

Growth Factors, Cytokines, Cell Cycle Molecules

# Corticotropin-Releasing Hormone Modulates Human Trophoblast Invasion through Carcinoembryonic Antigen-Related Cell Adhesion Molecule-1 Regulation

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**Abnormalities in the process of trophoblast invasion may result in abnormal placentation. Both the embryonic trophoblast and maternal decidua produce corticotropin-releasing hormone (CRH), which promotes implantation. Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), which is expressed in extravillous trophoblasts (EVTs) of normal human placenta, may also function in trophoblast/endometrial interactions. We investigated whether locally produced CRH plays a role in trophoblast invasion, primarily by regulating CEACAM1 expression. We examined cultures of freshly isolated human EVT, which express CEACAM1, and an EVT-based hybridoma cell line, which is devoid of endogenous CEACAM1. CRH inhibited EVT invasion in Matrigel invasion assays, and this effect was blocked by the CRH receptor type 1 (CRHR1)-specific antagonist antalarmin. Additionally, CRH decreased CEACAM1 expression in EVTs in a dose-dependent manner. After transfection of the hybridoma cell line with a CEACAM1 expression vector, the invasiveness of**

**these cells was strongly enhanced. This effect was inhibited by addition of blocking monoclonal antibody against CEACAM1. Furthermore, blocking of endogenous CEACAM1 in EVTs inhibited the invasive potential of these cells. Taken together these findings suggest that CRH inhibits trophoblast invasion by decreasing the expression of CEACAM1 through CRHR1, an effect that might be involved in the pathophysiology of clinical conditions, such as preeclampsia and placenta accreta. (Am J Pathol 2006, 168:141–150; DOI: 10.2353/ajpath.2006.050167)**

The complex developmental process of implantation involves a series of steps leading to an effective cross-talk between invasive trophoblast cells and the maternal endometrium. This dynamic process requires a precisely coordinated development of a hormonally primed adhesive endometrium and a blastocyst competent to implant. The trophoblast undergoes a number of distinct interactions with the underlying endometrial surface initiated by apposition, which involves close proximity between trophoblast and endometrial epithelium, followed by attachment, and concluded by invasion of trophoblast into the decidualized stroma.<sup>1</sup> However, the molecular interactions at the embryo-maternal interface during the time of adhesion and subsequent invasion are not fully understood.

The hypothalamic neuropeptide corticotropin-releasing hormone (CRH) is produced in several organs of the female reproductive system, including the endometrial glands, decidualized stroma, and trophoblast.<sup>2–6</sup> In addition, the gene encoding the CRH receptor type 1

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(*CRHR1*) is expressed in human endometrial and myometrial cells, indicating a local effect of uterine CRH.<sup>7,8</sup> Indeed, locally produced CRH promotes implantation and maintenance of early pregnancy.<sup>2</sup>

The trophoblast is the first tissue to differentiate in the mammalian conceptus, and its normal development and specific properties are crucial for both implantation and further survival of the embryo. Furthermore, the placenta is unique in its ability to proliferate and invade another tissue in a controlled manner. It is not surprising that similarities exist between trophoblast invasion and the invasion of cancer cells. The endometrium restricts trophoblast invasion, whereas the latter is highly invasive in the human when it implants in ectopic sites, such as the peritoneum. Thus, trophoblast invasion is a very interesting model for the study of molecular mechanisms involved in these processes.

Starting with the initial contact, which is made between the trophoblast and the apical plasma membrane of the endometrial surface epithelial cells, through the invasion of the decidua and the invasion of decidual vessels with gradual colonization of the arterial wall of the spiral arteries, cellular contacts mediated by cell adhesion molecules are essential. Cell adhesion molecules are important mediators of tissue architecture and cellular polarity and have also been shown to modulate proliferation and differentiation processes.

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a member of the carcinoembryonic antigen (CEA) family and the immunoglobulin superfamily.<sup>9</sup> Glycoproteins belonging to this family are expressed in epithelial tissues, such as the colonic mucosa, as well as in cells of the myeloid lineage.<sup>9</sup> CEACAM1 (CD66a, C-CAM, BGP)<sup>10</sup> is the human homologue of the adhesion molecule cell-CAM (C-CAM) of the rat<sup>11</sup> suggested to function as a ligand for the endothelial adhesion molecule E-selectin.<sup>12</sup> In contrast to most of the genes of the CEA family, the CEACAM1 gene predicts the presence of a cytoplasmic domain containing sequence motifs interacting with signal transduction molecules,<sup>13,14</sup> such as pp60, c-src.<sup>15</sup> CEACAM1 is expressed in the normal human placenta with a specific localization in the extravillous trophoblast (EVT).<sup>16</sup> Because the EVTs are implicated in the invasion of maternal tissue, CEACAM1 may be involved in trophoblast/endometrial interactions.<sup>16</sup> The present study was designed to investigate the potential implication of CRH and CEACAM1 in implantation and placentation. We tested the hypothesis that locally produced CRH plays a role in trophoblast invasion, primarily by regulating CEACAM1 expression.

## Materials and Methods

### Reagents, Drugs, and Antibodies

All chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO), unless otherwise stated. The CRH antibody (rabbit antiserum; Peninsula Laboratories, Baltimore, MD) was raised against human

CRH and affinity-purified; it exhibited 100% cross-reactivity to rat CRH and no cross-reactivity to ovine CRH, human ACTH, gonadotropin-releasing hormone (GnRH), or arginine vasopressin. Affinity-purified goat anti-human CRHR1 (A-15) and goat anti-human CRHR2 (N-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). The CRHR1 antagonist antalarmin was obtained from the Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). To investigate the expression of CEACAM1, we used the monoclonal antibody mAb 4D1/C2<sup>17,18</sup> (Laboratory of Clinical Chemistry, University Hospital Eppendorf, Hamburg, Germany), which was previously used to study expression of this molecule in several normal human tissues,<sup>19</sup> as well as in normal and malignant mammary gland tissues and endometrial neoplasias.<sup>20,21</sup>

### Isolation and Characterization of Human Invasive EVT Populations

Cultures of first trimester invasive trophoblast populations were established and characterized as previously described.<sup>22</sup> Ten placentas were obtained from healthy women after legal termination of pregnancy (5 to 10 weeks of gestation). Informed consent and institutional review board approval were obtained. Briefly, the placentas were washed in sterile phosphate-buffered saline (s-PBS) until the supernatant was nearly free of blood. Areas rich in chorionic villi were selected and minced between scalpel blades into small pieces. The minced tissues were washed in s-PBS and were subjected to a 10-minute treatment with 0.125% trypsin and 0.2 mg/ml DNase I (Boehringer Mannheim GmbH, Mannheim, Germany) per ml in s-PBS containing 5 mmol/L MgCl. Cells that were released from this treatment were pooled and filtered through two layers of muslin. Trypsin was inactivated with fetal calf serum (FCS) (Gibco Life Technologies, Paisley, UK). The filtrates were centrifuged and cell pellets were washed with s-PBS. Cells were then resuspended in 70% Percoll (Pharmacia, Uppsala, Sweden) at a density of  $2 \times 10^5$  cells/ml and put under 20 ml of 25% Percoll. Ten ml of s-PBS were put on top of the 25% Percoll and a gradient was established by centrifuging for 20 minutes at 2000 rpm/minute. Cells from the middle band (density, 1.048 to 1.062 g/ml) of the gradient were pooled, washed in s-PBS, and seeded at a density of  $1.0 \times 10^6$  cells/ml of keratinocyte growth medium (Gibco) supplemented with 10% FCS (Gibco).

Cells were identified as trophoblasts by flow cytometry and immunocytochemical staining with monoclonal antibodies (mAbs) to cytokeratin (mAbs MNF116 and 35 $\beta$ H11, diluted 1:100; DakoCytomation, Glostrup, Denmark) and E-cadherin (mAb HECD-1; Takara Shuzo Co., Shiga, Japan), which stain only trophoblasts in the placenta.<sup>23</sup> The isolated trophoblasts were further characterized as EVTs by staining positive for major histocompatibility complex class I molecules (using anti-MHC mAb W6/32, diluted 1:50, DakoCytomation) and negative for hyaluronic acid (using mAb NDOG1, diluted 1:10; Serotec, Kidlington, UK), as previously described.<sup>2,16,23</sup>

The cells were kept in keratinocyte growth medium supplemented with 10% FCS and 1% penicillin-streptomycin (Gibco) at 5% CO<sub>2</sub> and 37°C for three to five passages before experiments. Percentages of cells stained positively for the above peptides before cultured for three to five passages were as follows: 97 ± 2% for cytokeratin, 97 ± 2% for E-cadherin, and 96 ± 2% for MHC class I. The respective percentages after three to five passages in culture were as follows: 92 ± 2% for cytokeratin, 92 ± 2% for E-cadherin, and 91 ± 2% for MHC class I.

### *Cell Lines*

The cells used for the transfection of CEACAM1 are EVT-choriocarcinoma hybrid cells (clone AC1M88), whose generation was achieved in previous studies.<sup>24,25</sup> Briefly, the initial cells used are EVTs from human term chorion laeve.<sup>25</sup> Because these cells do not proliferate at all, they were immortalized by hybridoma formation with suitable malignant tumor cells, ie, choriocarcinoma cells.<sup>24</sup> To eliminate all unfused tumor cells, choriocarcinoma cells used were deficient for hypoxanthine-guanine-phosphoribosyl transferase (HGPRT-negative mutants). Unfused tumor cells were thus eliminated by treatment with azaserine, while hybridomas compensated for HGPRT deficiency by the normal activity of the enzyme in the wild-type (EVT) fusion partner.<sup>24</sup> The cells were kept in 44% Nutrient Mixture F-12 HAM (Sigma), 44% Dulbecco's modified Eagle's medium without Phenol red (Cambrex, Walkersville, MD), 10% FCS (Gibco), 1% L-glutamine 200 mmol/L (Gibco-BRL), 1% penicillin-streptomycin (Gibco-BRL) at 5% CO<sub>2</sub> and 37°C and split twice a week.

### *Transient Transfections*

Transfections of placental hybridoma cells were performed with Lipofectamine Plus reagent (Life Technologies, Karlsruhe, Germany). On the day before transfection, cells were plated in six-well culture plates at a density of  $5 \times 10^5$  cells/well. After 24 hours, the medium was replaced by 0.8 ml of FCS-free medium per well, and the cells were transfected with 1 μg of plasmid DNA with PLUS reagent and lipofectamine, as suggested by the manufacturer. After a 3-hour incubation with this mixture, 1-ml aliquots of medium containing 20% FCS were added per well. The cells were harvested after 24 hours.

The construction of the expression vectors containing the full coding region of the *CEACAM1/L* gene in the plasmid pcDNA3.1(-) was recently described.<sup>26</sup> As negative control for the transfection experiments (mock), the expression vector pcDNA3.1(-) (Invitrogen, Karlsruhe, Germany) was used. To demonstrate that the transfections were successful, cells were analyzed by Western blot and immunocytochemistry.

### *Immunocytochemistry*

For immunostaining of transfected cell lines, the cells were trypsinized 24 hours after transfection, transferred

to culture slides (BD Biosciences, Heidelberg, Germany), and incubated for another 24 hours. Then, the chambers of the culture slides were removed, the cells were fixed in cold methanol (5 minutes) and acetone (30 seconds), air-dried, and stored at -20°C. After thawing for 20 minutes, the slides were washed in Tris-buffered saline, blocked with normal goat serum at room temperature for 30 minutes (DAKO, Glostrup, Denmark), diluted 1:20 in Tris-buffered saline, and incubated overnight at 4°C with mAb 4D1/C2 diluted 1:200 in Tris-buffered saline for the detection of CEACAM1. Slides were then reacted with biotin-labeled anti-mouse immunoglobulin (IgG), incubated with preformed ABC-complex (Vectastain; Vector Laboratories, Burlingame, CA) and detected with alkaline phosphatase substrate kit or diaminobenzidine kit (for hPL) (Vectastain, Vector Laboratories). The slides were counterstained with hemalaun and mounted with glycerine/gelatin.

### *Western Blot Analysis*

Extraction of proteins for Western blot analysis was performed in PBS in the presence of 1% Nonidet P-40 and protease inhibitors, as previously described.<sup>16</sup> Briefly, 24 hours after incubation with additives, cells ( $3 \times 10^6$  cells per sample) were harvested and lysed in a lysis buffer containing 50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Nonidet P-40, and freshly added proteinase inhibitors (10 μg/ml phenylmethyl sulfonyl fluoride and 1 μg/ml aprotinin) for the detection of CEACAM1 and actin proteins. Lysis was performed for 30 minutes on ice with occasional vortexing. Lysates were collected and stored at -80°C. The protein concentration of each lysate was measured by a modification of the Bradford Coomassie Brilliant Blue G-250 method using bovine serum albumin fraction V as standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in a 7.5% polyacrylamide gel under reducing conditions, applying 60 μg of each sample of protein extract. After electrophoretic transfer to nitrocellulose and blocking in Tris-buffered saline containing 5% bovine serum albumin for 2 hours, CEACAM1-specific mAb 4D1/C2 was added to a final concentration of 1 μg/ml and incubated at 4°C overnight. Detection was performed with a goat anti-mouse alkaline phosphatase-coupled antibody applying the chemiluminescent substrate and enhancer kit (Dianova). Films (Hyperfilm, Amersham) were exposed for 5 minutes. To normalize for protein content, the blots were stripped in stripping buffer containing 62.5 mmol/L Tris-HCl, pH 6.7, 2% sodium dodecyl sulfate, 100 mmol/L β-mercaptoethanol, and stained with anti-actin antibody. The concentration of CEACAM1 protein was normalized to actin. The intensity of the bands was quantified using the Bio-Rad imaging system.

### *Flow Cytometric Analysis of Human EVT Cells*

Flow cytometry was done as previously described.<sup>27</sup> The isolated human first trimester EVTs were incubated with

various concentrations of CRH with or without antalarmin for 24 hours. Then, the cells were washed in PBS with 0.1% bovine serum albumin, precipitated by centrifugation, and incubated with the high-affinity purified CEACAM1 antibody at 4°C for 1 hour. After washing with PBS, the cell pellet was incubated with the specific fluorescein isothiocyanate-conjugated secondary IgG antibody in the dark at 4°C for 30 minutes. After washing in PBS, cells were analyzed with an EPICS XL flow cytometer (Coulter Corp., Hialeah, FL). Cells from seven separate placentas were used for the analysis. Cells from each placenta were analyzed once under all conditions (ie, different CRH concentrations, addition of antalarmin). Negative controls included substitution of the primary CEACAM1 antibody with nonspecific isotype-matched antibody at the same concentrations.

### *Invasion Assay*

The invasive potential of freshly isolated EVT cells (after treatment with 100 nmol/L CRH and/or 1  $\mu$ mol/L antalarmin or with 1  $\mu$ g/ml or 10  $\mu$ g/ml blocking anti-CEACAM1 antibody) and placental hybridoma cells (clone AC1M88) (after transfection with the expression vectors for CEACAM1/L and anti-CEACAM1 treatment), was tested with Matrigel invasion chambers (24-well plates; BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Twenty-four hours after transfection with the vector or the mock plasmid, the cells were trypsinized and resuspended in fresh medium, and identical cell numbers for each transfection ( $2.5 \times 10^4$  cells/well) were transferred to the rehydrated inserts of the invasion assay. For comparison, the same number of control inserts without Matrigel membrane (BD Biosciences) was filled with the transfected cells in the same way. All tests were done in parallel.

After incubation at 37°C in a 5% CO<sub>2</sub> atmosphere in the presence of CRH and/or antalarmin or anti-CEACAM1 antibody for 24 hours (EVTs) or 48 hours (hybridoma cells), noninvading cells were removed from the upper surface of the membrane with a cotton swab, and the cells on the lower surface were stained with components supplied in the kit. Then the membranes were removed from the inserts and fixed on microscope slides. The cells that had passed through the membranes were counted under a microscope in three identical square fields (excluding the membrane area at the periphery). The effect of the transfected gene on the invasive potential of the cells in the presence or absence of anti-CEACAM1 antibody was estimated by comparison with the cell numbers observed after transfection with mock plasmids (=100%). For EVT cells, the effect of CRH and/or antalarmin or anti-CEACAM1 treatment on their invasiveness was estimated by comparison with the cell numbers observed in untreated cells.

## **Results**

### *CRH Reduces EVT Invasion through CRH-R1*

To determine whether CRH has an effect on the invasiveness of cells of placental origin, freshly isolated EVT cells were used for *in vitro* invasion assay experiments. Addition of 100 nmol/L CRH for 24 hours resulted in a strong decrease in the invasiveness of EVT cells (by  $80 \pm 6.2\%$ ) compared to control cells. These results were reproducible in five separate experiments ( $P < 0.005$ ) (Figure 1).

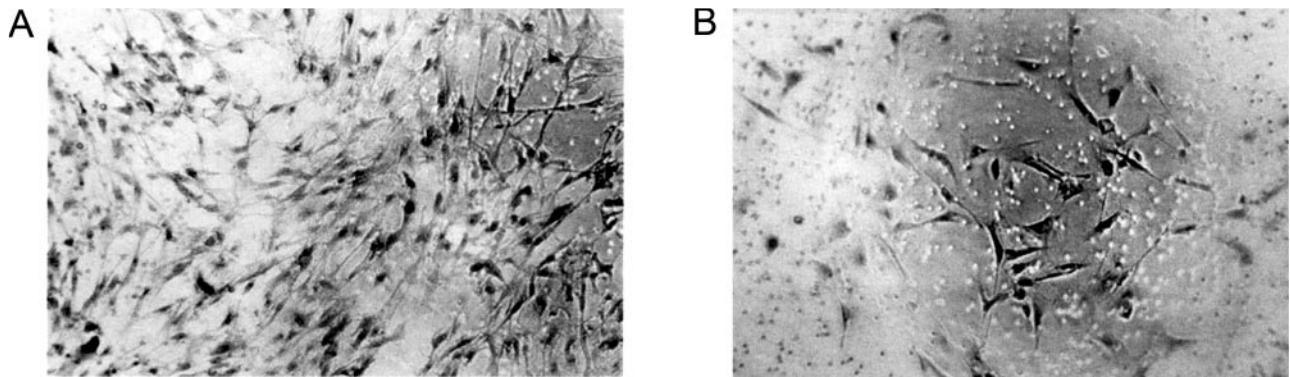
To determine whether the effect of CRH is mediated through the CRHR1 receptor, EVT cells were also treated with 100 nmol/L CRH plus the CRHR1-specific antagonist antalarmin (1  $\mu$ mol/L) for 24 hours. The latter blocked the inhibitory action of CRH in the invasion of EVT cells. Furthermore, because EVT cells produce and secrete CRH in culture, treatment with 1  $\mu$ mol/L antalarmin alone for 24 hours also resulted in an increased invasion of EVT (by  $61 \pm 5.4\%$ ) compared to control. These results were reproducible in five separate experiments ( $P < 0.005$ ) (Figure 1C). Cell cycle analysis and MTT assay revealed no statistically significant effect of the additives on the apoptosis and viability of the cells after 48 hours of incubation (data not shown).

### *CRH Reduces CEACAM1 Protein Expression in EVT through CRHR1*

To investigate the effect of CRH on the expression of CEACAM1 in EVT, flow cytometric analysis (Figure 2, A–D) was performed. Addition of CRH decreased CEACAM1 protein in isolated EVT cells in a dose-dependent manner after 24 hours of incubation. The maximum effect was observed at 100 nmol/L (Figure 2D). Indeed, incubation of EVT cells with 10, 50, or 100 nmol/L CRH for 24 hours decreased CEACAM1 mean fluorescent intensity by 2.5-, 3-, and 4-fold respectively ( $P < 0.005$ , analysis of variance with posthoc Student's *t*-test corrected by Bonferroni). This effect was confirmed by immunoblotting experiments (Figure 2E). This effect of CRH was mediated by its type 1 receptor (CRHR1) because incubation with 1  $\mu$ mol/L antalarmin completely reversed it ( $P < 0.005$ ) (Figure 2, D and E).

### *CEACAM1 Increases Invasion of Extravillous Hybridoma Cells*

To determine whether CEACAM1 has an effect on the invasiveness of cells of placental origin, extravillous hybridoma cells were transfected with an expression vector for the classic (long) form of CEACAM1 (CEACAM1/L) or with the corresponding mock plasmid. The effectiveness of transfectants was analyzed by Western blot and immunocytochemistry (Figures 3 and 4). The monoclonal antibody 4D1/C2, which does not cross-react with other members of the CEA family and the specificity of which has been studied in detail,<sup>17</sup> was used for immunocytochemical localization of CEACAM1 in placental hybridoma cells (clone AC1M88). Immunocytochemical expres-



**Figure 1.** *In vitro* invasion assay in isolated EVT cells. Effect of CRH and/or antalarmin. **A:** Control. **B:** Treatment with CRH. **C:** Note the decrease of invasion in EVT after treatment with 100 nmol/L CRH for 24 hours and the reversion of this effect after co-treatment with 1  $\mu$ mol/L antalarmin (Ant). Also note that when antalarmin was used alone (under similar experimental conditions), a significant increase in invasion of EVT cells was observed, because EVT cells produce and secrete CRH. Values represent mean  $\pm$  SE (percentage of control) of five separate experiments (\* $P < 0.005$ ). The pictures have been made on the lower surface of the invasion membrane. Original magnifications,  $\times 10$ .

sion analysis showed the presence of CEACAM1 immunoreactivity in CEACAM1/L-transfected cells, while untransfected and mock-transfected cells were negative. Twenty-four hours after transfection, the cells were used in our *in vitro* invasion assay experiments described in Materials and Methods.

Placental hybridoma cells (clone AC1M88) did not show spontaneous expression of CEACAM1 (as was shown by Western blots) and are of low invasive potential. Thus, these cells represent a useful control model for testing the effects of CEACAM1 transfection on cellular invasiveness. Transfection with the CEACAM1 expression vector resulted in strong expression of CEACAM1, confirmed by immunocytochemistry and Western blot, and a significant increase in invasiveness (compared to both untransfected and mock-transfected cells) (Figure 5). Indeed, transfection of hybridoma cells with the CEACAM1 expression vector increased their invasiveness by sevenfold compared to mock-transfected cells ( $n = 3$ ,  $P < 0.05$ ).

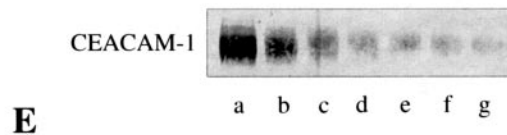
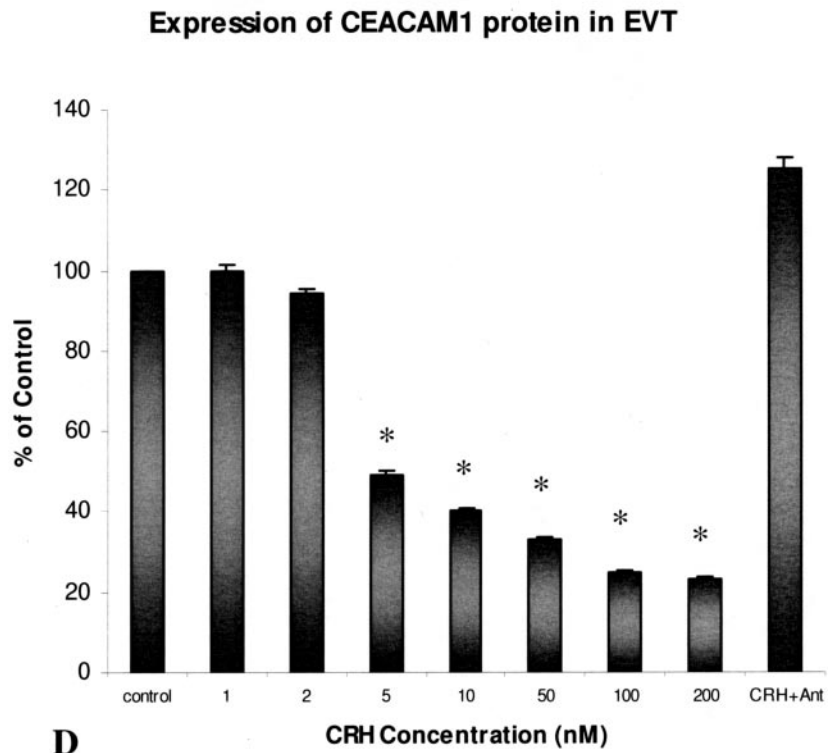
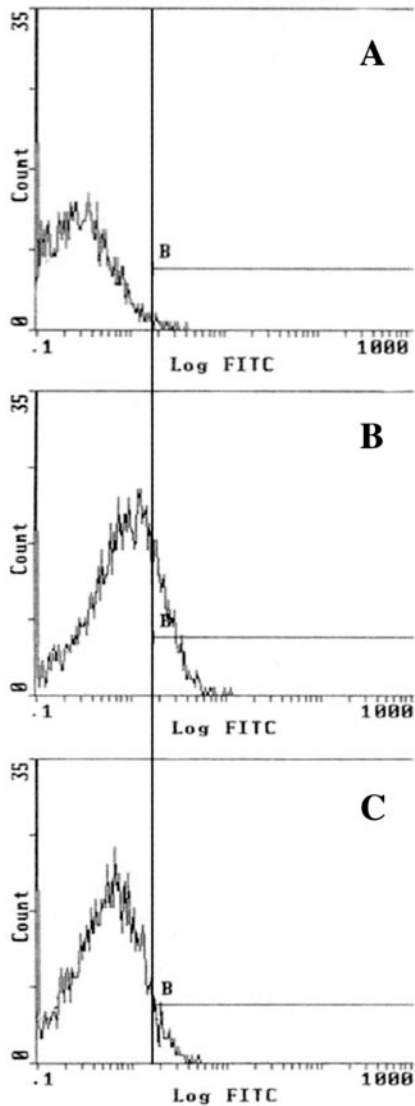
To confirm that CEACAM1 was responsible for the observed effect, transfected cells were also treated with different concentrations of an anti-CEACAM1-specific (blocking) antibody, which strongly inhibited the invasive potential of CEACAM1-transfected cells (Figure 5) ( $n = 3$ ,  $P < 0.05$ ). Cell cycle analysis and MTT assay revealed no statistically significant effect of the additives on the apoptosis and viability of the cells after 48 hours of incubation (data not shown).

### Blockade of Endogenous CEACAM1 in EVT Cells Results in Decreased Trophoblast Invasion

To determine whether endogenous CEACAM1 has an effect on the invasiveness of cells of placental origin, EVT cells were used for *in vitro* invasion assay experiments. EVT cells isolated from first trimester placenta showed spontaneous expression of CEACAM1 (as shown by flow cytometry and Western blot analysis; Figure 2, B and E) and have a high invasive potential. Thus, these cells are a useful model for testing the effects of endogenous CEACAM1 on cellular invasiveness. To determine whether endogenous CEACAM1 was responsible for invasion effect, EVT cells were treated with different concentrations of anti-CEACAM1-specific (blocking) antibody for 24 hours. The latter inhibited the invasive potential of EVT cells (by  $60 \pm 5.5\%$  of the control levels;  $P < 0.005$ ) (Figure 6). Cell cycle analysis and MTT assay revealed no statistically significant effect of the additives on the apoptosis and viability of the cells after 48 hours of incubation (data not shown).

### Discussion

In the present study, we propose a novel function for CRH produced locally at the maternal-fetal interface during early placentation. Matrigel invasion assays suggest that CRH inhibits the invasiveness of EVT cells. This action of CRH

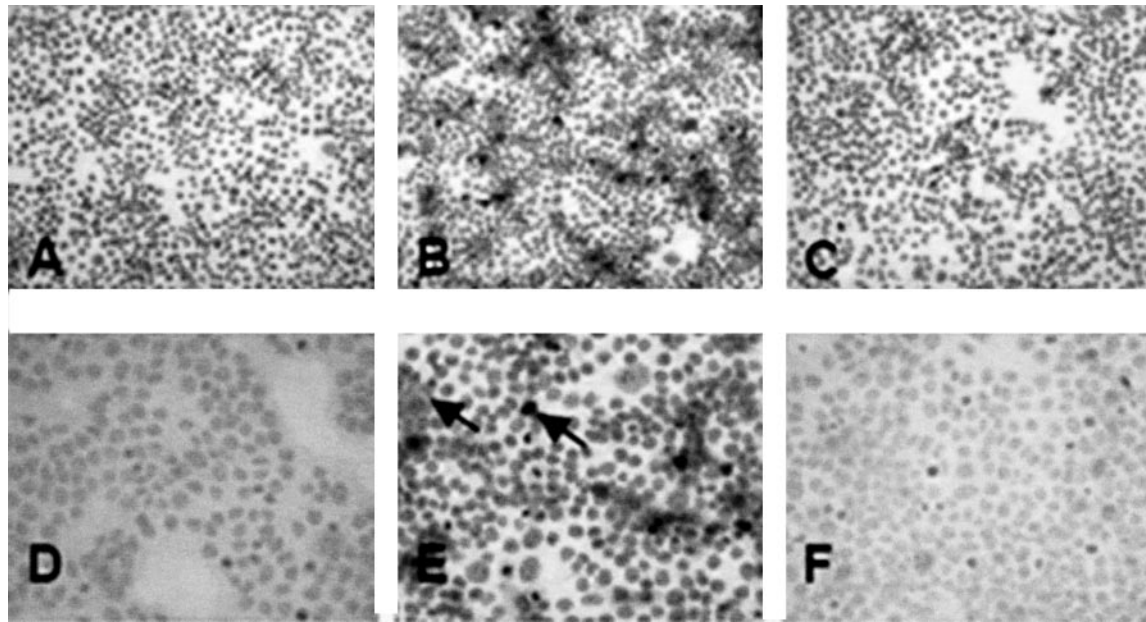


**Figure 2.** Regulation of CEACAM1 protein in EVTs by CRH through its R1 receptor. **A–D:** Flow cytometric analysis of CEACAM1. **A:** Negative control. **B:** CEACAM1 without treatment with any additive. **C:** CEACAM1 after treatment with 100 nmol/L CRH. **D:** Quantification of the dose-response analysis of the effect of CRH on CEACAM1 expression. Note the dose-dependent effect of CRH treatment on the expression of CEACAM1 peptide in EVTs. Maximum effect was observed after treatment with 100 nmol/L CRH for 24 hours. At this concentration CEACAM1 expression decreased by fourfold, an effect mediated through CRHR1 because addition of 1  $\mu$ mol/L antalarmin reversed it. Values represent mean  $\pm$  SE (percentage of control) of five separate experiments (\* $P < 0.005$ ). **E:** Western blot for CEACAM1 after 24 hours of incubation with additives. **Lanes a:** 100 nmol/L CRH + 1  $\mu$ mol/L antalarmin, **b:** control, **c:** 5 nmol/L CRH, **d:** 10 nmol/L CRH, **e:** 50 nmol/L CRH, **f:** 100 nmol/L CRH, **g:** 200 nmol/L CRH.

was specifically mediated through CRHR1 because the addition of antalarmin, a specific CRHR1 antagonist, completely reversed it. Incubation of trophoblasts with antalarmin alone partially increased EVT invasiveness, an effect most probably due to blockade of endogenous CRH produced by trophoblasts. Furthermore, we show that the effect of CRH in trophoblast invasiveness directly involved inhibition of CEACAM1 expression in EVTs.

The invasion of cytotrophoblasts to the proper depth of the uterus primarily determines the outcome of pregnancy. Excessive invasion can lead to abnormally firm attachment of the placenta directly onto the myometrium (a condition called placenta accreta), to the extension of the placenta into the myometrium (placenta increta), or to invasion through the myometrium to the uterine serosa and even into adjacent organs (placenta percreta). Ab-

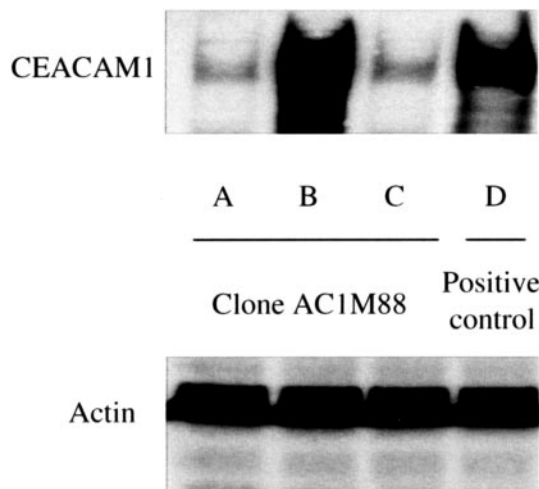
errant invasion has also been implicated in the pathophysiology of preeclampsia, in which the characteristic pathological lesion is the result of shallow interstitial invasion by cytotrophoblasts and, more consistently, limited endovascular invasion.<sup>28,29</sup> Interestingly, in pregnancies complicated by preeclampsia, the maternal plasma levels of CRH are significantly elevated, together with a concomitant reduction in CRHR1 expression.<sup>30,31</sup> Although the reason for this elevation is unknown, a defective CRH/CRHR1 system has been suggested to be involved in the pathophysiology of placental ischemia in preeclampsia.<sup>32</sup> Our findings expand further the potential role for CRH in the development of the disease, suggesting that aberrant expression of CRH may deregulate trophoblast invasion. For instance, excess of placental CRH may lead to shallow interstitial trophoblast invasion.



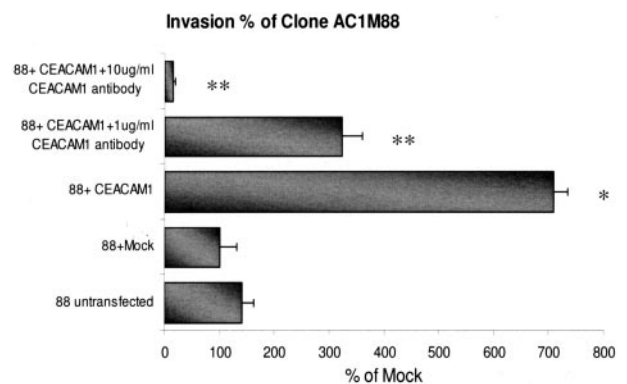
**Figure 3.** Immunocytochemical detection of CEACAM1 in placental hybridoma cells (clone AC1M88). **A:** Untransfected. **B:** CEACAM1-transfected, staining for CEACAM1. **C:** Mock-transfected. **D:** Untransfected. **E:** CEACAM1-transfected, staining for CEACAM1 (arrow). **F:** Mock-transfected. Original magnifications:  $\times 10$  (A–C);  $\times 20$  (D–F).

We have previously reported that CEACAM1 is specifically expressed on the EVT cells in first trimester human placentas.<sup>16</sup> Given the ability of CEACAM1 to form homo- and heterotypic cell-to-cell interactions,<sup>33,34</sup> a role for this adhesion molecule in mediating adhesion of invasive trophoblast to maternal tissues is anticipated. Indeed, CEACAM1 might mediate adhesion between trophoblasts and endometrial epithelial cells, which also express CEACAM1.<sup>21</sup> Furthermore, CEACAM1 binding to E-selectin expressed by endothelial cells<sup>12</sup> might participate in the adhesion of EVTs to the endothelial cells of maternal vessels, thus promoting endovascular invasion and replacement of endothelial cells by trophoblasts.<sup>35</sup>

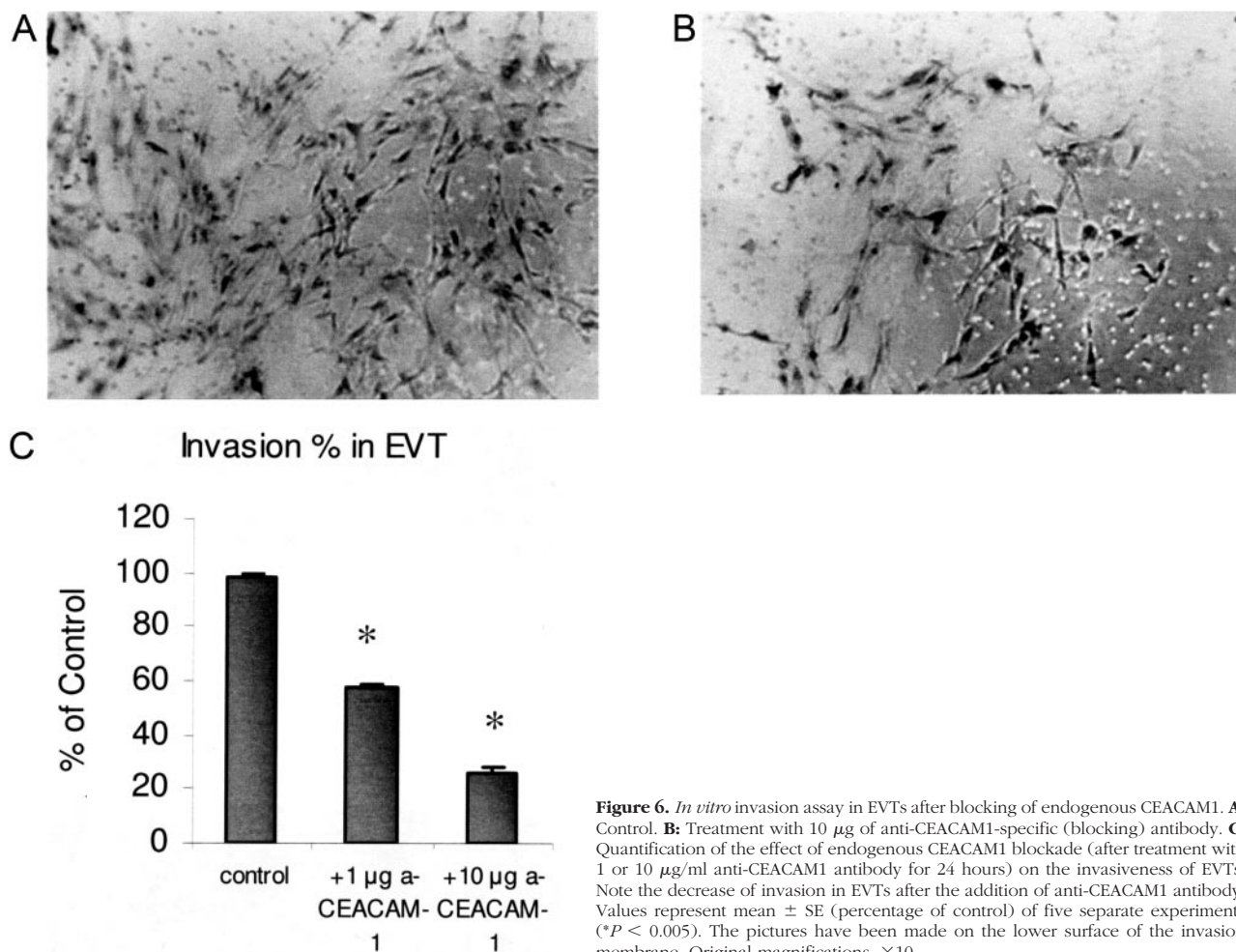
Our data provide experimental evidence that expression of CEACAM1 on an EVT-based hybridoma cell line enhances the invasive capacity of the cells in an *in vitro* system simulating the extracellular matrix (ECM). The importance of functional CEACAM1 molecules to trophoblast invasiveness is also demonstrated by the marked decrease of invasion after blocking of CEACAM1 with a specific anti-CEACAM1 antibody in isolated EVTs. Interstitial trophoblast invasion depends primarily on cell-ECM interactions, which in turn involve integrin-ECM interactions. Accumulating data suggest that CEACAM1 interacts with and modulates integrin signaling in several cell systems including EVT cells. Anti-CEACAM1 antibodies affect the function of  $\beta 1$  and  $\beta 2$  integrins in neutrophils



**Figure 4.** Western blot analysis of placental hybridoma cells (clone AC1M88) with mAb 4D1/C2 for CEACAM1 expression. **A–C:** Placental hybridoma cells (clone AC1M88). **A:** Untransfected. **B:** CEACAM1-transfected, staining for CEACAM1. **C:** Mock-transfected. **D:** Positive control (G361 cells).



**Figure 5.** *In vitro* invasion assay in placental hybridoma cells (clone AC1M88). *In vitro* invasion assay in placental hybridoma cells in the presence of CEACAM1, CEACAM1 and 1  $\mu\text{g}/\text{ml}$  anti-CEACAM1 antibody, or CEACAM1 and 10  $\mu\text{g}/\text{ml}$  anti-CEACAM1 antibody. Note the increase of invasiveness of CEACAM1-transfected hybridoma cells compared to untransfected, mock-transfected, and anti-CEACAM1 antibody-treated transfected cells. Values represent mean  $\pm$  SE (percentage of control) of three separate experiments (\* $P < 0.05$  in comparison with mock-transfected cells; \*\* $P < 0.05$  in comparison with CEACAM1-transfected cells).



**Figure 6.** *In vitro* invasion assay in EVT after blocking of endogenous CEACAM1. **A:** Control. **B:** Treatment with 10 µg of anti-CEACAM1-specific (blocking) antibody. **C:** Quantification of the effect of endogenous CEACAM1 blockade (after treatment with 1 or 10 µg/ml anti-CEACAM1 antibody for 24 hours) on the invasiveness of EVT. Note the decrease of invasion in EVT after the addition of anti-CEACAM1 antibody. Values represent mean ± SE (percentage of control) of five separate experiments (\**P* < 0.005). The pictures have been made on the lower surface of the invasion membrane. Original magnifications, ×10.

and B lymphocytes,<sup>36–38</sup> and direct interactions between β3 integrins and CEACAM1 have been observed in granulocytes, epithelial cells, and invading trophoblasts.<sup>39</sup> Similarly to our study, CEACAM1 was found to enhance the invasion of melanocytic and melanoma cells in Matrigel invasion assays. This effect was dependent on integrins, and a functional role of CEACAM1-integrin β3 interaction in cellular invasion was suggested.<sup>26</sup> Furthermore, paxillin, a key protein of focal adhesions, has been shown to associate with CEACAM1 in granulocytes, epithelial cells, and human umbilical vein endothelial cells.<sup>40</sup> CEACAM1 can also interact with the protein tyrosine phosphatases SHP-1 and SHP-2 and tyrosine kinases of the src-family,<sup>41–43</sup> which participate in integrin signaling.<sup>44–46</sup> In granulocytes and endothelial cells, CEACAM1 associates with talin, a major cytoskeletal protein implicated in integrin activation.<sup>40,47</sup> Recently, osteopontin, a secreted ECM glycoprotein, and CEACAM1 were suggested to act as a functional complex to enhance invasiveness of trophoblast cells.<sup>48</sup> Thus, CEACAM1 is able to interact and interfere with integrins or integrin-regulated cellular functions and therefore to affect the cellular interactions with the ECM.

Expression of the rat homologue of CEACAM1, cell-CAM105, was previously described at the trophoderm surface of rat blastocysts,<sup>49</sup> as well as at the apical

surface of rat uterine epithelium.<sup>50</sup> However, cell-CAM105 was lost or masked from the surface of the murine trophoblast cells of adhesive-stage blastocysts.<sup>49</sup> It was therefore suggested that decrease of cell-CAM105 in the mural trophoblasts could be linked to the acquisition of trophoblast invasiveness. Our study was focused on EVT, freshly isolated from first trimester human placenta. Thus, a direct comparison between the two experimental systems is not possible. Nevertheless, the presence of CEACAM1 at the implantation sites in first trimester human placentas and the stimulatory effect of CEACAM1 in trophoblast invasiveness suggest that in humans, the presence and not the absence of CEACAM1 is important for early placentation.<sup>16</sup>

In our study, antalarmin increased trophoblast invasion. However, this increase was partial (by 60%) compared to controls, suggesting that other redundant mechanisms may take part in trophoblast invasion as well. Therefore, CRH antagonists, which show a promising future in the treatment of depression and anxiety disorders, should be thoroughly examined for their effects on reproductive physiology. In conclusion, our findings show that CRH reduces EVT invasiveness. This effect is due to a CRHR1-mediated inhibition of CEACAM1 expression. Finally, CEACAM1 promotes trophoblast invasion. Deregulation of trophoblast inva-

sion due to a defective CRH/CRHR1 system may be involved in the pathophysiology of preeclampsia and placenta accreta.

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