest accumulation of COX-1 mRNA in uterine epithelial cells (data not shown). Consistent with the Northern blot results, none of the steroid treatments induced COX-2 mRNA at any time. le, luminal epithelium; ge, glandular epithelium; s, stroma; cm, circular muscle; lm, longitudinal muscle.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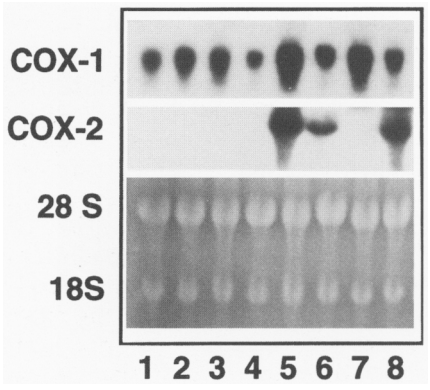

FIGURE 8. Northern blot analysis of COX-1 and COX-2 mRNAs in adult ovariectomized mice treated with E_2, LPS or E_2+LPS. Ovariectomized mice were injected and killed as follows: oil, 4 h (lane 1); E_2, 24 h (lane 2); LPS, 2 h (lane 3); E_2+LPS, 2 h (lane 4); LPS, 4 h (lane 5); E_2+LPS, 4 h (lane 6); LPS, 12 h (lane 7); and E_2+LPS, 12 h (lane 8). Mice treated with E_2 were given their LPS injection 24 h later. The mobilities of 28S and 18S rRNAs are indicated. Following hybridization, blots were subjected to radioimage quantitation using the Radioanalytic Image System (Ambis Systems, San Diego, CA, USA). These experiments were repeated twice with two separate sets of animals in each group and similar results were obtained.

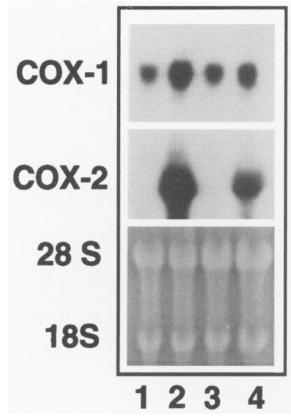

FIGURE 9. Northern blot analysis of COX-1 and COX-2 mRNAs in adult ovariectomized mice treated with LPS and/or Dex. Ovariectomized mice were injected with oil (lane 1), LPS (lane 2), Dex (lane 3) or LPS+Dex (lane 4), and killed 4 h after the last injection. The mobilities of 28S and 18S rRNAs are indicated. These experiments were repeated twice with two separate sets of animals in each group and similar results were obtained.

hybridization, E_2 or P_4 alone modestly upregulated the levels of COX-1 mRNA in the epithelial cells (data not shown). However, the most dramatic increase in the COX-1 mRNA levels was evident in the luminal and glandular epithelium after treatment with both E_2 and P_4 (Fig. 7c and d). In contrast, none of the steroid treatments had any effects on the induction of COX-2 mRNA in the uterus (Fig. 7e–h). This is also consistent with the Northern blot results described above. Uterine sections hybridized with the sense probes did not exhibit any positive signals for these mRNAs (data not shown).

Analysis of COX mRNAs in the E_2-treated uterus following LPS and/or Dex treatment

LPS upregulated the expression of COX-1 or COX-2 mRNA in the ovariectomized mouse uterus (Figs 8 and 9). The induction of COX-1 was most prominent 4 h (sixfold) after treatment and persisted through 12 h (fourfold). However, the same treatment in the E_2-primed uterus downregulated the expression of this gene (Fig. 8). In contrast, LPS induced COX-2 mRNA at 4 h (18-fold), but this expression disappeared by 12 h. LPS treatment in the E_2-primed uterus downregulated the LPS induction of this gene at 4 h, but the downregulation observed previously at 12 h by LPS alone did not occur; rather, elevated levels of COX-2 mRNA were noted at 12 h (Fig. 8). Dex alone did not influence the expression of either the COX-1 or COX-2 gene (Fig. 9). However, Dex downregulated the LPS induction of these genes, with the COX-2 gene being more susceptible to this treatment (Fig. 9).

DISCUSSION

The use of conventional PG synthesis inhibitors, and measurement of uterine PGs during early pregnancy provide evidence that PGs participate in implantation and decidualization processes (Lau *et al.* 1973, Kennedy 1977, Johnson & Dey 1980, Tawfik *et al.* 1987, Gupta *et al.* 1989). However, the recent identification and cloning of the two isoforms of COX (COX-1 and COX-2) raise important questions regarding the relative importance and differential regulation of these isoforms in the peri-implantation uterus. Our present results of temporal and cell-specific expression of these isoforms in the mouse uterus during this period and under steroid hormonal stimulation provide new insights to these issues. In general, COX-1 is expressed constitutively in many cell types, while COX-2 is induced by a variety of stimuli. However in the peri-implantation mouse uterus, the

expression of both the COX-1 and COX-2 genes is developmentally regulated. During the preimplantation period, the low levels of COX-1 mRNA, observed in the glandular epithelium on days 1–3, became highly but transiently abundant in both the luminal and glandular epithelia on day 4 of pregnancy. In contrast, the COX-2 gene that expressed highly on day 1 rapidly declined thereafter, reaching undetectable levels through most of day 4. These results indicate that both isoforms of COX are inducible in the pregnant mouse uterus, but with a different temporal sequence. The low levels of COX-1 expression in the day 1 glandular epithelium could be attributed to the effects of preovulatory estrogen on the uterus (Huet *et al.* 1989). This is consistent with modest induction of the COX-1 mRNA in ovariectomized mice after an E_2 injection. In contrast, heightened uterine expression of COX-1 through most of day 4 could be due to the combined effects of rising P_4 levels from newly formed corpora lutea and secretion of a small amount of ovarian estrogen (reviewed in Huet *et al.* 1989). The highest induction of the COX-1 mRNA in the ovariectomized uterus after a combined treatment with P_4 and E_2 is consistent with this assumption. Since treatments of ovariectomized mice with P_4 and/or E_2 failed to induce uterine COX-2 mRNA at any of the times examined, it is suggested that this gene is not inducible by ovarian steroids in the mouse uterus. The bacterial endotoxin LPS or cytokines are known to upregulate COX-2 expression in a variety of cells (Lee *et al.* 1992, Ristimaki *et al.* 1994, Jacobs *et al.* 1994). Thus, the heightened expression of COX-2 in the day-1 pregnant uterus perhaps is not due to the preovulatory estrogen effects, but rather is due to the presence of numerous bacteria or heightened cytokine expression in the uterus (Kover *et al.* 1995, Parr & Parr 1985). This is consistent with higher levels of COX-2 in mouse uterine cells exposed to interleukin-1α in culture (Jacobs *et al.* 1994). LPS upregulated the uterine expression of both the COX-1 and COX-2 genes in ovariectomized mice, though the induction of the COX-2 gene was more dramatic. The induction of these genes was maximal at 4 h after LPS injection. However, the accumulation of COX-1 mRNA persisted, while that of COX-2 disappeared by 12 h after LPS injection. Interestingly, an injection of E_2 24 h prior to an LPS injection downregulated the induction of COX-1. E_2 priming also downregulated the LPS-induced expression of COX-2 at 4 h but, unlike LPS alone, it sustained the expression through 12 h. These experiments were designed to simulate the conditions as observed on day 1 of pregnancy when the uterine expression of COX-2 was high compared with the modest levels of COX-1 expression. Indeed, the results are largely consistent with those observed in the day-1 pregnant uterus. The mechanism by which E_2 priming affects LPS induction of these genes remains unknown.

The expression of these two genes showed an interesting pattern with the initiation of the attachment reaction on day 4 of pregnancy. While the epithelial expression of COX-1 greatly diminished or disappeared at this time, the expression of COX-2 appeared exclusively in the luminal epithelial and adjacent subepithelial stromal cells surrounding the blastocyst at the antimesometrial pole. This pattern persisted through the morning of day 5. A previous report described accumulation of immunoreactive COX-2 in stromal cells at the implantation sites on day 6 of pregnancy (Jacobs *et al.* 1994). Although there are some differences between this and our present studies, collectively the results suggest that the blastocyst plays a critical role in inducing the COX-2 gene in the uterus. The absence of COX-2 mRNA from the pseudopregnant uterus during these times provides further support for this possibility. Cell–cell contact (trophectoderm–luminal epithelium) perhaps is not the inducer of this gene, since the COX-2 gene is not expressed in the luminal epithelium closely apposed to the zona-free dormant blastocysts in the P_4-treated delayed implanting mice. In contrast, COX-2 expression occurs in the uterus solely at the sites of blastocyst apposition after termination of the delay by E_2, suggesting an interaction of the active blastocyst with the hormonally prepared receptive uterus for this expression. Indeed, the activated state of the blastocyst should coincide with the receptive state of the uterus for successful implantation in the mouse (Paria *et al.* 1993*a*). Collectively, the results suggest that blastocyst-derived molecule(s) is most likely the inducer of this gene, the identification of which poses a major challenge. However, blastocyst-derived growth factors/cytokines (Rappolee *et al.* 1988, Rothstein *et al.* 1992, Wride & Sanders 1995) may well be the inducers of the uterine COX-2 gene. The induction of the EGF-like growth factors in the luminal epithelium at the sites of blastocyst apposition before or at the time of attachment reaction on day 4 (Das *et al.* 1994, 1995) could also be responsible for the localized induction of the COX-2 gene. Indeed, EGF stimulates PG synthesis in mouse uterine cells in culture (Paria *et al.* 1991). The COX-2 expression switched from the antimesometrial to the mesometrial pole on day 6 and persisted therein through day 8 of pregnancy. Although this transition is dramatic, the mechanism by which this

occurs is not yet clear. The pattern of interleukin-6 expression in the antimesometrial deciduum (Montro *et al.* 1990) is suggestive of a role for this cytokine in the induction of the COX-2 gene at this site. The evidence for the first decidual induction of the COX-1 gene was noted on day 7 as limited only to the secondary decidual zone. This was followed by a remarkable increase throughout the secondary deciduum both at the mesometrial and antimesometrial poles on day 8. Whether decidual growth factors influence the COX-1 expression will require further investigation.

The relative importance of COX-1 and COX-2 in generating PGs in the mouse uterus with respect to various events during early pregnancy can only be speculated upon at this time. The heightened COX-2 expression in the day-1 uterus could be responsible for its high contents of $PGF_{2\alpha}$ (our unpublished observation). If $PGF_{2\alpha}$ is secreted basolaterally from the epithelium and reaches the myometrium via the stroma, then the myometrial contractions for facilitating sperm transport at the fertilization site in the oviduct could be achieved via this PG. Indeed, $PGF_{2\alpha}$ is secreted basally by polarized murine uterine epithelial cells in culture (Jacobs *et al.* 1990). In contrast, the generalized uterine edema observed on day 4 could be due to higher levels of uterine PGE_2 (our unpublished observation) resulting from the heightened expression of the COX-1 gene. This could be important for the uterine luminal closure and subsequent attachment reaction. Since the attachment reaction coincides with increased localized endometrial vascular permeability, the uterine COX-2 expression at the sites of blastocysts at this time could be associated with the local production of PGE_2 that participates in increased vascular permeability. Dex is known to interfere with E_2-induced implantation in the P_4-primed delayed implanting rat and this inhibition is reversed by PGE_2 (Johnson & Dey 1980). This observation suggests that Dex inhibits the generation of PGs required for implantation. Indeed, Dex has been shown to inhibit COX-2 expression and PG synthesis induced by LPS, cytokines or serum (DeWitt & Meade 1993, Jacobs *et al.* 1994, Hempel *et al.* 1994*a*,*b*). However, the failure of Dex to interfere with E_2-induced implantation in the delayed implanting mouse is surprising. Results of *in situ* hybridization showed that the localized uterine COX-2 expression at the sites of blastocyst attachment was normal after Dex treatment. This suggests that blastocyst-induced uterine expression of the COX-2 gene in the mouse is not influenced by Dex. Alternatively, glucocorticoid receptors may be absent from the mouse uterus. However, the downregulation of the LPS-induced expression of the COX-2 gene by Dex suggests that the Dex treatment was effective and that glucocorticoid receptors are present in the mouse uterus. It should be noted that glucocorticoid receptors are present in the rat uterus (Panko *et al.* 1981, Bigsby 1993). Nonetheless, it is possible that the glucocorticoid receptors were absent or downregulated in the mouse uterus at the implantation sites.

PGs are implicated in decidualization in the rodent (Kennedy 1985, Tawfik *et al.* 1987). Thus, the decidual expression of COX-1 on days 7 and 8 of pregnancy could be involved in this process. PGs may also be important for continued increased vascular permeability at the sites of implantation. In contrast, COX-2 expression in the mesometrial deciduum from day 6 onward could be involved in angiogenesis for the establishment of placenta. The role of PGs in angiogenesis is well recognized (Form & Auerbach 1983, Diaz-Flores *et al.* 1994). However, because of overlapping expression of COX-1 and COX-2 at the mesometrial decidual bed, it is difficult to assess the relative importance of these two isoforms in angiogenesis. It is believed that the ratio of PGE_2 to $PGF_{2\alpha}$ determines the vascular and other biological effects of these mediators. However, it is not known whether there is any correlation between the status of the COX genes and attainment of physiological ratios of PGs *in vivo*. The definitive roles for the COX-1 and COX-2 genes are likely to be achieved by targeting these genes by homologous recombination.

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Note added in proof

While this article was in press, the phenotypes of COX-1 and COX-2 deficient mice produced by gene targeting were reported (Langenbach *et al.* 1995, Morham *et al.* 1995, Dinchuk *et al.* 1995). Although COX-$1^{(-/-)}$ female mice appear to be fertile, COX-$2^{(-/-)}$ females are infertile. Our observations suggest that the absence of the uterine COX-2 gene, which is normally expressed at sites of blastocyst implantation, from these COX-$2^{(-/-)}$ females could also be a contributing factor for this infertility. Future studies using these mice will provide important information in this regard.

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