INTRODUCTION

The role of histamine, a biogenic amine formed from L-histidine by histidine decarboxylase (HDC), in the processes of implantation and decidualization has been the subject of considerable research interest in the past. Although there was evidence that pharmacological interventions that interfere with histamine functions interrupt embryo development (Dey and Johnson, 1980; Hudgins et al., 1982), implantation (Shelesnyak, 1957; Nalbandov, 1971; Dey et al., 1979a,b; Johnson and Dey, 1980; Dey, 1981; Dey and Hubbard, 1981) and decidualization (Marcus et al., 1963, 1964), the lack of molecular approaches failed to establish definitive roles of this amine in these processes (DeFeo, 1967).

Because of its vasoactive nature, histamine has been considered to be an important mediator in this event. However, neither the sites of formation of histamine nor its mode of action in implantation has been defined clearly. The dogma was that histamine required for implantation is derived from the resident mast cells in the uterus by the preimplantation ovarian surge of estrogen (Shelesnyak, 1957; Padilla et al., 1990). However, the role of uterine mast cells in implantation in mice and rats has been challenged (Brandon and Bibby, 1979; Salamonsen et al., 1996). Furthermore, normal implantation and birth of live offspring in mast-cell-deficient mice further raised questions regarding the importance of mast cell histamine in implantation (Wordinger et al., 1986).

Collectively, the results suggest that, if histamine is involved in implantation, its source should be of uterine cell types other than the resident mast cells. Indeed, our recent investigation in the mouse demonstrated for the first time that uterine epithelial cells are the major sites of expression of HDC that produces histamine. Moreover, maximal uterine expression of HDC occurs on the day of implantation (day 4 pregnancy) (Paria et al., 1998a). Since information regarding histamine receptors either in the embryo or in the uterus was not available, the site and mode of actions of histamine during early pregnancy remained largely undefined.

Histamine mediates its action by binding to cell surface receptors, which are classified into three major subtypes, H₁, H₂ and H₃, based on their responses to selective agonists and antagonists (Haaksma et al., 1990; Hill, 1990). Recently, H₁ (De Backer et al., 1993; Fugimoto et al., 1993; Inoue et al., 1996a) and H₂ (Gantz et al., 1991; Kobayashi et al., 1996) have

Blastocyst H₂ receptor is the target for uterine histamine in implantation in the mouse

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SUMMARY

The process of implantation is a ‘two-way’ interaction between the blastocyst and uterus. It has long been suspected that histamine is an important mediator in embryo-uterine interactions during implantation, but its source, targets and mechanism of actions remained undefined. We have recently demonstrated that uterine epithelial cells are the source of histamine, which peaks on day 4 of pregnancy (the day of implantation) in the mouse. In searching for its target and site of action, we discovered that preimplantation blastocysts, which express histamine type 2 receptor (H₂), is the target for histamine action. Using multiple approaches, we demonstrate herein that uterine-derived histamine interacts with embryonic H₂ receptors in a paracrine fashion to initiate the process of implantation.

Key words: Histamine, H₂ receptors, Blastocyst, Mouse, Implantation, Uterus

INTRODUCTION

The role of histamine, a biogenic amine formed from L-histidine by histidine decarboxylase (HDC), in the processes of implantation and decidualization has been the subject of considerable research interest in the past. Although there was evidence that pharmacological interventions that interfere with histamine functions interrupt embryo development (Dey and Johnson, 1980; Hudgins et al., 1982), implantation (Shelesnyak, 1957; Nalbandov, 1971; Dey et al., 1979a,b; Johnson and Dey, 1980; Dey, 1981; Dey and Hubbard, 1981) and decidualization (Marcus et al., 1963, 1964), the lack of molecular approaches failed to establish definitive roles of this amine in these processes (DeFeo, 1967).

The initial attachment reaction for implantation results from an intimate ‘cross-talk’ between the trophectoderm of the active blastocyst and luminal epithelium of the receptive uterus. This event first occurs late on day 4 (2200-2300 hours) of pregnancy in the mouse (Das et al., 1994). The molecular basis of this initial interaction between the blastocyst and the uterus remains an enigma. One of the prerequisite events for the attachment reaction is an increased endometrial vascular permeability at the site of blastocyst apposition (Psychoyos, 1973). Ovariectomy before the preimplantation ovarian estrogen secretion results in implantation failure and blastocyst dormancy. This condition termed delayed implantation can be maintained by progesterone (P₄) treatment, but is terminated by an injection of estrogen with blastocyst activation and initiation of the attachment reaction (Yoshinaga and Adams, 1966; Psychoyos, 1973; Paria et al., 1993a).
been cloned and sequenced. The deduced amino acid sequence of H1 and H2 reveals the existence of seven putative transmembrane domains, indicating that these receptors are members of the large family of G-protein-coupled receptors (Hill, 1990). H1 mediates histamine-induced activation of phospholipase C via Gq class of heterotrimeric G proteins resulting in increased cytosolic Ca²⁺ concentration and protein kinase C activity in target cells (Tilly et al., 1990; Schwartz et al., 1991). In contrast, activation of H2 stimulates adenyl cyclase activity via Gs, thereby increasing intracellular cAMP levels (Schwartz et al., 1991). Stimulation of H2 can also release Ca²⁺ from intracellular stores. H3 functions as an autoreceptor modulating histamine synthesis and release. Thus, H3 receptors are primarily present in histamine-synthesizing cells, but are also located on target cells where H3 receptors act as heteroreceptors (Arrang et al., 1983). Since the H3 receptor gene has not been cloned, the molecular structure of this receptor is unknown. In the present investigation, we sought to examine whether HDC, H1 and H2 receptors are expressed in the preimplantation mouse embryo and whether histamine ligand-receptor signaling is important for blastocyst function and implantation. Our results demonstrate that, while HDC and H1 receptors are not expressed, H2 receptors are expressed in the blastocyst. Further, we provide evidence that uterine histamine interacts with blastocyst H2 receptors in a paracrine manner to initiate the process of implantation.

MATERIALS AND METHODS

Animals

Virgin CD-1 female mice (48-60 days old, 20-25 g, Charles River Laboratories, NC) were mated with fertile or vasectomized males of the same strain to induce pregnancy and pseudopregnancy, respectively. The morning of finding a vaginal plug was designated day 0. Embryos at different stages of development were recovered in Whitten's medium by flushing the reproductive tracts of pregnant mice (Paria and Dey, 1990). To induce and maintain delayed implantation, pregnant mice were ovarioctomized at 0830-0900 hours on day 4 of pregnancy before the preimplantation estrogen secretion and received daily injections of P4 (2 mg/mouse) from days 5-7. To retrieve activated blastocysts (Paria et al., 1993a,b), P4-primed delayed implanting mice were given an injection of estradiol-17β (E2; 25 ng/mouse) on the third day of delay (day 7). Mice were killed 12 hours after treatments with the respective steroid hormones, and their uteri were flushed to obtain dormant and activated blastocysts.

Analysis of HDC, H1 and H2 mRNAs

To examine the expression of mRNAs for HDC, H1 and H2 in preimplantation embryos (85 one-cell; 82 two-cell; 80 four-cell; 80 eight-cell/morulae and 80 and 80 day 4 blastocysts), dormant (85 blastocysts) and activated (75 blastocysts) blastocysts, reverse transcription coupled PCR (RT-PCR) was employed (Paria et al., 1993b). For RT-PCR analysis, the following primers were used: 5'-CGCG-CTACTATCGGTCCGTCC-3' (sense) and 5'-CCCTGTTGGCT-TGCTCTGTC-3' (antisense) for HDC (Yamamoto et al., 1990), 5'-CCTGATTTGAGGGCAACATG-3' (sense) and 5'-GCCTAC-ACACATTGAGATC-3' (antisense) for H1 (Inoue et al., 1996a) or 5'-GAGGCCAAAGGGCAAGCT-3' (sense) and 5'-CCCTCTG-GTCCATTTTC-3' (antisense) for H2 (Kobayashi et al., 1996). The sense and antisense primers for ribosomal protein L-7 (rpl7, a house-keeping gene, GenBank Accession No. M29016) were: 5'-TCAATGGGAGTATCCCAAGG-3' and 5'-CAAGGACCGAC- GCAATCAAG-3', respectively (Das et al., 1997; Meyuhas and Klein, 1990; Paria et al., 1998). The internal oligonucleotides, 5'GAGCA- AGAAAGAACAACATC, 5'-GAGGTATCAGACCAAGCC-3', 5'-CACTCTGCTCTCAGTGGAG-3' and 5'-GATTGCCTTGCA- GATAATCC-3' were used for Southern blot analysis of HDC, H1, H2 and rpl7, respectively. Total RNA (1 μg) from the brain and stomach or 25% of the embryonic RNA was reverse transcribed by using specific antisense primers. One-third of the RT products was PCR-amplified using sense and antisense primers as described (Paria et al., 1993b). PCR cycle parameters were as follows: 94°C for 4 minutes, 55°C for 1.5 minutes and 72°C for 2.5 minutes for the first cycle followed by 94°C for 1 minute, 55°C for 1.5 minutes and 72°C for 2.5 minutes for 30 cycles. One-tenth of the amplified product was electrophoresed on agarose gels (1%), stained with ethidium bromide, and analyzed by Southern blot hybridization (Paria et al., 1993b). Experimental and negative controls were run simultaneously.

Ligand-binding assay and autoradiographic detection of ligand-binding sites

This technique essentially followed the protocol as previously described by us (Paria and Dey, 1990; Paria et al., 1993b, Yang et al., 1996). We used [³H]tiotidine (NEN, Boston, MA; specific activity, 81.10 Ci/mmol) as a radioligand instead of [³H]cimetidine normally considered as a selective H2 receptor blocker. However, [³H]tiotidine was recently introduced as a substituent for [³H]cimetidine, because tiotidine is more selective and displaced by various H2-selective ligands (Haaksma et al., 1990; Hill, 1990). [³H]B-naphthyl-alpha-methyl-histamine (NEN, Boston, MA; specific activity, 81.500 Ci/mmol), a potent and selective H3-agonist, was used to examine H3 receptor-binding sites in the blastocyst (Hill, 1990). Normal day 4, dormant and activated blastocysts were collected in Whitten's medium. Normal day 4 blastocysts were freed of zona pellucidae and washed (Paria and Dey, 1990). Blastocysts were then incubated for 1 hour in Whitten's medium containing 4 nM [³H]pyrilamine (NEN, Boston, MA; specific activity, 20.00 Ci/mmol) whose high binding affinity is saturable and dispensable by nonlabeled H1 antagonists (Haaksma et al., 1990; Hill, 1990). [³H]B-naphthyl-alpha-methyl-histamine (NEN, Boston, MA; specific activity, 81.500 Ci/mmol), a potent and selective H3-agonist, was used to examine H3 receptor-binding sites in the blastocyst (Hill, 1990). For autoradiographic studies, blastocysts were washed in the same medium (cold), fixed in 2% paraformaldehyde, air dried and subjected to autoradiography with an exposure time of 5-10 days. Slides were poststained with Eosin and Hematoxylin (Paria and Dey, 1990; Paria et al., 1993b, 1995). Black grains under the bright field indicated the binding sites.

Isolation of the inner cell mass (ICM) from the blastocyst

To isolate ICMs from blastocysts, immunosurgery was performed using the procedure previously described by us (Paria et al., 1992). Day 4 blastocysts were collected in M2 medium and removed of their zona pellucidae by a brief exposure to a 0.5% pronase solution. Zona-free blastocysts were incubated in M2 medium for 30 minutes followed by incubation in rabbit anti-mouse T-cell serum (1:30 in M2 medium) for 60 minutes at 37°C in a CO2 incubator. After incubation, blastocysts were washed in fresh M2 medium three times. They were exposed to a guinea pig complement (1:3 in M2 medium) for 15-30
minutes and observed under a dissecting microscope. With the onset of lysis reaction, the blastocysts were removed from the complement solution and placed in fresh M2 medium. Blastocysts were then passed through a small glass pipet (inner diameter of the pipet is little smaller than the blastocyst diameter) several times to remove the lysed outer layer of cells. ICMs were collected and cultured in fresh M2 medium for 60 minutes before processing them for binding studies.

**Measurement of cyclic adenosine-3',5'-monophosphate (cAMP) accumulation in the blastocyst**

To study the effects of H2 receptor agonist and/or antagonist on cAMP accumulation, day 4 blastocysts in a group of 10 were cultured in 25 μl of Whitten’s medium at 37°C in an humidified atmosphere of 5% CO2:95% air for 2 hours in the absence or presence of H2 agonist, impromidine (Smith Kline & French Laboratories, PA), with or without H2 antagonist, famotidine (Sigma Chemical Co.). Impromidine is considered to be a preferred agonist to H2 since it is 4810 times more active than histamine and a weak agonist for the histamine H3 receptors. However, its potency is tissue type dependent. The ED50 value for impromidine in stimulating beats/minute in isolated guinea pig right atrium is 25 nM (Duran et al., 1978). Famotidine is also a potent H2 receptor antagonist with a Kd values in nM range (reviewed in Hill, 1990; Krielaart et al., 1990). Thus, impromidine and famotidine are useful agonist and antagonists, respectively, to study H2 receptor functions. Blastocysts in a batch of 35-50 in each group were boiled and sonicated in 20 μl of Whitten’s medium containing 0.3% H2 (tiotidine, famotidine and ranitidine) on preimplantation embryo development, H1 or H2 receptor mRNA in the periimplantation mouse uterus (Krielaart et al., 1990). Thus, to ascertain the reversibility of H2 receptor functions. Blastocyst supernatants were used for cAMP measurement by a competitive protein-binding assay as described (Paria et al., 1995).

**Blastocyst zona hatching in vitro**

To study the role of H2 receptor on zona hatching (complete escape of the blastocyst from its zona pellucida) in vitro, blastocysts recovered on day 4 (0900 h) of pregnancy were cultured in groups (6-10 embryos/group) for 72 hours in 25 μl of Whitten’s medium at 37°C in an humidified atmosphere of 5% CO2:95% air in the presence of antagonist (0-20 nM impromidine) of H2 and/or antagonists (20 nM) of H2 (tiotidine, famotidine and ranitidine, Sigma Chemical Co.) and H1 (pyrilamine, Sigma Chemical Co.). The control cultures contained the same concentration of the vehicle. Embryonic development was monitored every 12 hours. The number of blastocysts showing complete hatching were recorded at the end of the culture period (72 hours) (Das et al., 1994). Radioligand-binding and displacement studies have reported that tiotidine, famotidine and ranitidine are almost equipotent H2 receptor blockers (Cavanagh et al., 1983; Krielaart et al., 1990). Thus, to ascertain the reversibility of H2 receptor-mediated zona hatching by impromidine, we used these three H2 antagonists.

**Culture of preimplantation embryos**

To examine the effects of H2 receptor antagonists (tiotidine, famotidine and ranitidine) on preimplantation embryo development, 2-cell embryos were recovered on day 2 (1200-1300 hours) from several mice and were pooled in Whitten’s medium containing 0.3% bovine serum albumin. Embryos were cultured in groups of 8-10 in 25 μl of Whitten’s medium under silicon oil in an atmosphere of 5% CO2:95% air at 37°C for 72 hours (Paria and Dey, 1990) in the absence or presence of 20 nM tiotidine, famotidine or ranitidine. The control cultures contained the same concentration of the vehicle. The embryos were observed every 24 hours to monitor their development. After termination of cultures, the number of embryos that had formed blastocysts was recorded.

**Blastocyst culture and transfer**

Normal blastocysts were collected on day 4 of pregnancy (0830-0900 hours). They were cultured in a group of 10-12 in 25 μl of Whitten’s medium at 37°C in an humidified atmosphere of 5% CO2:95% air for 6 hours in the presence or absence of H2 antagonists, famotidine or ranitidine or an H1 antagonist pyrilamine. For reversal experiments, blastocysts were cultured with H2 antagonists together with H2 agonist, impromidine. After termination of cultures, blastocysts were washed several times in the same medium. They were then transferred into uteri of day 4 pseudopregnant recipients. Implantation sites (increased vascular permeability at the site of blastocyst apposition) were determined by intravenous injections (0.1 ml/mouse) of a Chicago Blue B dye solution (1% in saline) 24 hours after transfer of blastocysts. They were killed 5 minutes later to examine implantation sites as demarcated by discrete blue bands along the uterus (Paria et al., 1993a). If implantation sites were absent, uterine horns were flushed with saline to recover unimplanted blastocysts. Mice without implantation sites and blastocysts were excluded from the experiments.

**Systemic infusion of HDC inhibitor**

Miniosmotic pumps (Alza Corporation, Palo Alto, CA; mean pumping rate 0.5 μl/hour and mean fill volume 93 μl) containing either the vehicle or the irreversible inhibitor of DL-α-fluromethylhistidine (DL-α-FMH, 2 mg/ml, Marck Sharp & Dohme Research Lab., Rahway, NJ) were placed subcutaneously under the back skin of mice at the time of induction of delayed implantation on day 4 of pregnancy and continued through the day 8 of delay. Delayed implantation was maintained with daily injection of P4 (2 mg/mouse) from days 5-7. To initiate implantation, P4-primed delayed pregnant mice were given an injection of E2 (10 mg/mouse). Implantation sites were determined 24 hours after an E2 injection by the blue dye method. If implantation sites were absent, uterine horns were flushed with saline to recover unimplanted blastocysts. Mice without implantation sites and blastocysts were excluded from the experiments.

**RESULTS**

**H2 gene, but not HDC or H1 gene, is expressed in the blastocyst**

We previously demonstrated the expression of HDC mRNA, protein and its activity in mouse uterine epithelial cells (Paria et al., 1998a). However, it was not known whether HDC is expressed in preimplantation embryos. Using RT-PCR, we failed to detect HDC mRNA in preimplantation embryos (1-cell through blastocyst), suggesting the uterine epithelial cells are the primary local source of histamine during implantation. We then sought to identify the targets for histamine actions. We detected H2, but not H1, mRNA exclusively in blastocysts during preimplantation embryonic development (Fig. 1). Mouse stomach and brain RNAs served as positive controls for these experiments. RNA integrity was confirmed by the detection of rpL7 mRNA in these samples. We failed to detect H1 or H2 receptor mRNA in the preimplantation mouse uterus by northern and in situ hybridization, although low levels of these receptors were detected in the uterus by RT-PCR (data not shown). These results provide evidence that uterine histamine is available to interact with blastocyst H2 receptors.

**H2-binding sites are present in the blastocyst**

To examine whether H2 mRNA expressed in blastocysts is translated and whether this receptor binds to its ligand, [3H]tiotidine (a selective H2 antagonist) binding was performed in day 4 blastocysts. Indeed, [3H]tiotidine binding was evident
in day 4 blastocysts (Fig. 2). This binding was specific, since unlabeled tiotidine successfully displaced the binding of \[^{3}H\]tiotidine. In contrast, day 4 blastocysts did not show any binding with \[^{3}H\]pyrilamine (a selective H\(_{1}\) antagonist). These results are consistent with our RT-PCR results. The data demonstrate that H\(_{2}\) receptors are solely expressed at the blastocyst stage and are capable of binding in a ligand-specific manner. In contrast, negligible binding was noted for H\(_{3}\) (data not shown).

\[^{3}H\]tiotidine-binding sites appeared to be displayed in trophoderm cells of day 4 blastocysts (Fig. 3A). This may reflect the inaccessibility of the ligand to the inner cell mass (ICM) cells. Therefore, binding studies in immuno-surgically isolated ICM was performed. Autoradiographic signals were very low in isolated ICM cells (Fig. 3C), suggesting \[^{3}H\]tiotidine-binding sites are primarily present in trophoderm cells.

**H\(_{2}\) receptor-binding sites are absent in dormant blastocyst**

To determine the level of H\(_{2}\)-binding sites in dormant and activated blastocysts, autoradiographic \[^{3}H\]tiotidine-binding sites in dormant blastocysts were compared with those in day 4 normal and estrogen-activated blastocysts using the delayed implantation model. Specific \[^{3}H\]tiotidine-binding was virtually undetectable in dormant blastocysts (Fig. 3D). In contrast, the binding sites were readily detected within 12 hours after termination of the delay with blastocyst activation by E\(_{2}\) (Fig. 3E). The binding was specific, since unlabeled tiotidine successfully competed in displacing \[^{3}H\]tiotidine (Fig. 3F).

**H\(_{2}\) receptor gene is not expressed in dormant blastocysts**

The delayed implantation model was used to determine whether H\(_{2}\) receptor expression in the blastocyst is regulated. Consistent with the H\(_{2}\) receptor autoradiographic studies, we observed reduced levels of H\(_{2}\) mRNA in day 7 dormant blastocysts as compared to normal day 4 blastocysts (Fig. 4). Considering
the increased cell numbers during the 3-day delay (129±3.2/blastocyst) as opposed to those in normal day 4 blastocysts (32±0.5 /blastocyst) (Paria et al., 1993b), reduced H2 mRNA levels in dormant blastocysts is remarkable. An injection of E2 in P4-treated delayed mice rapidly upregulated the expression in activated blastocysts, suggesting that E2, its metabolites or E2-induced uterine factors regulate H2 receptor expression in the blastocyst (Fig. 4). Mouse brain and stomach served as positive controls. RNA integrity was confirmed by the detection of rpL7 mRNA in these samples.

**Activation of H2 receptors stimulates cAMP accumulation in the blastocyst**

To examine whether H2 receptors in the blastocyst are G-protein coupled and functional, cAMP accumulation in the blastocyst was measured after exposure to a specific H2 agonist. Indeed, incubation of day 4 blastocysts with 20 nM impromidine (a selective H2 agonist) for 2 hours significantly (P<0.01) stimulated cAMP accumulation as compared to vehicle-treated controls (Fig. 5). Impromidine-induced cAMP accumulation was inhibited by 20 nM famotidine (an H2 antagonist). These results suggest that blastocyst H2 receptors are specific and biologically functional.

**Activation of H2 receptors promotes blastocyst zona hatching in vitro**

The escape of blastocysts from zona-pellucidae is essential for their implantation in the uterus. We examined whether activation of the H2 receptor is important for zona hatching in vitro (Fig. 6). The zona-hatching rate was significantly (P<0.001) high in the presence of impromidine compared with that of vehicle-treated controls and this effect was dose dependent. Impromidine-induced accelerated zona-hatching rate was impaired by the addition of equimolar H2-selective antagonists, tiotidine, famotidine or ranitidine. Zona-hatching rate of blastocysts after exposure to any of these antagonists alone was similar to controls. However, the zona-hatching rate in the presence of famotidine or ranitidine with impromidine was a little lower, but not statistically different (P>0.05), from the controls. The morphological appearance of these blastocysts was normal. The inhibitory effects of these antagonists were specific to H2, since pyrilamine (a selective H1 antagonist) did not impair the zona-hatching rate induced by impromidine (Fig. 6). As shown above, embryos at the preblastocyst stages do not express H2 receptors and thus they should not respond to H2 receptor antagonists. Indeed, about 82% of the 2-cell embryos (32 embryos in each group) cultured in vitro in the absence of presence of tiotidine, famotidine or ranitidine developed to blastocysts, suggesting these drugs at 20 nM did not have any toxic effects toward embryo development (data not shown). These results demonstrate that histamine ligand-receptor signaling via H2 receptors is important for blastocyst functions.
Neutralization of H₂ receptor activation attenuates blastocyst implantation in the receptive uterus

In order to determine whether activation of blastocyst H₂ receptors is important for implantation, we incubated day 4 blastocysts with famotidine or ranitidine (H₂ antagonists), or pyrilamine (an H₁ antagonist) for 6 hours in vitro and then transferred them to day 4 pseudopregnant recipients. Implantation was examined on day 5 by the blue dye method. Uterine horns that did not exhibit any implantation sites were flushed with saline to recover blastocysts. Mice without implantation sites or blastocysts were excluded from experiments. The numbers within the parentheses indicate the number of recipients showing implantation/total number of recipients. The numbers at the top of each bar indicate the number of recipients showing implantation/total number of recipients in each group. Numbers inside the bars indicates number ± s.e.m. *P<0.01 (χ² and Fisher exact tests) compared with other groups. About 40-60% of transferred blastocysts were recovered from uterine flushings of the mouse having no implantation sites. About 47% of the recovered blastocysts from the H₂ receptor antagonist-treated group were zona-encased.

Systemic inhibition of HDC activity interferes with implantation

To determine whether local synthesis of histamine by uterine epithelial cells is important for implantation, the delayed implantation model was used. This model was used to eliminate any effects that could be mediated via the pituitary-ovarian axis. P₄-treated delayed implanting mice were infused with DL-α-FMH (an irreversible HDC inhibitor) at a constant rate (1 μg/hour) via the miniosmotic pumps at the time of induction of delayed implantation on day 4 of pregnancy and continued through day 8 of the delay. To initiate implantation, P₄-primed delayed pregnant mice were given an injection E₂ (10 ng/mouse). Implantation sites were determined 24 hours after an E₂ injection by the blue dye method. Uterine horns that did not exhibit any implantation sites were flushed with saline to recover blastocysts. Mice without implantation sites or blastocysts were excluded from experiments. The numbers at the top of each bar indicate the number of recipients showing implantation/total number of recipients. Numbers inside the bars indicate number ± s.e.m. DL-α-FMH, DL-α-fluromethylhistidine.
abnormalities in the number of embryos or their viability, although it prevented the increase in kidney HDC activity on day 18 of pregnancy (Maeyama et al., 1982). Thus, α-FMH is a useful compound for depleting histamine and provides an experimental tool for studying histamine functions under a physiological condition. Our observation of failure of blastocyst implantation by DL-α-FMH suggests that histamine effects are stage-specific.

**DISCUSSION**

The novelty of the present investigation is that the blastocyst is the target for uterine-derived histamine in mediating blastocyst zona hatching and implantation. The exclusive expression of H2 receptors in the trophectoderm of the blastocyst, but not at other stages of the preimplantation embryo, also suggests a unique developmental regulation of this receptor gene. The downregulation of H2 receptors in dormant blastocysts during the P4-treated delayed implantation and their upregulation in activated blastocysts with the termination of the delay with E2 suggest that this steroid influences the expression of the H2 gene in the blastocyst in uterus. However, it is not known whether estrogen has a direct effect on the blastocyst or whether other factors of uterine or blastocyst origin resulting from the activation process are involved in this regulation in a paracrine or an autocrine manner. There is no evidence for direct effects of primary estrogens on blastocyst activation in vitro, although catecholestrogens have been shown to activate dormant blastocysts in vitro to become implantation-competent (Paria et al., 1998b). Alternatively, downregulation or upregulation of H2 receptors in dormant or activated blastocysts could be a consequence of the blastocyst’s state of activity.

Participation of histamine in blastocyst implantation has long been considered important. However, its site of formation and mode of action in this process is still not clearly defined. We have recently shown that epithelial cells are the primary source of histamine in the mouse uterus (Paria et al., 1998a). Our present findings establish that the blastocyst, but not the uterus, is a primary target for uterine histamine for implantation. Thus, the luminal epithelial expression of HDC (Paria et al., 1998a) and expression of H2 receptors in the trophectoderm on day 4 prior to the initiation of implantation suggest a potential ligand-receptor signaling with histamine in this process. This suggestion is consistent with the interference of zona hatching and implantation of blastocysts exposed to H2-specific antagonists, as well as impediment of implantation by systemic administration of an HDC inhibitor. In addition, the stimulation of zona hatching by H2 agonists further attests to the importance of this receptor subtype in blastocyst functions. The presence of HDC in the luminal epithelium, but its absence in the blastocyst, provides evidence that blastocysts are the targets of uterine histamine. This is consistent with the observation of failure of detecting any cell-specific expression of histamine receptors in the uterus. Further, the absence of H1 and negligible presence of H3 in blastocysts as well as the nonresponsiveness of blastocysts to H1 agonists or antagonists place H2 as the primary target for histamine action during implantation. This assumption is consistent with the report that H1-receptor-deficient mice have normal embryo development and implantation (Inoue et al., 1996b). Thus, it appears that the expression of H2 in the blastocyst is important for further blastocyst growth and implantation.

The nature of the signal(s) emanating from histamine interactions with blastocyst H2 receptors in regulating the process of blastocyst functions and embryo-uterine interactions during implantation is not yet clear. However, the increased rate of zona hatching by H2 agonist is suggestive of increased plasminogen activator activity in trophectoderm cells (Whisnant et al., 1998). Alternatively, histamine-induced prostaglandin (PG) synthesis could be involved in this event, since PGs are known to be involved in fluid accumulation in the mouse blastocyst and zona hatching (Biggers et al., 1978). Histamine stimulates cell proliferation via activation of H2 receptors (Cricco et al., 1994). These receptors are coupled to adenylate cyclase and their activation increases the intracellular cAMP concentration (Schwartz et al., 1991). cAMP is an important intracellular second messenger whose formation in the blastocyst stimulates gene expression, blastocoel expansion and implantation (Manejwala and Schultz, 1989; Poueymirou and Schultz, 1989; Johnson, 1996). Thus, stimulation of cAMP formation in the blastocyst by H2 receptor agonists points toward these functions. The stimulation of cAMP accumulation in the mouse blastocyst by the H2 receptor agonist and its blockade specifically by H2, but not H1, receptor antagonist, reinforce our contention that local uterine histamine action on the blastocyst is mediated specifically via H2 receptors. An attenuation in blastocyst growth and zona hatching resulting from reduced cAMP levels by endocannabinoids has previously been documented (Paria et al., 1995). This investigation showed that Gi-protein coupled brain-type (CB1) cannabinoid receptors in the blastocysts are activated by endocannabinoids resulting in reduced cAMP levels and blastocyst growth. Although very low levels of H3 receptors are expressed in the blastocyst (data not shown), it is not known whether this receptor subtype has any functions in the blastocyst.

Successful implantation is dependent upon an intimate ‘cross-talk’ between the blastocyst and the uterus. It is postulated that histamine ligand-receptor signaling is one of the key components in the implantation process. Once the blastocyst escapes from the zona pellucida, its trophectoderm gains the ability to attach with the uterine luminal epithelium. Thus, a blastocyst’s escape from the zona is a prerequisite for its implantation. Higher levels of uterine histamine on day 4 of pregnancy and H2 receptor expression in the blastocyst are consistent with a possible role of this amine in early pregnancy. This suggestion is consistent with our observation that H2 agonist facilitates zona hatching of blastocysts in vitro and that these stimulatory effects of H2 agonist are neutralized by H2 antagonists. Furthermore, transfer of H2 antagonist-treated blastocysts to pseudopregnant day 4 recipients prevented implantation when examined on day 5. The recovery of both zona-encased and zona-free unimplanted blastocysts suggests that failures in both zona dissolution and attachment reaction contributed to implantation failure. These inhibitory effects were reversed by cotreatment with imipramidine suggesting involvement of H2 receptors as mediators of the inhibitory effects of famotidine and ranitidine. Famotidine, ranitidine and tiotidine are almost equipotent with respect to their interactions with H2 receptors (Cavanagh et al., 1983; Krielaart, 1990). The
effects of H₂ antagonists on blastocyst zona hatching and implantation are not the result of their non-specific toxic effects, since tiotidine, famotidine or ranitidine had no adverse effects on the formation of blastocysts from 2-cell embryos, which do not express histamine receptors. The infusion of an HDC inhibitor α-FMH via miniosmotic pumps in delayed implanting mice prevented estrogen-induced initiation of implantation. Since dormant blastocysts were zona-free and remained closely apposed to the uterine luminal epithelium during the delay, inhibition of estrogen-induced implantation by α-FMH and recovery of morphologically normal blastocysts suggest that histamine synthesis by the uterus is also required for blastocyst attachment reaction. Since histamine can cause increased vascular permeability (reviewed in Malathy et al., 1986), it is possible that implantation failure by the HDC inhibitor was due to the reduced uterine vascular permeability. However, our failure to detect H₁ and H₂ receptors in any major uterine cell types or uterine endothelial cells argue against this possibility. In contrast, histamine synthesis in the uterine epithelium and the presence of H₂ receptors in the blastocyst suggest that the blastocyst is a target for uterine histamine during implantation with respect to zona dissolution and initiation of the attachment reaction. Since HDC expression is not detected at the implantation sites after day 5 (Paria et al., 1998a), it is unlikely that histamine plays a role in the attachment process. Nonetheless, the inhibition of uterine HDC by α-FMH may have effects on the uterus itself to influence the implantation process.

In conclusion, our molecular, pharmacological and physiological approaches clearly provide evidence that uterine histamine targets blastocysts for implantation, although histamine effects on the uterus cannot be ruled out. Further evidence for this signaling pathway in these events requires generation of mice deficient in HDC or histamine receptor genes by null mutation.

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