Calcitonin Down-Regulates E-cadherin Expression in Rodent Uterine Epithelium during Implantation

Quanxi Li¹, Jun Wang³, D. Randall Armant³,

Milan K. Bagchi² & Indrani C. Bagchi¹*

¹Dept. of Veterinary Biosciences, ²Dept. of Molecular & Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL and ³Dept. of Ob/Gyn, C. S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, Detroit, MI

Running Title: Regulation of uterine E-cadherin expression by calcitonin

Key words: Calcitonin, Implantation, E-cadherin, Uterus

*To whom correspondence should be addressed:

Indrani C. Bagchi, Ph. D.
Dept. of Vet. Biosciences
University of Illinois at Urbana-Champaign
Urbana, IL 61802
Phone (217) 333-7986
ABSTRACT

Previous studies indicated that calcitonin (CT), a peptide hormone involved in calcium homeostasis, is transiently expressed in the receptive rat and human endometrial epithelia within the window of implantation. Attenuation of uterine CT expression using antisense methods severely impaired implantation in the rat. The molecular pathway of CT in the pregnant uterus, however, remains unknown. In the present study, we investigated the cellular events following the binding of CT to its membrane receptors in human endometrial epithelial cell line Ishikawa. We observed that CT treatment triggers a transient rise in intracellular calcium in these cells. Most interestingly, CT treatment also led to the disappearance of E-cadherin, a critical cell adhesion molecule, from cell-cell contact sites. Blockade of intracellular calcium release by BAPTA-AM prevented the CT-induced disappearance of E-cadherin. Our studies further revealed that CT treatment markedly down-regulates the level of E-cadherin mRNA in Ishikawa cells. We also examined whether CT influences the expression of E-cadherin mRNA in intact rat uterine tissue during implantation. In pregnant rats, high levels of E-cadherin mRNA was expressed during the first three days of gestation when the CT mRNA in uterine epithelial cells is undetectable. Concomitant with a transient burst of CT expression during days 4-5 of pregnancy, the level
of E-cadherin mRNA sharply declined. Furthermore, administration of exogenous CT to animals on day 2 of pregnancy led to a premature suppression of E-cadherin mRNA level on day 3, indicating a direct link between elevated levels of uterine CT and the down regulation of E-cadherin expression in the surface epithelium. Collectively, our results are consistent with the hypothesis that CT-induced reduction in E-cadherin expression may remodel the adherens junctions between epithelial cells and this change in epithelial cell phenotype might be a critical event during the implantation of the blastocyst.

**INTRODUCTION**

Implantation involves complex and progressively intimate interactions between the blastocyst and the uterine epithelium (1-2). In humans and rodents, the blastocyst initially adheres to and penetrates the uterine epithelium and subsequently invades the uterine stroma (1-2). At the initial stages of implantation, the uterine epithelium undergoes pronounced changes in cell proliferation and remodeling, which prepares it to be "receptive" to invasion by the embryo (1-6). The specific modifications leading to the acquisition of the "receptive" state of the uterine epithelium are regulated by a complex interplay of a variety of effectors including the steroid hormones, growth factors and cytokines (2, 7-9). However, the precise nature of the molecular mechanisms through which these effectors promote uterine
receptivity remains unclear.

Our previous studies identified calcitonin (CT), a peptide hormone known to regulate calcium homeostasis in bone and kidney cells, as a potential regulator of implantation (10-11). The expression of CT was transiently induced in rat endometrium epithelium precisely at the onset of implantation (10-11). Our studies also showed that the expression of CT is induced in the human endometrial epithelium specifically during the mid-secretory phase (days 19-24) of the menstrual cycle, which closely overlaps with the putative window of implantation (12). Most importantly, suppression of the steady-state level of the calcitonin mRNAs in the preimplantation rat uterus by antisense ODNs resulted in a dramatic reduction in the number of implanted embryos (13). These results suggested strongly that a transient surge of CT expression in the preimplantation rat uterus is crucial for blastocyst implantation. The precise function of CT in rat and human endometrium during implantation, however, remains unknown.

In order to gain an understanding of the function of CT during implantation, we investigated the regulation of cellular functions by this hormone in a human endometrial epithelial cell line Ishikawa. We observed that binding of CT to its cell surface receptor in Ishikawa cells leads to a transient rise in intracellular calcium, which in turn suppresses the expression of calcium-dependent cell adhesion glycoprotein, E-cadherin, at cell-cell contact sites. Consistent with this in vitro observation, we found that administration of exogenous CT
down regulates E-cadherin expression in the endometrial epithelium of pregnant rats without altering the expression of ZO-1, a marker of tight junctions. Based on these results, we favor the hypothesis that uterine CT plays a critical role during implantation by modulating adherens junctions. We postulate that the hormone-induced down regulation of E-cadherin expression results in the reorganization of the adherens junctions between epithelial cells, thereby facilitating implantation of the blastocyst.

MATERIALS AND METHODS

Reagents

Salmon CT, calcitonin gene related peptide (CGRP), and CGRP antagonist 8-37, were purchased from Peninsula Laboratories (Belmont, CA). Antibodies against E-cadherin (human) and ZO1 (rat) were purchased from Zymed (Burlingame, CA). An antibody against E-cadherin (rat) was purchased from BD Transduction Company (Lexington, KY).

Animals

All experiments involving animals were conducted according to NIH standards for the care
and use of experimental animals. Virgin female rats (Sprague-Dawley, from Charles River, Wilmington, MA; 60-75 days of age), in proestrus, were mated with adult males. The different stages of the cycle in the non-pregnant rats were ascertained by examining vaginal smears. The presence of a vaginal plug after mating was designated as day 1 of pregnancy. The animals were killed at various stages of gestation and the uteri collected. In some experiments, animals were injected intravenously or intramuscularly with salmon CT (100 µg) as described in the Results section. The rats were killed 24 h after final injection.

**CT binding assay**

Previous studies have shown that T47D cells harbor abundant CT receptors. Two forms of human CT receptors have been cloned from expression cDNA library generated from these cells (15). We have therefore used T47D as control cells. Ishikawa and T47D cells were grown in calcium containing DMEM and 10% fetal bovine serum. 0.5 ml culture medium containing Ishikawa or T47D cells were placed in test tubes. Each tube received 50,000 cpm [125I]sCT (Peninsula Laboratories). Unlabeled sCT was added to the tubes to produce a cold displacement curve. The tubes were incubated overnight with gentle agitation at 4 C, washed twice with 2 ml culture medium, and counted in a γ-counter. The results were subjected to Scatchard analysis to determine the dissociation constant (Kd) and number of binding sites per cell.
**Measurement of Intracellular calcium**

Intracellular calcium was monitored in Ishikawa cells by fluorescence microscopy. 1000 Ishikawa cells per well were cultured in 96 well plate overnight, and loaded with 10µM fluo-3 acetoxyethyl ester (fluo-3 AM) at 37 C for 1 h. The attached cells were rinsed 3 times with medium and cultured in 100 µl of fresh medium. Some of the cells were preloaded for 1 h with 10 µM BAPTA-AM in addition to fluo-3-AM. Fluorescent images were generated by excitation of fluo-3 at 450 to 490 nm with a mercury lamp and detecting the light emitted at 525 nm. A GenIISys image intensifier (DAGE-MTI, Inc., Michigan city, IN) was used to enhance the signal. All images were video-taped every 5 sec using a Panasonic AG-7350 video recorder. The video camera (CCD 72, DAGE-MTI, Inc.) was set to reverse the image so that a computer-based image analysis system (MCID M4, Image Research, St. Catherines, Ontario, Canada) could be utilized to determine the fluorescence intensity. [Ca^{2+}]_i was estimated using the following formula: [Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F), where K_d is the dissociation constant for [Ca^{2+}]_i (316nM), F is the fluorescence intensity, F_{min} is the background fluorescence, and the F_{max} is the maximal fluorescence obtained by equilibrating cytoplasmic and extracellular Ca^{2+} using 5 µM ionomycin. 10 cells in each well were monitored to determine the average fluorescence intensity for calculation of [Ca^{2+}]_i.
Immunofluorescence

Ishikawa cells were grown in a calcium-containing DMEM (Gibco BRL, Grand Island, NY) medium to 60-70% confluency. Cells were then treated with either CT (10 nM/100 nM) or vehicle. Twenty four hours following treatment with CT, cell surface distribution of E-cadherin was examined by immunofluorescence using a monoclonal antibody that specifically recognizes E-cadherin. For certain experiments cells were also treated with CGRP (100 nM) or CGRP antagonist (8-37) for 24 h. In other experiments, cells were pretreated with BAPTA-AM (33.3 µM) for 1 h prior to the addition of CT. The experiments were repeated at least three times.

Northern Blot Analysis

For Northern analysis 20 µg of total RNA was separated by formaldehyde agarose gel electrophoresis and transferred to Duralon membrane (Stratagene). After transfer, the membranes were baked at 80 C for 2 h. Blots were prehybridized in 50 mM NaPO₄, pH 6.5 / 5X SSC / 5X Denhardt’s / 50% formamide / 0.1% SDS and 100 µg/ml salmon sperm DNA for 4 h at 42 C. Hybridization was carried out overnight in the same buffer containing 10⁶ cpm/ml of a ³²P-labeled E-cadherin cDNA fragment. The filters were washed twice for 15 min in 1X SSC/0.1% SDS at room temperature, then twice for 20 min in 0.2X SSC/0.1%
SDS at 55°C and the filters were exposed to X-ray films for 24-72 h. The intensities of signals on the autoradiogram were estimated by densitometric scanning. To correct for RNA loading, the obtained signals were normalized with respect to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) signal in the same blot. For this the filters were stripped of the radioactive probe by washing for 10 min in 0.5% SDS at 95°C. The blots were then re-probed with a 32P-labeled GAPDH probe as described above.

**In Situ Hybridization**

Uterine tissues from pregnant animals were collected and frozen. Tissues were fixed in 4% paraformaldehyde at 4°C. Cryostat sections were cut at 8 µm and attached to 3-aminopropyl triethyl silane (Sigma) coated slides. *In situ* hybridization was performed with digoxygenin (DIG)-labeled sense or antisense RNA probes complimentary to E-cadherin gene. DIG-labeled RNA probes were synthesized from E-cadherin cDNA using T3 or T7 RNA polymerase and DIG-labeled nucleotides according to manufacturer’s specifications (Boehringer Mannheim). Prehybridization was carried out in a damp chamber at 37°C for 60 min in hybridization buffer (50% formamide, 5X SSC, 2% blocking reagent, 0.02% SDS, 0.1% N-laurylsarcosine). Hybridization was carried out at 42°C overnight in a damp humidified chamber. To develop the substrate, sections were sequentially washed in 2X SSC,
1X SSC, and 0.1X SSC for 15 min in each buffer at 37 C. Sections were then incubated with anti-DIG alkaline phosphatase conjugated antibody. Excess antibody was washed away and the color substrate (Nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indoylphosphate, NBT-BCIP) was added. Slides were allowed to develop in the dark and the color was visualized under light microscopy until maximum levels of staining were achieved. The reaction was stopped and the slides counterstained in Nuclear Fast Red for 5 min. The slides were washed in water, dehydrated, and coverslipped. Control incubations utilized a DIG-labeled RNA sense strand and were performed under identical conditions.

**Immunohistochemistry**

Polyclonal antibodies against rat E-cadherin and ZO1 were diluted 1:1000 for immunohistochemistry. Frozen uteri were sectioned at 7 µm, mounted on slides, and then fixed in 5% formaldehyde in phosphate-buffered saline (PBS). Sections were washed in PBS for 20 min and then incubated in a blocking solution containing 10% normal rabbit serum for 10 min before incubation in primary antibody overnight at 4 C. Immunostaining was performed using a Streptavidin-Biotin kit for rabbit primary antibody (Zymed, Burlingame, CA). Red deposits indicate the sites of immunostaining.

**Reverse Transcription-Polymerase Chain Reaction**
Total RNA was subjected to reverse transcription reaction using a Stratascript RT-PCR kit. Briefly, the RNA samples were mixed with oligo (dT) primer, incubated at 65°C for 5 min and annealed at room temperature. First strand cDNA was synthesized using MMLV reverse transcriptase at 37°C and the reaction was stopped by heating the tubes at 95°C for 5 min. PCR reaction was then performed in 100 µl total volume using 35 ng of CT, E-cadherin, and GAPDH-specific primers, 200 µM each of dATP, dGTP, dCTP and dTTP, 1.5 mM Mg++ and 0.5 µl of Taq DNA polymerase (Perkin-Elmer). The conditions for PCR were 94°C, 30 sec; 1 cycle followed by 94°C, 30 sec; 65°C, 30 sec; and 68°C, 2 min; 25 cycles. PCR products were electrophoresed on agarose gels and processed for Southern blot analysis.

Total RNA was prepared from Ishikawa cells as well as from T47D breast cancer cells. The T47D cells are known to express abundant CTRs and served as a positive control (15). The RNA samples were reverse transcribed and amplified by PCR using CTR-specific primers that flank the region of the insert containing 16 amino acids. The PCR products were then subjected to Southern blot analysis employing a radiolabeled CTR cDNA fragment as a probe.

**Southern Blot Analysis**

PCR products (2 µl each) were run on 1% agarose gel. After electrophoresis, the gel was
transferred to Duralon membrane (Stratagene). The membrane was prehybridized in 6X SSC, 5X Denhardt’s, 0.5% SDS, and 100 µg/ml salmon sperm DNA for 2 h at 68°C. Hybridization was performed in the same buffer containing 10^6 cpm/ml of ^32P-labeled cDNA fragments of CT, E-cadherin, and GAPDH overnight at 68°C. The membrane was washed with 2X SSC and 0.1% SDS for 15 min at room temperature, in 0.1X SSC containing 0.5% SDS at 68°C for 45 min and exposed to x-ray film for 8 h.

**SDS-PAGE and Immunoblot Analysis of Uterine Extracts**

Uteri were collected from rats on days 1, 4, and 6 of pregnancy, homogenized, and extracted in chilled buffer containing 0.5% Triton X-100, 25 mM KCl, 120 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1 mM DTT, 0.015 mM pepstatin A, 0.085 mM bestatin and 0.011 mM E-64 in 10 mM Tris-HCl, pH 7.5. The extract was centrifuged and the supernatant was loaded (50-80 mg of total protein in each lane) onto 7.5% SDS-PAGE gel. The gel was then blotted onto PVDF membrane. The PVDF membrane was transferred to a solution of 5% dried nonfat milk in TBS (0.1 M Tris-buffered saline Ph 7.5) and incubated overnight with primary antibody (rabbit anti-E-cadherin polyclonal antibody or rabbit anti-ZO-1 polyclonal antibody). After washes, the membrane was probed with secondary antibody coupled to horseradish peroxidase, washed extensively before incubation in enhanced chemiluminescence reagent and exposed to autoradiographic film.
Treatment of animals with ODNs

Pregnant rats were treated with sense and antisense ODNs targeted to calcitonin mRNA as described previously (13). Briefly, rats were deeply anesthetized and an incision was made in the lower abdomen. The sense or antisense ODNs were mixed with DOTAP (Boehringer Mannheim, Indianapolis) and 20% F127 pluronic gel (Sigma Chemical Co., St. Louis, MO). The solution was maintained in liquid form at 40 C prior to injection. 20 μg of this ODN solution was taken in pre-chilled syringes and injected into each uterine horn on day 2 of pregnancy. After surgery, the animals were returned to their cages. Forty-eight hours following the surgery, the animals were killed, uteri were collected, and mRNAs were isolated for Northern blot analysis. The blot was hybridized with 32P-labeled probes containing calcitonin, E-cadherin, and GAPDH cDNA sequences.

RESULTS

Ishikawa cells contain abundant cell surface receptors for CT

To investigate the regulation of cellular functions by CT and its receptor, human endometrial
adenocarcinoma (glandular origin) Ishikawa cells present a convenient model system. These cells, like normal preimplantation uterine epithelial cells, harbor estrogen and progesterone receptors and retain response to these steroid hormones. Since the responsiveness of a target cell to CT is dependent upon the presence of the cognate receptors on the cell surface, we first ascertained that CTRs are indeed expressed in Ishikawa cells. The human CTR is expressed in two molecular forms, which differ slightly in size (14). The larger isoform contains a 16 amino acid insert that the shorter form lacks. We initially analyzed the expression of CTR mRNAs in Ishikawa cells by RT-PCR. Since previous reports indicated that T47D cells harbor abundant functional calcitonin receptors (14), these cells were used as positive control cells. As shown in Figure 1, significant amounts of the two isoforms of CTR mRNAs were detected in Ishikawa cells (lane 2) and the levels of these mRNAs were comparable to those in T47D cells (lane 1).

We also determined the number of CT binding sites in Ishikawa cells by using radiolabeled CT. The results of the Scatchard analysis are shown in Table 1. The dissociation constants for [125I] CT binding in T47D and Ishikawa cells were statistically equivalent. The number of CT binding sites in T47D and Ishikawa cells were also similar. Whereas T47D cells exhibited 3.76x10^4 CT binding sites per cell, the Ishikawa cells harbored 2.78x10^4 CT binding sites per cell. These results indicated that Ishikawa cells are likely to be responsive to CT.
Binding of CT to its cell surface receptor increases intracellular calcium in Ishikawa cells

Binding of CT to CTR is known to elevate intracellular calcium levels in a variety of cell types, including bone and kidney cells (16, 17). We, therefore, investigated the effect of CT on intracellular calcium levels in Ishikawa cells using fluorescence microscopy. In this experiment, the cells were plated at a density of $10^4$ cells/well in a 96 well plate and loaded with the fluorescent calcium indicator fluo-3 acetoxyethyl ester (fluo-3-AM). Some of the cells were preloaded with BAPTA-AM, an intracellular calcium chelator. 10 cells in each well were monitored to determine the average fluorescence intensity for calculation of $[\text{Ca}^{2+}]_i$. As shown in Fig. 2 (top panel), no intracellular calcium elevation was observed when cells were treated with vehicle alone. Treatment of the cells with 3 or 5 nM CT led to a marginal rise in intracellular calcium (Fig. 2, middle panel, left and center images, respectively). Addition of 10 nM CT, however, elicited a sharp rise in intracellular calcium level within 5 sec (Figure 2, middle panel, right image). This rise in intracellular calcium level was blocked when cells were preloaded with BAPTA-AM (Figure 2, bottom panel), indicating that the increased fluorescence of fluo-3 was specific for calcium. Taken together, these results demonstrated that Ishikawa cells respond to CT through receptor-mediated calcium signaling.

CT treatment triggers disappearance of E-cadherin protein from cell-cell contact sites
An alteration in cellular calcium is known to control adhesiveness and polarity of epithelial cells (18). Our observation that CT alters the level of intracellular calcium in Ishikawa cells raised the possibility that such alterations may influence cellular adhesiveness by changing the expression or triggering redistribution of critical cell adhesion molecules or junctional complexes. E-cadherin is a cell surface glycoprotein that mediates calcium-dependent cell-cell adhesion and is believed to be critical for the establishment and maintenance of adherens junctions in epithelial cells (19, 20). We, therefore, investigated whether treatment with CT altered expression or distribution of E-cadherin in Ishikawa cells.

Ishikawa cells grown in a calcium-containing medium to 60-70% confluency were treated with either CT (100 nM) or vehicle. Twenty four hours following treatment with CT, cell surface distribution of E-cadherin was examined by immunofluorescence using a monoclonal antibody that specifically recognizes E-cadherin. E-cadherin was clearly localized at points of cell-cell contact in the untreated Ishikawa cells (Figure 3A, panels B, and C, and Figure 3B, panel A). Upon treatment with CT, E-cadherin immunostaining at the cell surface was dramatically reduced after 24 hours (Figure 3A, panels b, and c, and Figure 3B, panel B). We also performed a time course to monitor the decline in E-cadherin after 2, 6, 12 and 24 hour of CT treatment. Our studies detected a marked decline in the level of E-cadherin in response to CT only after a 24-hour of hormone treatment (data not shown).

We next examined the role of CTR in CT-induced disappearance of E-cadherin from
contact sites of Ishikawa cells. We observed that when the cells were treated with another peptide hormone, CT gene related peptide (CGRP), which does not bind to CTR, E-cadherin expression at the contact sites of epithelial cells remained unaffected (Figure 3B, panel C). Interestingly, when cells were treated with a modified CGRP (8-37), which binds to CTR and functions as an agonist (40), E-cadherin expression at the points of cell-cell contact declined (Figure 3B, panel D), indicating that the effect of CT is dependent on CTR-mediated signaling.

**Calcium signaling is critical for CT regulation of E-cadherin expression**

We next tested the hypothesis that the CT-induced rise in intracellular calcium is a critical upstream event for the observed loss of E-cadherin from the cell-cell junctions. We, therefore, treated the cells with BAPTA-AM, which effectively chelates intracellular calcium. We found that in the presence of BAPTA-AM, CT failed to induce a loss of E-cadherin from the cell-cell contact sites (Figure 3B, panel E). These results suggested that this effect is indeed triggered by an intracellular calcium spike induced by CT. Collectively, these data are consistent with the view that CT acts through its cell surface receptor to initiate calcium signaling, which through a cascade of events eventually alters the expression of E-cadherin in epithelial cells.

**CT down-regulates E-cadherin mRNA in Ishikawa cells**
We next investigated whether the CT-induced change in expression of E-cadherin in Ishikawa cells is due to a down-regulation of E-cadherin mRNA. Ishikawa cells were grown to 60-70% confluency and treated with CT or vehicle. Twenty-four hours after treatment, cells were harvested to isolate mRNA for Northern blot analysis. Our results showed that significant amounts of E-cadherin mRNA were present in Ishikawa cells treated with vehicle alone (Figure 4, lanes 1 and 2). The addition of CT to Ishikawa cells led to a marked decline in the level of E-cadherin mRNA (Figure 4, lanes 3 and 4). The relative levels of expression of E-cadherin mRNA in Ishikawa cells treated with or without CT were estimated by densitometric scanning followed by normalization with respect to the control GAPDH mRNA signal. By our estimate, the magnitude of E-cadherin mRNA reduction in Ishikawa cells upon CT administration was greater than 60%. The calcium signaling induced by CT is, therefore, transduced by an unknown second messenger pathway(s) to down regulate expression of E-cadherin gene in Ishikawa cells.

**E-cadherin mRNA and protein declines in rat uterine epithelium at the onset of implantation**

Our previous studies showed that CT is transiently induced in rat endometrial epithelium within the implantation window (10). Since CT induced down regulation of E-cadherin expression in our *in vitro* cell culture studies, we sought to determine whether it exerts a similar effect on E-cadherin expression in the uterine tissue *in vivo.*
As a first step towards this goal, we analyzed the profile of E-cadherin mRNA expression in the uterus during early pregnancy. RNA was isolated from uteri of animals on days 1 to 6 of pregnancy and subjected to RT-PCR analysis using both CT- and E-cadherin-specific primers. Our results revealed significant amount of E-cadherin mRNA expression in the uterus during days 1 and 2 of pregnancy when CT mRNA expression remained undetectable or low (Figure 5). As the CT mRNA level started to rise on day 3 of gestation, we noted a progressive decline in E-cadherin mRNA level. Concomitant with the high levels of CT mRNA in rat uterus on days 4 and 5 of pregnancy, there was a sharp decline in the level of E-cadherin mRNA on these days. As CT mRNA level dropped on day 6, we observed that E-cadherin mRNA expression returned to a level, which is approximately 50% of that on day 1 of gestation.

We also performed immunoblot and immunohistochemical analyses to monitor the expression of E-cadherin protein in rat uterus on days 1, 4, and 6 of pregnancy. Figure 6A, shows immunoblotting of equal amounts of uterine extracts obtained from pregnant rats on days 1, 4, and 6 of pregnancy, employing antibodies against E-cadherin (top panel) or tight junction protein ZO-1 (bottom panel). A robust E-cadherin signal was observed in the endometrial extracts of day 1 pregnant rats (lane D1). The intensity of this signal declined considerably on day 4 of gestation (lane D4) and then recovered slightly on day 6 (lane D6). The level of ZO-1, however, did not alter significantly between days 1, 4, and 6 of pregnancy (Fig. 6A). Consistent with the immunoblot analysis, we found an intense staining
for E-cadherin, both at the lateral and apical junctions between the epithelial cells, on day 1 of pregnancy (Figure 6B, panel A). However, on day 4 of gestation, there was an overall decline in the E-cadherin staining in the epithelium. While almost no E-cadherin staining was observed at the lateral interfaces, very little staining was found at the apical junctions. On day 6 of gestation, the E-cadherin staining increased predominantly in the apical junctions compared to day 4 (Figure 6B, panels B and C). Collectively, these results indicated that the level of E-cadherin at the cell-cell junctions in the uterine epithelium declines at the onset of implantation.

**Inhibition of CT expression in preimplantation uterus by antisense ODN results in up-regulation of E-cadherin**

We had previously demonstrated that administration of antisense ODN targeted against CT mRNA led to an inhibition of CT gene expression in the preimplantation rat uterus (13). We, therefore, investigated whether the suppression of CT mRNA expression upon antisense ODN treatment influenced E-cadherin level during early pregnancy. As described previously (13), we administered either the antisense or the corresponding sense CT ODNs into both uterine horns of rats on the afternoon of day 2 of pregnancy. The animals were killed on day 4 of gestation, uteri were collected, and mRNAs were isolated for Northern blot analysis. The blot was hybridized with \(^{32}\text{P}\)-labeled probes containing CT, E-cadherin, and GAPDH.
cDNA sequences. The results of these experiments are shown in Figure 7. As shown in the top panel of the figure, the uterine horns that were treated with antisense ODNs exhibited significantly reduced calcitonin mRNAs on day 4 compared to the horns that were injected with the same doses of corresponding sense ODNs (compare lanes 1 and 3 with lanes 2 and 4, respectively). Interestingly, the uterine horns that exhibited reduced level of CT expression upon administration of antisense ODN showed higher level of E-cadherin compared to the horns that were treated with sense ODNs (Fig. 7, middle panel, compare lanes 1 and 3 with lanes 2 and 4, respectively). Hybridization of the blot with GAPDH probe indicated no significant alteration in the intensity of this signal in the antisense or sense ODN-treated uterus (Figure 7, bottom panel). These results suggested that in the pregnant rat uterus CT and E-cadherin expression exhibit a reciprocal relationship and are likely to be functionally linked.

**Administration of exogenous CT leads to precocious down-regulation of E-cadherin during early pregnancy**

We reasoned that if the transient expression of CT and the down-regulation of E-cadherin in the surface epithelium are functionally linked events, administration of exogenous CT to pregnant rats will be expected to lead to a further decline in the level of E-cadherin in the uterus. Specifically, we examined whether premature elevation of CT levels in the pregnant rat uterus leads to a concomitant reduction in the level of E-cadherin in the epithelial cells.
Animals on day 2 of pregnancy, when the level of endogenous CT level is extremely low and the level of E-cadherin is high, were injected intravenously or intramuscularly with CT or vehicle. Twenty-four hours after administration of the peptide hormone, uterine mRNA was isolated and subjected to Northern blot analysis using a radiolabeled fragment of E-cadherin cDNA as a probe. Figure 8 shows that in comparison to animals treated with vehicle only, the level of uterine E-cadherin mRNA on day 3 of pregnancy was markedly reduced following administration of CT (compare lanes 1 and 2; 3 and 4).

We also performed in situ hybridization and immunohistochemistry to further confirm the down-regulation of E-cadherin mRNA and protein in uterine epithelium in response to exogenous CT. In situ hybridization with antisense E-cadherin RNA probe revealed an intense signal of E-cadherin mRNA in the surface epithelial cells of vehicle-treated (control) uteri of day 3 pregnant animals (Figure 9A, panel A). Control uterine sections hybridized with the corresponding sense RNA probe of equal length did not exhibit any signal demonstrating the specificity of the hybridization reaction (Figure 9A, panel C). A remarkable decline in the level of E-cadherin mRNA was observed in the luminal epithelium of day 3 pregnant animals following exogenous administration of CT on day 2 of pregnancy (Figure 9A, panel B).

Essentially similar results were obtained by immunohistochemical analysis. As shown in Figure 9B, a marked E-cadherin-specific staining was observed in the luminal epithelium of
uterine sections of animals treated with vehicle only on day 2 and killed on day 3 of pregnancy (panel a). However, a dramatic reduction in the level of E-cadherin was observed in the epithelial cells of uterus following administration of CT (panel b). The specificity of this down regulation of E-cadherin in response to CT was demonstrated by the fact that the expression of the tight junction protein ZO-1 in the uterine epithelial cells was unaffected upon administration of CT (Figure 9C). Consistent with these results, immunoblot analysis of uterine extracts obtained from day 2 pregnant rats treated with CT, showed a dramatic decline in the level of E-cadherin compared to vehicle-treated controls 24 h after treatment (Fig. 9D, top panel, compare lanes 1 and 3 with lanes 2 and 4, respectively). The level of ZO-1 in the uterine extracts, however, did not change significantly upon administration of CT (Fig. 9D, bottom panel, compare lanes 1 and 3 with lanes 2 and 4, respectively). Taken together, these studies strongly support our hypothesis that uterine CT functions during implantation by specifically remodeling the adherens junctions in the endometrial epithelium.

**DISCUSSION**

This study addresses the mechanism of action of CT as a paracrine or autocrine effector in the uterus during implantation. Our previous studies revealed that the uterine epithelium of
species as diverse as rat and human transiently produces CT within the window of implantation (10, 12). Targeted suppression of CT mRNAs in the preimplantation uterus upon administration of antisense ODNs led to a block of implantation (13). These results prompted us to speculate that CT is secreted into the uterine lumen at the time of implantation and its principal function in the preimplantation uterus is to regulate blastocyst implantation in a paracrine manner. Consistent with this prediction, we detected significant amounts of CT in the luminal secretions of rat uterus collected on days 4-5 of pregnancy, indicating that this hormone is secreted at or near the implantation bed (11). It was speculated that the secreted CT would bind to CTR of surface epithelial cells and modulate their function at the time of implantation. Here, we describe that CT indeed alters cell adhesiveness in the surface epithelium by reprogramming the expression of E-cadherin, a critical adhesion molecule.

CT functions through its cell surface receptor, CTR, which belongs to a class of seven transmembrane G-protein-coupled receptors (21). Previous studies have shown that binding of CT to CTR on the target cell leads to a rise in intracellular calcium and/or cAMP, which in turn regulate cellular functions (16, 17, 22). Depending upon the target cell, the CTRs are known to couple to multiple heterotrimeric G proteins, leading to the activation of adenyl cyclase and/or phospholipase C (16, 17, 22). Signaling through both cAMP and intracellular calcium are important in CT action in osteoclasts, chondrocytes, and renal cells. In brain, however, CT apparently causes no change of adenylate cyclase activity and there is some
evidence that calcium acts as a second messenger in this tissue (23-25). Likewise, in hepatocytes CT-induced adenylate cyclase activity has not been shown, but CT, even at very low concentration, is capable of increasing intracellular calcium levels (26). Although we do not know whether binding of CT to its receptor activates cAMP production in Ishikawa cells, we did observe a rapid elevation in intracellular calcium in response to this hormone, presumably via phospholipase C activation. It is likely that inositol phosphate (IP3), which is produced following the activation of phospholipase C by CT, stimulates release of calcium from intracellular stores.

As intracellular calcium level rises in response to CT, one or more calcium-sensitive signal transduction cascades are likely activated, leading to regulation of cellular functions. Three well-established calcium-sensitive pathways involve protein kinase C (PKC), calmodulin-dependent kinase (CaM kinase), and mitogen-activated protein kinase (MAPK). In each of these pathways, the kinase is activated either directly or indirectly in response to calcium. The activated kinase phosphorylates the transcription factors that recognize specific cis-acting elements in the control regions of a specific network of target genes. Alternatively, calcium-dependent kinases can influence assembly or disassembly of junctional complexes through direct phosphorylation or dephosphorylation of undefined protein targets.

Our study shows that CT-induced upsurge in intracellular calcium suppresses the level of E-cadherin mRNA in Ishikawa cells. This effect is manifested in the loss of E-cadherin protein
from cell-cell contact sites in *in vitro* cell cultures as well as in pregnant uterine epithelium at the time of implantation. E-cadherin, a transmembrane linker protein of the intercellular adherens junctions, plays a key role in the maintenance of the adhesive and polarized phenotype of uterine and other epithelial cells (19, 20, 37). Cell-cell adhesion results from head-to-head contact between cadherin homodimers in adjacent cell membranes. It is important to note that many previous studies hinted at a role of calcium in reprogramming of cell-cell interactions in the surface epithelium during implantation, although the precise molecular basis of this process eluded us (27-29). The present study, for the first time, clearly links changes in calcium homeostasis to specific reorganization of a master cell adhesion molecule in the surface epithelium at the time of implantation.

A number of recent studies have reported down regulation of E-cadherin expression during the acquisition of metastatic potential at late stages of epithelial tumor progression (30-33). In majority of these cases the decline in the level of E-cadherin has been attributed to a transcriptional event in which trans-acting regulators, such as multi-zinc finger proteins *snail* and SIP1, bind directly to E-cadherin promoter and repress RNA synthesis (30-32). However, down regulation of E-cadherin is also known to occur via endocytosis following ubiquitination of the E-cadherin complex (33). Our studies in Ishikawa cells and in intact rat uterine epithelium seem to suggest that down regulation of E-cadherin in response to CT is a transcriptional event. It is likely that a CT-induced cascade modulates the activity of transcription factors that regulate the E-cadherin promoter. The chain of events is initiated
by binding of CT to its receptor, resulting in a surge of intracellular calcium. The blockade of the elevation in intracellular calcium by BAPTA-AM prevented down regulation of E-cadherin, indicating that signaling downstream of calcium release is critical for this effect. The elevated intracellular calcium activates one or more kinase signaling pathways, leading to the phosphorylation of a transcription factor, which then regulates E-cadherin gene expression. We, however, cannot rule out the possibility that post-transcriptional/translational modifications in response to CT also contribute to the down regulation of E-cadherin mRNA.

The identity of the kinase cascade(s) that is activated by CT-induced calcium signaling and is operative in down regulating E-cadherin expression in the endometrial epithelial cells remains unknown. Interestingly, a recent study has shown that CTR can activate parallel independent signaling pathways in the same target cell (34). In cultured cells stably expressing the CTRs, CT induced time- and concentration-dependent increases in MAPK phosphorylation and activation. The study further showed that besides increasing MAPK phosphorylation, CT also induces the activation of other signaling effectors, including PKC. Thus, multiple signaling pathways are operative downstream of an increase in intracellular calcium for full CTR-dependent cellular response. Taken together, these studies point to the complexity of CT/CTR mediated signaling pathway in a target cell.

CT-induced down regulation of E-cadherin in the pregnant uterus may have important
implications for the process of implantation. Biochemically distinct junctional complexes hold the uterine luminal epithelial cells together. Whereas tight junctions occur between apical surfaces, adherens junctions at basolateral membranes mediate stable cell-cell adhesion. A remodeling of various junctional complexes in the uterine epithelial cells during implantation is well documented (35-39). It is thought that as the uterus enters the receptive phase under the influence of steroid hormones, the epithelial cells acquire adhesiveness for trophoblast. The molecular basis of this switch from a non-adhesive state to an adhesive state is not clear. It is, however, conceivable that during acquisition of adhesiveness, alterations in both apical and basolateral junctional complexes in the surface epithelium take place in a concerted way and multiple steroid hormone-induced factors regulate this process. CT, which specifically alters the adherens junctions without affecting the ZO1 expression at the apical interfaces, is clearly one of these factors. Most interestingly, we have previously shown that the expression of CT in the receptive uterus is induced by progesterone. CT, therefore, represents one of the molecular links between steroid hormone action and the functional reprogramming of epithelial cell adhesiveness during implantation.

A CT-induced decline in the level of E-cadherin would disrupt lateral adhesiveness between the surface epithelial cells. This scenario may have functional consequences for the process of implantation in rodents in which the trophoblast invasion occurs via a “displacement” mechanism (2). The trophectoderm cells of the embryo need to penetrate between the intact luminal epithelial cells of the uterus. One can, therefore, envision that the down regulation of
E-cadherin at the time of implantation may lead to a plastic epithelium in which the intercellular space can be penetrated more easily by invading trophoblast cells. After penetration of the trophoblast cells between the lateral surfaces of the epithelium, the cells of the lining epithelium are displaced and appear to undergo apoptosis. While apoptosis is restricted to the cells adjacent to the embryo, the reduction in E-cadherin occurs throughout the epithelium. The disruption of cell-cell contact, which Glasser (6) suggested to be the initial signal that ultimately leads to the loss of uterine epithelial integrity and sloughing of cells during implantation, could in part be due to the CT-mediated down regulation of E-cadherin in epithelial cells. Interestingly, our previous studies have shown that addition of CT to embryos cultured in vitro advances their development (41). One can, therefore, envision a role for CT in coordinating the developmental programs of both embryo and uterus during implantation. In response to this hormone, the trophoblast cells are ready to invade as the epithelium becomes receptive and the junctions begin to open up. Future development of an in vitro model system to study the interactions between trophoblast and uterine epithelial cells would allow us to rigorously test this hypothesis.

Acknowledgements: The authors would like to thank Michelle Macaraig for expert technical assistance. The advice and help of Dr. Li-Ji Zhu in the immunostaining and immunofluorescence studies are also gratefully acknowledged. This work was supported by
NIH grants R01 HD-34527 and R01 HD-39291 to I.C.B. M. K. Bagchi is supported by NIH Grants R01-DK-50257 and U54-HD-13541.

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FIGURE LEGENDS

Figure 1. Expression of CTR in Ishikawa cells. Total RNA (0.2 µg) was isolated from T47D (lane 1) or Ishikawa (lane 2) cells and subjected to RT-PCR using CTR- (30 cycles) or GAPDH- (15 cycles) specific primers. The authenticity of the amplified products was confirmed by Southern blotting using 32P-labeled CTR or GAPDH probes. The experiment
was repeated twice for reproducibility. The results of a representative experiment are shown.

**Figure 2.** Profile of CT-induced calcium influx in Ishikawa cells. Cells preloaded with fluo-3 AM were treated with vehicle (top panel, all three images), CT (middle panel, left image, 3 nM, center image, 5 nM, and right image, 10 nM), or after pretreatment of BAPTA-AM (bottom panel, all three images). Fluorescent images were generated by excitation of fluo-3 at 450 to 490 nm with a mercury lamp and detecting the light emitted at 525 nm.

**Figure 3A.** Changes in E-cadherin in Ishikawa cells with or without CT treatment. Phase-contrast images of semiconfluent cultures of Ishikawa cells without (panel A) or with CT treatment for 24 hours (panel a). Panels B, b, C, and c show expression of E-cadherin by immunofluorescence. Ishikawa cells were treated without (panels B, C) or with CT (b, and c) for 24 hours. Magnification: Panels A, a, B, and b; 40X, C and c; 100X

**Figure 3B.** CT-induced change in E-cadherin expression is dependent upon CTR-mediated intracellular calcium signaling. Analysis by immunofluorescence the expression of E-cadherin in Ishikawa cells treated with vehicle (panel A), CT (panel B), CGRP (panel C), CGRP(8-37) (panel D), BAPTA-AM prior to the addition of CT (panel E).

**Figure 4.** CT down regulates E-cadherin mRNA in Ishikawa cells. Northern blot analysis of E-cadherin expression in cells untreated (lanes 1 and 2) or treated with CT (lanes 3 and 4).
for 24 hours. 20 µg of total RNA was loaded per lane. GAPDH was used as an internal control.

**Figure 5.** Profile of expression of CT and E-cadherin mRNA in rat uterus between days 1 and 6 of early pregnancy. Total RNAs (1 µg) were reverse transcribed using oligo dT primer and RNase H-reverse transcriptase. The first strand cDNA products were amplified using either CT-specific primers (top panel) or E-cadherin primers (bottom panel).

**Figure 6.** Expression of E-cadherin in rat uterus by immunoblotting and immunocytochemistry. A. E-cadherin (upper panel) and ZO-1 (lower panel) were detected in uterine extracts of rats on days 1 (lane 1), 4 (lane 2), and 6 (lane 3) of pregnancy by immunoblotting. B. Immunocytochemistry was performed employing polyclonal rabbit anti-E-cadherin using sections from day 1 (panel A), day 4 (panel B), and day 6 (panel C) pregnant rat uterus as described in the Materials and Methods. Red deposits indicate sites of specific immunoreactivity.

**Figure 7.** Effects of antisense or sense calcitonin ODNs on CT and E-cadherin mRNA expression in pregnant rat uterus. Rats on day 2 (afternoon) of pregnancy were injected in both uterine horns with either antisense or the corresponding sense CT ODNs. The uteri were collected 48 h after treatment. Total RNAs (20 µg/lane) were prepared from uteri collected from ODN-treated animals and analyzed by Northern blotting. S and As represent
samples from sense and antisense ODN-treated animals, respectively. The upper panel shows the pattern of signals obtained after hybridization with a $^{32}$P-labeled CT probe. The middle and lower panels show the same blot after hybridization with $^{32}$P-labeled E-cadherin and GAPDH probes, respectively.

**Figure 8.** Administration of exogenous CT leads to a down regulation of E-cadherin mRNA in the uteri of pregnant rats. RNA (20 µg per lane) was subjected to Northern blot analysis and hybridized with a $^{32}$P-labeled E-cadherin (upper panel) or GAPDH (lower panel) probe. Lanes 1 and 3 represent RNA from uteri of animals injected with vehicle only; 2: RNA from uteri of animals injected with CT (intravenous); 4: RNA from uteri of animals injected with CT (intramuscular). All the injections were performed on day 2 of pregnancy and the uteri were isolated 24 hour later on day 3 of gestation.

**Figure 9.** Expression of E-cadherin and ZO-1 in the uterus of animals treated with CT during early pregnancy. A: *in situ* hybridization analysis of uterine sections from pregnant (day 3) rats treated with vehicle (panel A) or CT (panel B) on day 2 of pregnancy. Hybridization was performed employing a cRNA probe specific for E-cadherin. Panel C represents hybridization of the CT-treated uterine section with a sense E-cadherin probe. B: Immunohistochemistry was performed employing polyclonal rabbit anti-E-cadherin using uterine sections from day 3 pregnant rats injected with vehicle (panel a) or CT (panel b) on
day 2 of pregnancy. C: Immunohistochemical analysis of ZO-1 in the uterine sections of rats injected with vehicle (panel A) or CT (panel B). D: Immunoblot detection of E-cadherin (upper panel) and ZO-1 (lower panel) in uterine extracts of pregnant rats treated with vehicle (lanes 1 and 3) or CT (lanes 2 and 4). The animals were injected on day 2 of gestation and the uteri were collected on day 3.
|            | $K_d$ (nM) | Binding sites (no./cell) |
|------------|------------|--------------------------|
| Ishikawa   | 1.94       | $2.78 \times 10^4$       |
| T47D       | 1.27       | $3.76 \times 10^4$       |
Calcitonin down-regulates E-cadherin expression in rodent uterine epithelium during implantation

Quanxi Li, Jun Wang, D. Randal Armant, Milan K. Bagchi and Indrani C. Bagchi

J. Biol. Chem. published online September 16, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203555200

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