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Effects of E-cadherin on mouse embryo implantation and expression of matrix metalloproteinase-2 and -9

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Abstract

E-cadherin is a cell surface glycoprotein, which is responsible for adhesion between epithelial cells. Whether it is involved in embryo implantation is still unknown. In a mouse intrauterine horn injection model, one uterine horn in each mouse was injected with different doses of E-cadherin antibody on day 3 of pregnancy. The results showed that embryo implantation was significantly inhibited in the mice injected with 3 μg E-cadherin antibody. The mouse uteri in this group were collected on days 5, 6, and 7 of pregnancy and expressions of MMP-2 and -9 were studied. In situ hybridization and RT-PCR results showed that the expression of MMP-2 and -9 mRNAs in uteri of E-cadherin antibody treated group was increased on days 5–7. The results of gelatin zymography of MMPs showed that the activities of pro-MMP-2, MMP-2, and pro-MMP-9 were increased significantly on days 5 and 6, and pro-MMP-9 activity was increased on day 7. The present study suggested that E-cadherin was involved in embryo implantation through decreasing the expressions and activities of MMP-2 and -9.

Keywords: E-cadherin; Mouse; Implantation; MMP-2; MMP-9

E-cadherin is a 120–130 kDa transmembrane, calcium-dependent glycoprotein, and linked to the cytoskeleton via intracellular ligands termed catenin α, β, and γ, respectively. It is responsible for the organization, maintenance, and morphogenesis of epithelial tissues [1–4]. E-cadherin has been studied extensively in carcinomas and has been thought to be involved as tumor suppressor gene in carcinogenesis [4,5]. The loss of E-cadherin expression or activity favors tumor cell invasion, and transfection of E-cadherin-encoding cDNA into invasive cancer cells inhibits their invasiveness [4].

Embryo implantation is a highly regulated event, which is critical for the establishment of pregnancy. Successful embryo implantation depends on the synchronized development of both the invasiveness of the embryo and the receptivity of the endometrium.

E-cadherin has been identified in some human trophoblast populations and is believed to mediate homophilic interactions between cytotrophoblasts [6]. In mice, it was indicated that E-cadherin was expressed in the peri-implantation embryo and uterine epithelial cells, as well as at focal contacts between trophectoderm and uterine epithelium during the attachment stage of implantation [7,8]. However, there is no direct evidence that E-cadherin was involved in embryo implantation.

Embryo implantation is a very complicated process, which is accompanied by extensive degradation and remodeling of extra cellular matrix (ECM). Numerous studies have implicated that matrix metalloproteinases (MMPs), which are responsible for the degradation of ECM, are regulators of blastocyst implantation [9–11]. Among these MMPs, MMP-2 (gelatinase A) and MMP-9

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(gelatinase B) are the main, rate-limiting enzymes in ECM remodeling during implantation [12–14]. The activities of MMP-2 and MMP-9 are tightly regulated by their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs) [15]. The elaborated balance between the activation of MMPs and their inhibition by TIMPs is important for the regulation of embryo implantation [16].

It has been reported that E-cadherin-transfected prostatic adenocarcinoma cells have reduced MMP-2 activity and display a reversion of an invasive to a stationary phenotype [17], while E-cadherin down-regulation results in increased MMP-9 activity, motility, and metastasis of skin carcinoma cells [18]. Blastocyst invasion shares many invasive characteristics with tumors, and MMPs are extensively involved in uterus tissue remodeling during embryo implantation [19,20]. Thus, studies in E-cadherin of tumor invasion will provide insight into the effects of E-cadherin on embryo implantation.

In the present study, a mouse uterine horn injection model was employed to investigate the effect of E-cadherin on mouse embryo implantation and on the expression of MMP-2 and MMP-9 in order to explore the possible relationships between E-cadherin and MMP-2 and MMP-9 during embryo implantation.

Materials and methods

Animals and tissue preparation. All procedures involving animals were carried out in accordance with the Policy on the Care and Use of Animals of the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Adult male and female mice of Kunming white strain were provided by the Experimental Animal Center of Institute of Zoology, Chinese Academy of Sciences. They were bred randomly at room temperature (about 25 °C) in a constant photoperiod (light/dark cycle, 12L:12D) and allowed free access to food and water. To set up mating, two female mice were caged with a male overnight. The following morning, females were checked for the presence of a vaginal sperm plug. The day of vaginal plug was designated as day 1 of pregnancy.

The method of intrauterine horn injection was detailed previously by Wu and Gu [21]. On the morning of day 3, pregnant mice were anesthetized and injected into one uterine horn with 1, 2, and 3 μg of rabbit polyclonal E-cadherin antibody (H-108) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in saline, respectively, while the contralateral uterine horn received equal volume of normal rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and served as controls. On day 7 of pregnancy, the treated animals were killed and the number of implanted embryos in two uterine horns was counted, respectively.

On the morning of day 3, mice in the group of which embryo implantation was inhibited significantly were anesthetized. The implantation sites were defined by tail intravenous injection of 0.5 ml of 1% trypan blue and uteri were dissected 5 min later. On days 6 and 7, the implantation sites were visually distinct. Animals were sacrificed on the morning of days 5, 6, and 7 of pregnancy and the uteri were excised, trimmed, and appropriate implantation sites were separated. The tissues were divided into two parts. One part was embedded in embedding medium (Triangle Biomedical Sciences, Durham, NC, USA) for frozen tissue specimens and the other was snap-frozen in liquid nitrogen and stored at −80 °C until used.

Reverse transcriptional-polymerase chain reaction. Total RNA was extracted from mouse uteri with Trizol reagent (Gibco-BRL Life Technologies Inc., Rockville, MD, USA) according to the manufacturer's instructions. The cDNA was synthesized using Superscript II reverse transcriptase (Gibco-BRL Life Technologies Inc., Rockville, MD, USA) and oligo dT from 2 μg of total RNA. The reaction was carried out at 42 °C for 50 min and 70 °C for 15 min. In the PCR analysis, the cDNA was amplified by 27 cycles (denaturing at 94 °C for 45 s, annealing at 55 °C for 45 s, and elongating at 72 °C for 45 s) using E-cadherin primers (5′-GACAACGCTCCCATCCCA-3′ and 5′-CCACCTCCTTCTTCATCA TAG-3′, with the expected size of 515 bp), by 26 cycles using MMP-2 primers (5′-GCTGATACTGACACTGGTACTG-3′ and 5′-CAATC TTTTCTGGGAGCTC-3′, giving a PCR product of 216 bp) and by 29 cycles using MMP-9 primers (5′-TGAGTCCGGCAGACAATCC-3′ and 5′-CCTTATCCACGCGAATGACG-3′, with the anticipated size of 432 bp). The 25 μl PCR system contained 2 μl of RT products, 200 μmol/L dNTP, 2 mmol/L $MgCl_2$, 1 U *Taq* polymerase, and 10 pmol of each primer. The PCR system devoid of template cDNA was included as negative control. β-Actin was amplified as internal control (primers were 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGT CACGCACGATTTC-3′) with the expected size of 548 bp. The PCR products were electrophoresed on a 1% (w/v) agarose gel containing 0.5 μg/mL of ethidium bromide, and the sizes of the products were determined by comparison with a 2-kDa DNA marker (Takaka, Corp. Dalian, China). The band intensities were analyzed using Meta View image analyzing system (version 4.50, Universal Imaging Corp., USA).

In situ hybridization. The 515 bp amplified fragment for E-cadherin was recovered from the agarose gel and purified using CONCERTTM Rapid Gel Extraction system (Gibco-BRL Life Technologies Inc., Rockville, MD, USA). Then the fragment was inserted into pGEM®-T easy vector and sequenced (Sangon Corp., Shanghai, China). To generate antisense cRNA probe, the plasmid was linearized with *Nde*I and in vitro transcribed with T7 RNA polymerase (Promega Corp., Madison, WI, USA); while the sense probe was synthesized using *Sma*I and SP6 RNA polymerase (Promega Corp., Madison, WI, USA). The cRNA probes were labeled with digoxigenin (DIG) RNA labeling mix (Roche Molecular Biochemicals, Mennheim, Germany).

The process of in situ hybridization was performed as described previously [22]. Briefly, cryosections (10 μm) on poly-L-lysine coated slides were quickly thawed and fixed in 4% paraformaldehyde for 15 min at room temperature. The slides were washed for 2× 15 min in PBS containing 0.1% active DEPC and then in 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 15 min. Prehybridization was carried out at 56 °C for 2 h in prehybridization solution containing 50% deionized formamide, 5× SSC, and 120 μg/mL salmon-sperm DNA (Sigma Chemical Co., St. Louis, MO, USA). The slides were then hybridized with 400 ng/mL of Dig-labeled probes in prehybridization buffer overnight at 50 °C in a moist chamber. Then the slides were serially washed in 2× SSC at room temperature for 30 min, in 2× SSC at 65 °C for 1 h, and in 0.1× SSC at 65 °C for 1 h. The slides were incubated with anti-DIG-alkaline phosphatase (diluted 1:5000) for 2 h at room temperature and then rinsed twice for 15 min each in washing buffer (100 mM Tris, 150 mM NaCl, pH 7.5). Color development was carried out using NBT-BCIP (Boehringer–Mannheim, Indianapolis, IN, USA). The sense probes were used as negative controls for background levels. The results were recorded with SPOT digital camera system (Diagnostic Instruments, Inc., USA).

Gelatin zymography. Gelatin zymography was performed as described previously [23]. Protein extracts were mixed with sample buffer (0.25 M Tris–HCl, 40% glycerol, 0.04% bromophenol blue, and 8% SDS pH 6.8), and then incubated for 30 min at 37 °C. Protein samples were subjected to electrophoresis in a 10% polyacrylamide gel containing 0.5 mg/mL gelatin. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 (2.5% Triton X-100, 0.05 mol/L Tris–HCl, pH 7.5) for 30 min each time and subsequently incubated for 18 h in a calcium buffer (0.05 mol/L Tris–HCl, 0.2 mol/L NaCl, 0.01 mol/L $CaCl_2$, 1% Triton X-100, and 1 μmol/L $ZnCl_2$, pH 7.5). The gel was stained with 0.2% Coomassie brilliant blue R-250 and destained with 10% acetic acid. Then, the clear bands resulting from digestion of the substrate by gelatinase enzymes were visualized. Activities of MMP-2 and -9 detected by gelatin zymography were quantified by computer-aided densitometry (Personal Densitometer SI; Molecular Dynamics Inc., Sunnyvale, CA).

Statistical analysis. The intensity of each band amplified by RT-PCR was analyzed using Meta View image analyzing system (version 4.50, Universal Imaging Corp., USA). The relative levels of MMP-2 and MMP-9 mRNAs normalized to β-actin mRNAs were calculated. Activities of MMP-2 and MMP-9 detected by gelatin zymography were quantified by densitometric analysis of the intensities of the bands. All values are presented as means ± SEM. Statistical comparisons among groups were analyzed by one-way ANOVA followed by LSD test using SPSS software package (version 10.0.1, SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered significant.

Results

Effect of E-cadherin on mouse embryo implantation

On day 3 of pregnancy, 1, 2, and 3 μg, E-cadherin antibody was injected into one of the uterine horns, respectively, and the contralateral horn was injected with an equal volume of normal IgG, the control solution. On day 7 of pregnancy, mice were killed and the number of implanted embryos in each uterine horn was counted. As shown in Fig. 1, the number of implanted embryos was decreased in the mice injected with 3 μg E-cadherin antibody ($P < 0.05$). In subsequent studies on the modulatory effect of E-cadherin on the expression of MMP-2 and MMP-9, mice in the group of injection with 3 μg of E-cadherin antibody were used.

Effect of E-cadherin on the localization and expression of MMP-2 and MMP-9 mRNAs

Mouse uterine horns were collected on days 5, 6, and 7 of pregnancy after intrauterine horn injection of 3 μg E-cadherin antibody on day 3 of pregnancy. In situ hybridization and RT-PCR were employed to investigate the changes in localization and expression of MMP-2 and MMP-9 in uterine horns treated with E-cadherin antibody.

The results of in situ hybridization showed that clear signals for MMP-2 mRNAs were detected in both E-cadherin antibody treated uteri and the corresponding control uteri on days 5, 6, and 7 of pregnancy (Fig. 2). MMP-2 mRNA was localized in the stroma, especially the subepithelial stroma on day 5 of pregnancy (Fig. 2A and B). With the progression of decidualization, the signals of the MMP-2 mRNA were primarily localized in the secondary decidual zone on day 6 (Fig. 2C and D). On day 7 of pregnancy, MMP-2 transcripts accumulated in blastocyst and deciduas adjacent to myometrium (Fig. 2E and F). As shown in representive Fig. 2, the signals for MMP-2 mRNA were strong in uterine horns treated with E-cadherin antibody during peri-implantation period.

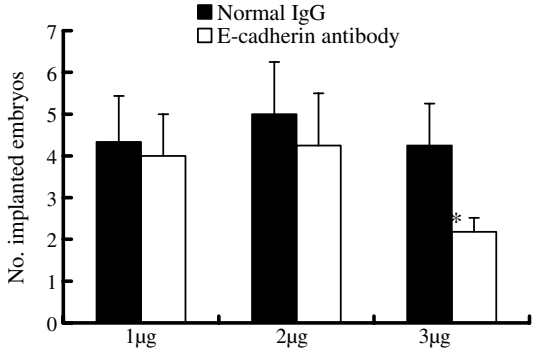

Fig. 1. Effects of intrauterine horn injection of different doses of E-cadherin antibody on mouse embryo implantation. *$P < 0.05$.

The results of RT-PCR in MMP-2, as shown in Fig. 3A, were consistent with those of in situ hybridization. Statistical analysis showed that MMP-2 mRNA was significantly increased in the E-cadherin antibody treated uterine horn, compared with that in the control uterine horn on days 5, 6, and 7 of pregnancy ($P < 0.05$) (Fig. 3B).

Different from MMP-2, MMP-9 showed different expression pattern. On day 5 of pregnancy, signals of MMP-9 mRNA were hardly detected in both E-cadherin antibody treated and the control uteri (Fig. 4A and B). However, MMP-9 mRNA was detected on days 6 (Fig. 4C and D) and 7 of pregnancy (Fig. 4E and F). The transcripts of MMP-9 were mainly localized in a network of cells at the periphery of the embryo in contact with the adjacent decidual cells, and the E-cadherin antibody treated uteri showed stronger hybridization signals of MMP-9 mRNA than the control uteri on days 6 and 7 of pregnancy (Fig. 4C–F).

RT-PCR-amplified products of MMP-9 mRNA in mouse uteri treated with E-cadherin antibody or in the control solution are representatively shown in Fig. 5A. Statistical analysis showed that the expression of MMP-9 mRNA in the E-cadherin antibody treated uterine horns was significantly increased on days 5, 6, and 7 of pregnancy ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively), compared with that in the control side (Fig. 5B).

Effect of E-cadherin on the activities of MMP-2 and MMP-9

In order to investigate the activities of MMP-2 and MMP-9 during peri-implantation period, the protein extracts of the uteri treated with E-cadherin antibody and the corresponding control uteri on days 5, 6, and 7 of pregnancy were subjected to gelatin zymographic assay. Three bands of gelatin activity at 92, 72, and 64 kDa represent latent (pro) MMP-9, latent MMP-2, and active form of MMP-2, respectively. As shown in Fig. 6, MMP-2 and MMP-9 were detected in tissue extracts of both the control and E-cadherin antibody treated uterine horns at these time points examined. On day 5 of pregnancy, the bands at 92, 72, and 66 kDa in uterine horns injected with E-cadherin antibody were increased significantly ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively), compared with the corresponding controls. On day 6, three bands at 92, 72, and 66 kDa were detected in both groups, and statistical analysis showed that the activities of MMP-2 and MMP-9 in E-cadherin antibody treated uterine horns increased significantly ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). On day 7, there was no significant difference

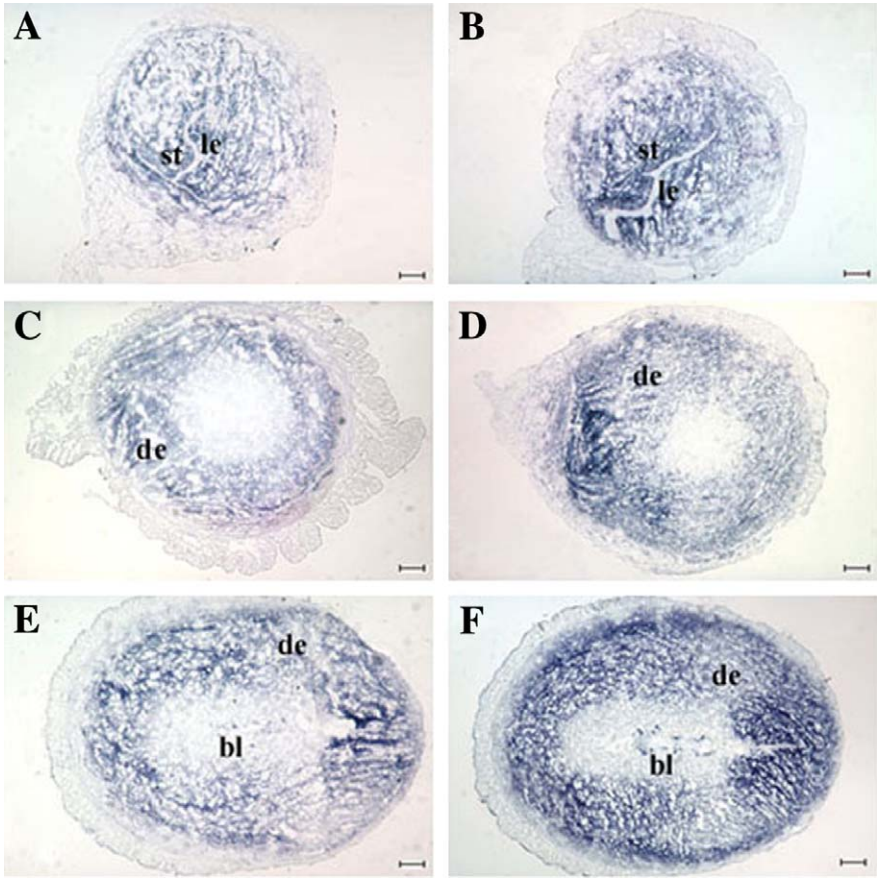

Fig. 2. In situ hybridization of MMP-2 in mouse uteri injected with E-cadherin antibody during peri-implantation period. (A), (C), and (E), Control uterine horns; (B), (D), and (F), E-cadherin antibody treated uterine horns. (A,B), day 5; (C,D), day 6; (E,F), day 7. le, Luminal epithelium; st, stroma; bl, blastocyst; de, decidua. Bar, 200 μm.

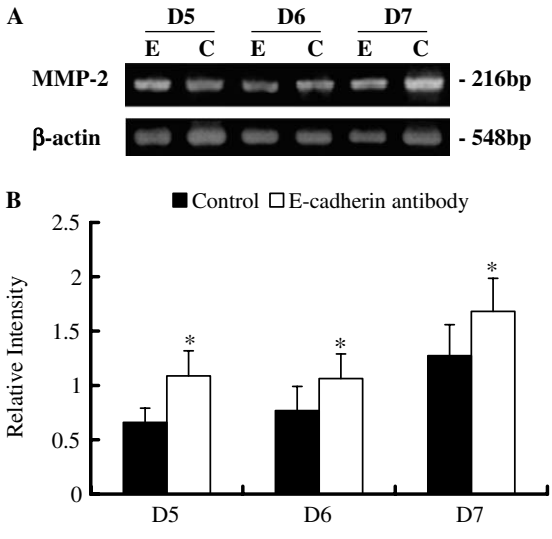

Fig. 3. Relative expression of MMP-2 mRNA in mouse uteri injected with E-cadherin antibody during peri-implantation period. (A) Representing RT-PCR results. (B) Graphic representation of the relative MMP-2 mRNA expression levels. Values are presented as ratio of densitometric readings of MMP-2 samples to corresponding β-actin samples. E, E-cadherin antibody treated uterine horn; C, control uterine horn; D5–D7, days 5–7 of pregnancy; bp, base pair. *$P < 0.05$, vs. control.

in the gelatinolytic activities of pro-MMP-2 and MMP-2 between the E-cadherin antibody treated group and control group ($P > 0.05$), and only the band at 92 kDa was significantly increased ($P < 0.05$).

Discussion

Embryo implantation involves complicated and progressively intimate interactions between the blastocyst and the uterine endometrial epithelium. In human and rodents, the blastocyst initially adheres to and then penetrates the uterine epithelium and subsequently invades the uterine stroma. At the initial stages of implantation, the uterine epithelium undergoes dramatic changes in cell proliferation and remodeling, which prepares it to be "receptive" to invasion by the embryo [24,25].

E-cadherin is an adhesion molecule and plays an important role in the adhesion between epithelial cells. Previous results found that E-cadherin mRNA in mouse uterus was at the highest level on day 1 of pregnancy, followed by a marked decline from day 5–8 of pregnancy [8], but there is no direct evidence so far for the effect of E-cadherin on embryo implantation. In the present study, a mouse

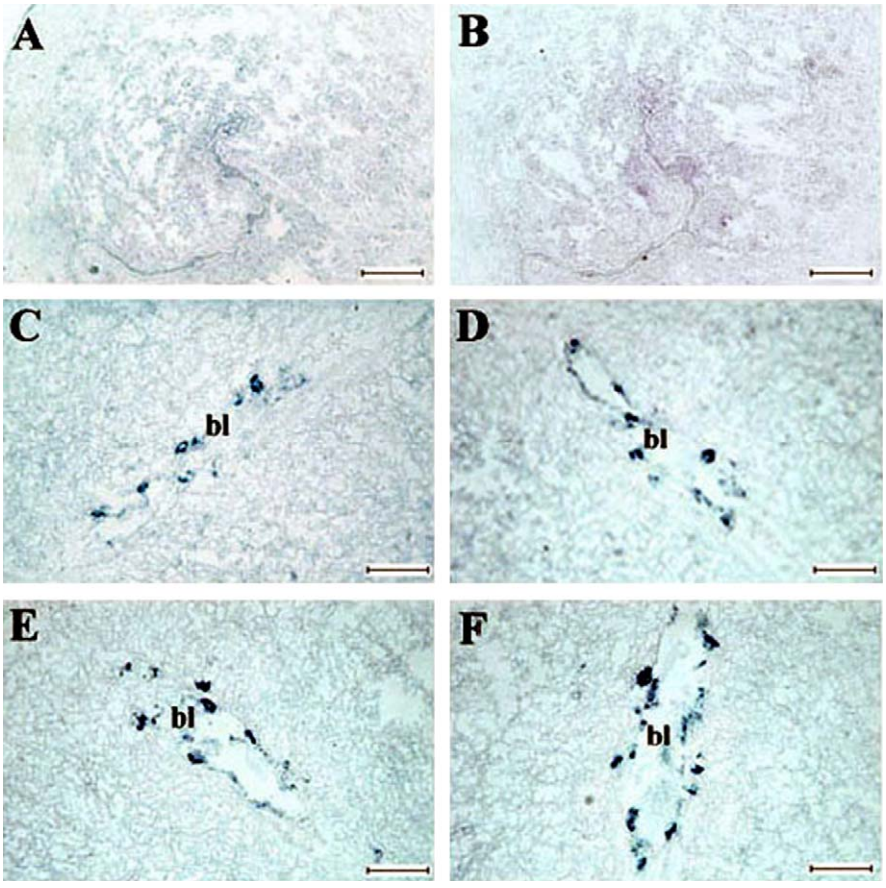

Fig. 4. In situ hybridization of MMP-9 in mouse uteri injected with E-cadherin antibody during peri-implantation period. (A), (C), and (E), Control uterine horns; (B), (D), and (F), E-cadherin antibody treated uterine horns. (A,B), day 5; (C,D), day 6; (E,F), day 7. Bar, 200 μm.

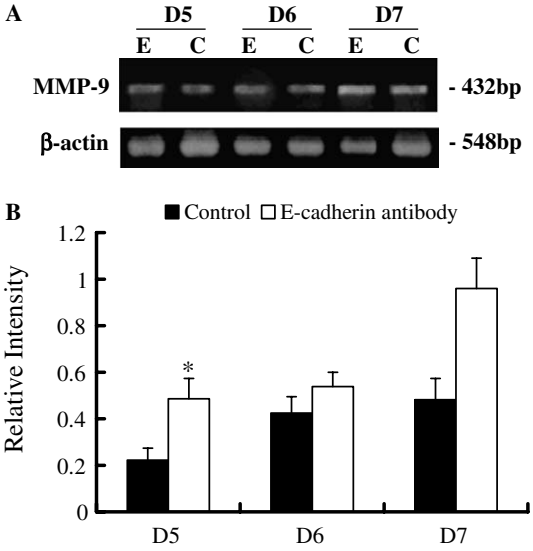

Fig. 5. Relative mRNA expression levels of MMP-9 in mouse uteri injected with E-cadherin antibody during peri-implantation period. (A) Representing RT-PCR results. (B) Graphic representation of the relative MMP-9 mRNA expression levels. Values are presented as ratio of densitometric readings of MMP-9 samples to corresponding β-actin samples. E, E-cadherin antibody treated uterine horn; C, control uterine horn; D5–D7, days 5–7 of pregnancy; bp, base pair. $*P < 0.05$, vs. control.

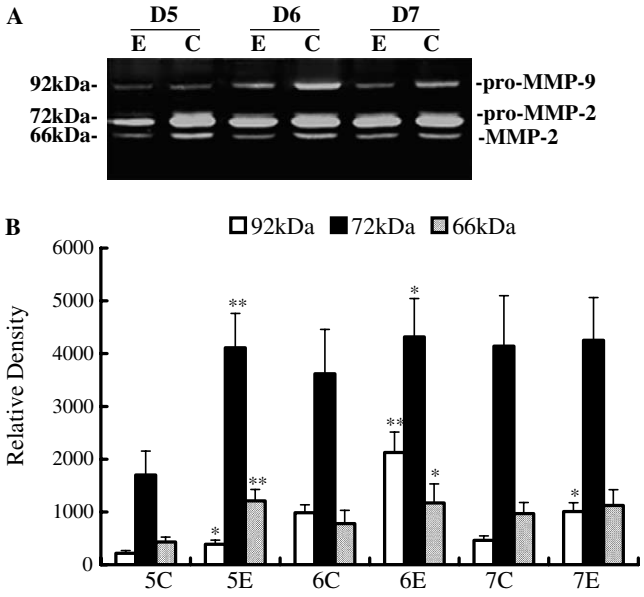

Fig. 6. The effects of E-cadherin antibody on activities of MMP-2 and MMP-9 in mouse uteri injected with E-cadherin antibody during peri-implantation period. (A) Zymography results. (B) Quantified density analysis of activities of MMP-2 and -9 detected by gelatin zymography. Values are means ± SEM of three experiments. E, E-cadherin antibody treated uterine horn; C, control uterine horn; D5–D7, days 5–7 of pregnancy. $*P < 0.05$, $**P < 0.01$, vs. control.

intrauterine horn injection model was used to study the effect of the E-cadherin on embryo implantation.

The antibody against extracellular domain of E-cadherin was injected into mouse uterine horns, and its effect on mouse embryo implantation was examined. The results of statistical analysis indicated that the numbers of implanted embryos in the 3 μg E-cadherin antibody treated horns were fewer than that in the control side, suggesting that mouse embryo implantation is inhibited by E-cadherin antibody. This result provides strong evidence that E-cadherin is involved in mouse embryo implantation, and it is necessary for successful embryo implantation.

However, the mechanism of E-cadherin in embryo implantation is unknown. It has been proved that MMP-2 and MMP-9 play important roles in mammalian embryo implantation. Previous study has indicated that induction of E-cadherin function decreased activity of MMP-2 in prostate carcinoma cells [17] and down-regulated MMP-2 and MMP-9 in bronchial tumor cells [26]. Furthermore, E-cadherin decreased synthesis of MMP-9 in premalignant human oral keratinocytes [27], the E-cadherin-mediated cell–cell contacts were involved in the down-regulation of MMP-9 expression in mouse skin carcinoma cell lines [18], decreased expression of E-cadherin was associated with up-regulation of MMP-9 in mouse keratinocytes [15], and down-regulation of E-cadherin was observed to induce the expression of MMP-9 in tumor cell lines [28]. Hence, it is possible that E-cadherin may also function in regulating MMP expression during embryo implantation. In this study, the expression of MMP-2 and MMP-9 mRNAs and the gelatinolytic activities of MMP-2 and MMP-9 in the pregnant mouse uteri injected with E-cadherin antibody were examined. The results showed that when E-cadherin was inhibited during peri-implantation period, the expression of MMP-2 and MMP-9 was increased at the transcriptional level, suggesting that E-cadherin is involved in the regulation of the gene expression of MMP-2 and MMP-9 during embryo implantation.

The activities of MMP-2 and MMP-9 are regulated at several levels including gene expression, stability of mRNA, and activation of zymogen [29]. Regulation of MMP-2 and MMP-9 at the zymographic level was also examined in this study. The activity of MMP-2 and -9 was significantly increased in the uterine horn treated with E-cadherin antibody, suggesting that E-cadherin is involved in regulating the gelatinolytic activity of MMP-2 and MMP-9 during mouse embryo implantation.

The detailed signal transduction pathway through which E-cadherin regulates proteinase gene expression is still unknown. In normal epithelial tissues, E-cadherin combines with cytoplasmic β-catenin to form cell adhesion junctions that maintain the functional characteristics of epithelia. In addition, β-catenin is found independent of cadherins within the cytoplasm and nucleus of the cell. The pool of free β-catenin is the key effector of the Wnt signaling pathway that regulates cell fate during development [30,31]. β-Catenin can translocate to the nucleus and form a complex with proteins of the T cell factor (TCF) family and regulates gene expression [32]. TCF proteins have been shown to activate genes, including MMP-7 and MMP-26 [33–35]. A number of other MMP promoters, including MMP-9, have TCF-4-binding sites and consequently, may be regulated by β-catenin [33,35]. However, the mechanism of E-cadherin regulating MMP-2 and -9 in embryo implantation remains to be further investigated.

In summary, E-cadherin is involved in mouse embryo implantation, and its effect on embryo implantation is mediated in part by regulating MMP-2 and MMP-9 mRNA expression and the gelatinolytic activity of MMP-2 and MMP-9.

Acknowledgments

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