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Blockade of Adhesion Molecule CD146 Causes Pregnancy Failure in Mice

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Unexplained pregnancy loss and recurrent miscarriage seriously impair human fecundity. However, the underlying molecular mechanisms remain elusive. Recent studies suggest that the adhesion molecule CD146 may be involved in unexplained recurrent miscarriage. Here, we investigate the effect of CD146 on early pregnancy. Using in situ hybridization and immunohistochemistry, we found that CD146 was specifically expressed in the receptive maternal uteri and invasive embryonic trophoblasts during the early stages of pregnancy, but it was completely absent in the non-pregnant uterus. Our in vitro studies demonstrated that blocking CD146 with a function-perturbation antibody AA98 significantly inhibited the attachment of blastocysts onto the receptive uterine luminal epithelial monolayer, the trophoblastic outgrowth of blastocysts and ectoplacental cones, and the secretion of matrix metalloproteinases. Animal experiments showed that applying this antibody before embryo implantation caused pregnancy failure in mice. Our data present direct evidence for the role of CD146 in mediating embryonic attachment and trophoblastic invasion, and provide new insight into the molecular mechanism underlying unexplained pregnancy loss and recurrent miscarriage.

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Recurrent miscarriage, defined as three or more consecutive, spontaneous pregnancy losses, affects approximately 1–2% of fertile women. However no apparent cause can be found in ~50% of these cases (Brigham et al., 1999; Rai and Regan, 2006). The spontaneous pregnancy loss often (~75%) occurs during the peri-implantation period (Wilcox et al., 1988; Zinaman et al., 1996). Better understanding the molecular events underlying embryo implantation will greatly promote the etiological studies of unexplained spontaneous pregnancy loss and recurrent miscarriage.

Embryo implantation is a critical process for successful pregnancy, which requires an active dialog between embryo and mother. This dialog is initiated by the attachment of an embryo onto the receptive uterine epithelium during a restricted period called the "implantation window." This window begins on 4.5 days postcoitum (dpc) in mice (Cross et al., 1994) and approximately on days 6–7 in human (Norwitz et al., 2001). During the peri-implantation period, a receptive uterus undergoes dramatic changes including over-expression of specific adhesion molecules, such as L-selectin and integrin $\alpha v\beta 3$, which actively promote embryo implantation (Cross et al., 1994; Norwitz et al., 2001; Lessey, 2002; Paria et al., 2002; Genbacev et al., 2003). Once an embryo attaches onto the maternal uterus, the embryonic trophoblasts are extremely invasive, cross the uterine epithelium, and invade the decidualized endometrium as well as spiral arteries. The invasive ability of trophoblasts, like malignant tumor cells, is mainly dependent on two factors. Firstly, trophoblasts produce a few proteinases, especially matrix metalloproteinases (MMPs) that are capable of digesting collagens in the extracellular matrix during invasive processes (Librach et al., 1991). Secondly, trophoblasts specifically express certain adhesion molecules, including VE-cadherin, platelet-endothelial adhesion molecule-1, and integrin $\alpha 4\beta 1$, which facilitate their invasion (Zhou et al., 1997). Clinical studies have shown the absence of certain adhesion molecules, such as integrin $\alpha 5\beta 3$, in impaired fertility (Lessey et al., 1992; Ordi et al., 2003).

CD146, a new member of the immunoglobulin superfamily, was initially identified as a melanoma cell adhesion molecule, termed Mel-CAM, MUC18 (Lehmann et al., 1989), and A32 (Shih et al., 1994). It has been demonstrated that the enforced expression of CD146 increases melanoma growth and metastasis (Xie et al., 1997), while decreased CD146 expression results in reduced tumorigenicity (Satyamoorthy et al., 2001; Mills et al., 2002). Beside its pro-metastasis role in tumor development, CD146 is also involved in trophoblast invasion during pregnancy establishment. We and others have reported that CD146 is selectively expressed in invasive trophoblasts, but not in non-invasive trophoblasts (Shih and Kurman, 1996; Liu et al., 2004a). However the CD146

Abbreviations: dpc, days postcoitum; EPC, ectoplacental cones; Ig, immunoglobulin; mIgG, mouse IgG; MMP, matrix metalloproteinase; PBS, phosphate-buffered solution; SSC, saline-sodium citrate.

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expression in trophoblasts is remarkably reduced in the placentas of patients with pre-eclampsia (Liu et al., 2004a), a pregnancy complication closely associated with deficient trophoblast invasion. Further studies showed that blocking CD146 inhibited trophoblastic invasion in vitro, providing evidence for the function of CD146 in facilitating trophoblast invasion (Liu et al., 2004b). Recently, Pasquier et al. (2005) reported that soluble, plasma CD146 is significantly increased in women with unexplained spontaneous pregnancy loss and recurrent miscarriage. Soluble adhesion molecules modulate the function of membrane adhesion molecules in cell adhesion and migration, and their abnormal expression are often observed in pathological conditions (Pigott et al., 1992). Indeed, besides unexplained pregnancy loss, increased soluble CD146 was also detected in idiopathic inflammatory myopathies (Figarella-Branger et al., 2006), renal failure (Bardin et al., 2003) and rheumatoid arthritis (Neidhart et al., 1999), diseases which are all closely associated with decreased blood vessel endothelial cohesion. As membrane CD146 is involved in endothelial cohesion (Bardin et al., 2001), these studies suggest that excessive soluble CD146 may perturb the normal function of membrane CD146 and induce pathological conditions. Therefore, in unexplained pregnancy loss and recurrent miscarriage, the increased soluble CD146 may similarly perturb the function of CD146 on embryo implantation and induce pregnancy failure. To address this possibility, we examined the expression of CD146 during early pregnancy in mice, and the effect of a CD146-perturbation antibody on pregnancy establishment. Our results reveal the CD146 was specifically expressed in the receptive maternal uteri and invasive embryonic trophoblasts during the early stages of pregnancy, but it was completely absent in the non-pregnant uteri. Results also demonstrated that blocking CD146 function caused pregnancy failure in mice, which may be mediated by inhibition of the blastocyst attachment onto the maternal uterus and subsequent trophoblastic invasion. Our data provide new insights into the function of CD146 on embryo implantation, suggesting that CD146 may be used as a diagnostic and therapeutic target in reproductive medicine.

Materials and Methods

Animals

All experiments were done under the Beijing guide for care and use of laboratory animals and standards of humane animal care. Adult female mice of the outbred ICR white strain (5–6 weeks of age, 25–30 g in weight) were purchased from Vital River Corp. (Beijing, China). The mice were housed in the animal care facility at the Institute of Biophysics, Chinese Academy of Sciences. Female mice were caged with fertile males of the same strain (2:1) overnight. The following morning, the female mice with a vaginal plug were designated as 0.5 dpc.

Antibodies

Rabbit anti-CD146 polyclonal Ab and mouse monoclonal Ab AA98 were generated in our laboratory (Yan et al., 2003). Rat anti-mouse CD31/PECAM-1 Ab were from BD Biosciences Pharmingen (San Jose, CA). HRP-conjugated goat-anti-rabbit IgG were from SuperSignal West Dura Extended Duration substrate kit (Pierce Biotechnology, Rockford, IL). HRP-conjugated goat-anti-mouse κ were obtained from Southern Biotechnology Associates (Birmingham, AL). AP-labeled anti-digoxin IgG were from Roche Corp. (Basel, Switzerland). The ABC kit was from Vector Laboratories, Inc. (Burlingame, CA).

In situ hybridization

Both pregnant and non-pregnant mouse uteri were examined with in situ hybridization (Schaeren-Wiemers and Gerfin-Moser, 1993), using digoxin-labeled sense and anti-sense cRNA as probes. These probes contained the nucleotide sequences at 612–861 of murine CD146. In order to localize implantation sites in uteri, 0.1 ml 2% tyrpan blue in 0.01 M phosphate-buffered solution (PBS, pH 7.4) was intravenously injected into the pregnant mice on 4.5 dpc. After 5 min, the mice were sacrificed, and their uteri were dissected for the blue stained implantation sites. All tissues were embedded in paraffin, sectioned (6 μm), and mounted on the slides. The sections were then dewaxed, digested by Proteinase K (Roche Corp.), and fixed in 4% paraformaldehyde. After pre-hybridization in 50% formamide in 2 × SSC at room temperature for 2 h, the sections were hybridized at 68°C overnight in hybridization buffer containing Digoxin-labeled sense or anti-sense CD146 cRNA probes. After washing, the slides were incubated with AP-labeled anti-digoxin antibody and color reactions were observed after adding the substrate NBT/BCIP.

Western blot

Cell extracts and tissue lysates were separated by 8% SDS–PAGE, and then electrically transferred to nitrocellulose membrane. The membranes were blocked with 5% milk in PBS, incubated with primary Ab and then HRP-conjugated secondary Ab. ECL detection according to the manufacturer's instructions followed (Amersham Biosciences, Buckinghamshire, England).

Immunohistochemistry

Fresh tissues were cryosectioned (10 μm), fixed in acetone at 4°C for 5 min, and then incubated with 0.3% H_2O_2 in methanol for 30 min to quench endogenous peroxidases. The sections were blocked with 5% normal goat serum for 1 h, and incubated with a primary Ab at 4°C overnight. For the negative controls, the primary Ab was omitted. Immunoreactivity was detected with ABC kit according to the manufacturer's instructions (Vector Laboratories, Inc.).

Co-culture of blastocysts with epithelial cells

As described by Lavranos and Seamark (1989), uteri from the pregnant mice on 3.5 dpc (082–084 h) were split longitudinally to expose the epithelial surface, digested with 0.6% trypsin at 4°C for 2 h, and then at 25–30°C for another 0.5 h. Tissues were gently shaken to dislodge the epithelial cells from the endometrial bed. The uterine epithelial cells were collected by centrifugation at 500*g* for 10 min, and washed three times with Ham's F-12 medium containing 2.20 mM calcium lactate, 2.05 mM glutamine, 12.5 mM $NaHCO_3$ and 400 IU/ml gentamicin sulfate. The cells were then suspended in Ham's F-12 medium with 10% fetal calf serum (FCS, Hyclone, South Logan, UT), and cultured in 24-well plates (1×10^6 cells/well) at 37°C, 5% CO_2.

Blastocysts were flushed from the pregnant uteri on 3.5 dpc with Ham's F-12 medium. Blastocysts were then co-cultured onto a uterine epithelial cell monolayer in 24-well plates with Ham's F12 medium containing 4 mg/ml BSA in the presence of either 20 μg/ml AA98 or isotype-matched mouse IgG (mIgG). The cultures were observed every 12 h, and attached blastocysts and subsequent outgrowth of primary giant trophoblasts from blastocysts were recorded.

Isolation and culture of ectoplacental cones

Ectoplacental cones (EPC) were isolated from pregnant uteri on 7.5 dpc, and washed with Ham's F12 medium to remove decidua and any surrounding maternal blood. EPC were then cultured in Ham's F-12 medium containing 3% FCS in the presence of either 20 μg/ml AA98 or mIgG in 24-well plates pre-coated with 10 μl laminin (1 mg/ml). EPC were observed every 12 h to record the number of attached and outgrowing EPC and the outgrowth area of secondary giant trophoblasts from the EPC.

Zymography

Collagenase activity of the EPC culture supernatant was analyzed by zymography using 10% SDS–PAGE containing 1 mg/ml gelatin under non-reducing conditions, as described (Gogly et al., 1998). Samples were adjusted to the same protein concentration before being loaded onto the gels. Collagenase activity was visualized by negative staining, and abolished by incubation of parallel gels with 20 mM EDTA. Collagenase activity was quantified by scanning the densitometry of the gels using software Quantity One (Bio-Rad, Hercules, CA).

Animal experiments

After being caged with male mice and documentation of a vaginal plug, female mice (n = 18) underwent intrauterine injection of AA98 (2 μg/10 μl PBS) into the left horn adjacent to the oviduct on 3.5 dpc, and the right horn was injected with the same amount of mIgG or vehicle PBS as control, using the method described by Zhu et al. (1995), Catalano et al. (2005), and Liu et al. (2006). During 7.5–9.5 dpc, the mice were sacrificed, their uteri were removed, and the number of implants was counted in each uterine horn.

TUNEL assay

Tissue sections were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, followed by immersion in 3% H_2O_2 in methanol for 10 min. Sections were then permeated with 0.1% Triton X-100/0.1% sodium citrate for 2 min on ice. After blocking with 3% BSA and 5% normal bovine serum, the sections were incubated with the TUNEL reaction mixture for 60 min at 37°C in the dark, according to the manufacturer's instructions (Roche Corp.). After washing, the slides were incubated with substance POD for 30 min at 37°C, rinsed with PBS and observed under the light microscope. Positive cells were counted to quantify apoptosis.

Statistical analysis

All of the results are shown as the mean ± the standard error of the mean (s.e.m.). To obtain *P*-value, data were analyzed using Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Results

CD146 is selectively expressed in the pregnant uteri

To examine whether CD146 is expressed in the pregnant uteri during the implantation window, we performed in situ hybridization and immunohistochemistry. The results from in situ hybridization demonstrate that CD146 mRNA was selectively expressed in the pregnant uteri, but was absent in the non-pregnant uterus (Fig. 1A). Interestingly, the CD146 expression was dynamic in the pregnant uteri. During the implantation window (on 4.5 dpc), the CD146 signal was widely distributed throughout the luminal epithelium, glands, and stromal cells in the receptive uteri (Fig. 1B). However, this signal gradually disappeared from the uterine luminal epithelium and glands after the implantation window (on 6.5 dpc), but persisted in the stromal cells (Fig. 1C). CD146 mRNA was also detected in embryonic trophoblasts (Fig. 1D), which would invade into the maternal decidua and establish a maternal–fetal interface. In contrast, negative controls using CD146 sense probe for hybridization displayed no signal (data not shown), indicating that the CD146 mRNA was specifically detected by CD146 anti-sense probe.

The above results were confirmed by immunohistochemical studies using rabbit anti-CD146 polyclonal antibodies. The specificity of these antibodies was first determined by Western blot (Fig. 1E). Results showed that antibodies specifically recognized murine CD146/Gicerin in mouse placenta lysates, as well as human CD146 (known as ~97 kDa) from human melanoma cell A375, but they did not cross-reacted with any other proteins. The immunohistochemical study using these antibodies showed that CD146 was specifically expressed in the maternal–embryonic interface during the implantation window period, but was dramatically downregulated when the implantation window closed (Fig. 1F–I). These results are consistent with in situ hybridization studies, showing the specific expression of CD146 in both the receptive maternal

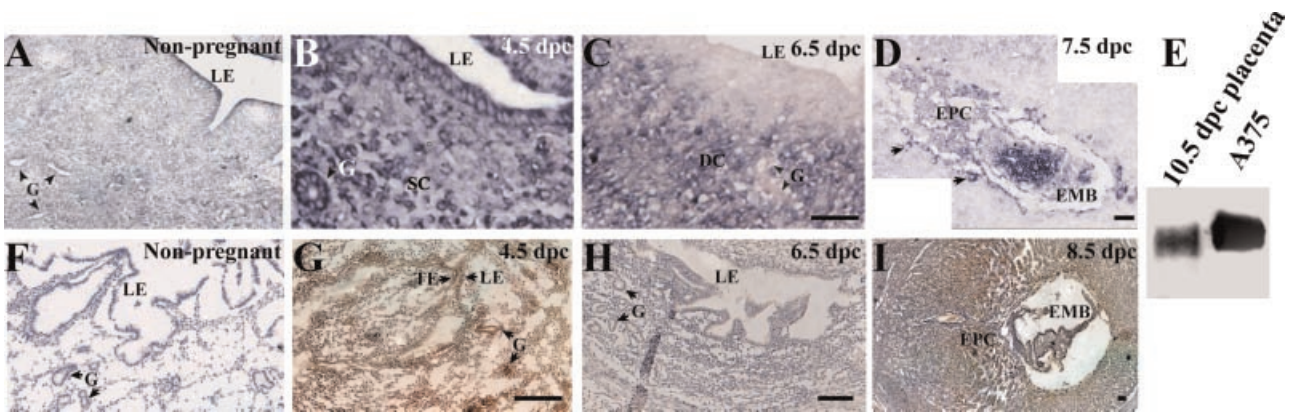

Fig. 1. Selective and dynamic expression of CD146 in the mouse uteri. Mouse uteri were analyzed by in situ hybridization using a CD146 anti-sense probe (A–D) or immunohistochemical staining using rabbit anti-CD146 polyclonal antibodies (F–I). A,F: No CD146 expression was detected in the non-pregnant uteri (G indicates gland; LE indicates luminal epithelium). B,G: Strong CD146 signal was detected at an implantation site of a maternal uterus within the implantation window (4.5 dpc; SC indicates stromal cells). C,H: The expression of CD146 disappeared from the uterine epithelium and glands following the implantation window (6.5 dpc; DC indicates decidual cells). D,I: CD146 was detected in invasive trophoblasts of the ectoplacental cones (EPC), as well as in the embryo (EMB). The arrows in D indicate the invasive trophoblastic cells. The expression patterns of CD146 protein are similar to those detected by in situ hybridization. All the immunohistochemical staining was counter-stained with hematoxylin (F–I). E: Western blot confirmed the specificity of the rabbit anti-CD146 polyclonal antibodies. Note that the anti-CD146 antibodies reacted specifically with CD146 (~97 KDa) in the lysates of mouse placentas on 10.5 dpc. As a positive control, the antibodies also reacted with human CD146 from A375, a melanoma cell line. All scale bars stand 100 μm. The bar in (C) is the same for the part (A) and (B); the bar in (G) is the same for the part (F). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

uterine epithelium and embryonic trophectoderm during the implantation window.

CD146-perturbation antibody AA98 inhibits embryo implantation in vitro

To investigate the role of CD146 in the interaction between the receptive maternal uterine epithelium and the embryonic trophectoderm during implantation, an in vitro embryo implantation model was employed in this study. In this model, blastocysts were cultured onto a uterine epithelial cell monolayer, both of which were isolated from the pregnant mice on 3.5 dpc (82–84 h). To determine whether CD146 was involved in the attachment of blastocysts onto the uterine epithelial monolayer, we added a CD146-pertubation monoclonal antibody, AA98 (Yan et al., 2003). Isotype matched mIgG, as negative control, was added to the control cultures. The specificity of AA98 for murine CD146/Gicerin was determined by Western blot (Fig. 2A). The cultures were observed for the blastocyst attachment and subsequent outgrowth of primary giant trophoblasts. AA98 significantly inhibited the attachment of blastocysts onto the uterine epithelial cell monolayer, as compared with the mIgG control group (Fig. 2B). Furthermore, fewer outgrowths occurred in the attached blastocysts in the AA98-treated group than in the mIgG control group (Fig. 2C). These observations suggest that CD146 promotes both blastocyst attachment and trophoblast invasion during the implantation window.

To provide direct evidence for the function of CD146 on trophoblast invasion without the interference from blastocyst attachment, we isolated EPC, a structure predominantly composed of invasive giant trophoblastic cells, from the pregnant uteri on 7.5 dpc. EPC were cultured in the presence of either AA98 or isotype matched control mIgG. The results showed that the trophoblastic outgrowth and the outgrowth area around the EPC were significantly reduced in the AA98-treated group, compared to the control groups (Fig. 2D–F).

Next, we determined whether the effects of CD146 on trophoblast invasion are due to alterations in trophoblastic secretion of MMP, a key factor in trophoblast invasion. Zymography was used for detecting the activity of MMP in the culture supernatants of EPC. As shown in Figure 2G, the collagenase activities of both MMP9 and MMP2 were remarkably reduced in the cultures treated with CD146-perturbation Ab AA98, whereas the cultures in normal medium or in the presence of mIgG maintained strong MMP activity. These results demonstrate that CD146 is required for blastocyst attachment and trophoblast outgrowth in vitro.

CD146 perturbation causes pregnancy failure in mice

To test whether CD146 was involved in implantation in vivo, Ab AA98 was injected into the left uterine horn of pregnant mice on 3.5 dpc to block CD146 function. In each case, the right uterine horn received the same amount of isotype matched control mIgG or PBS as an internal control. On 7.5–9.5 dpc, the treated uteri were removed and examined under a dissecting

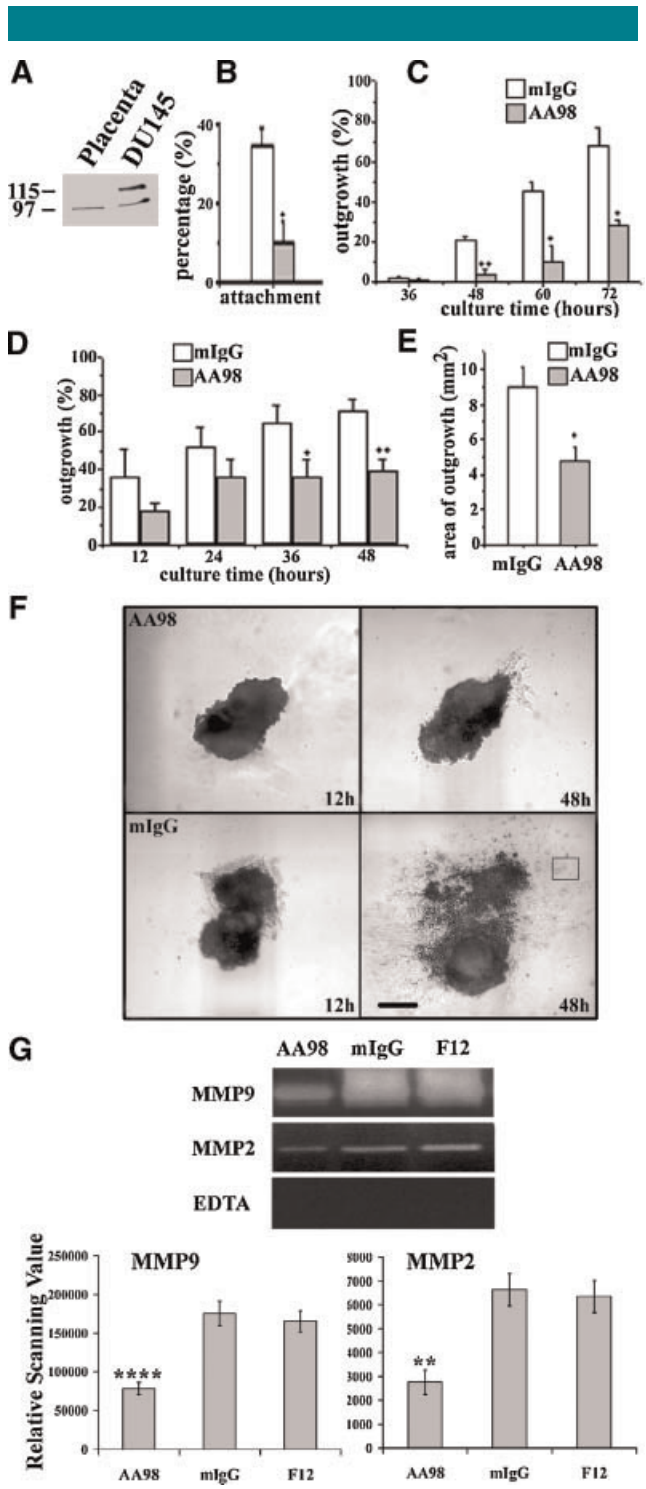

Fig. 2. The function of CD146 on blastocyst attachment and trophoblastic outgrowth. A: Western blot analysis of the specificity of monoclonal antibody AA98. Note that AA98 specifically reacted with CD146 (~97 kDa) from the mouse placenta lysates (10.5 dpc). As a positive control, the antibody also recognized the two isoforms (~115 and ~97 kDa) of CD146 in the lysates of DU145, a human prostate cell line. B,C: Blastocysts and uterine luminal epithelial cells were isolated from pregnant uteri on 3.5 dpc (82–84 h), and co-cultured in the presence of CD146-perturbation Ab AA98 or isotype matched mIgG as a negative control. Note the inhibitory effect of AA98, but not the control mIgG, on the attachment of blastocysts onto the uterine luminal epithelial cell monolayer (B) and on the trophoblastic outgrowth that occurred in attached blastocysts (C). D–F: Ectoplacental cones (EPC) were isolated from the pregnant uteri on 7.5 dpc and cultured in the media with AA98 or mIgG. Note that, compared to treatment with mIgG, the outgrowth percentages of EPC treated with AA98 were significantly decreased (D); even where outgrowth occurred, the mean outgrowth area was dramatically reduced (E). Each point represents the mean of triplicate determinations ($^{*}P < 0.05$; $^{**}P < 0.01$). F: The morphologic observation of EPC in culture with Ab AA98 or control mIgG at different time points. The small box in the mIgG-treated EPC at 48 h shows the outgrowing trophoblasts from the EPC. The bar stands 200 μm. G: The zymography study shows that the secretion of MMP9 and MMP2 in the EPC culture supernatant was also inhibited by AA98, compared with the mIgG or F12 medium as negative controls. The enzyme activities were abolished after the gels were incubated with EDTA. Collagenase activity was quantified by scanning densitometry using software Quantity One (n = 6–11; $^{**}P < 0.01$; $^{****}P < 0.0001$).

microscope. As shown in Figure 3A,B, 5–7 implanted embryos were found in the right horn of each uterus treated with vehicle or control mIgG, whereas the left uterine horns treated with AA98 had either no embryos or only 1–2 poorly developed embryos. These observations clearly demonstrated that the pregnancy failure in these mice was specifically induced by the CD146 perturbation, but not caused by surgery injury or by non-specific effect of Ab.

To study the pathological changes underlying the pregnancy failure induced by AA98, we performed histological study on the antibody-treated uterine horns. In AA98 treated horns, we observed small and disintegrated embryos in poorly developed decidual capsules (Fig. 3C), as compared to the mIgG treated horns, which contained normal embryos in well-developed decidual capsules (Fig. 3D). Using TUNEL assay, we found that excessive apoptotic cells were detected in the uterine decidua of horns treated with AA98 (Fig. 3E,I). However, few apoptotic cells were detected in the contralateral uterine horn receiving mIgG (Fig. 3F,I).

Using anti-CD31 antibodies, we then examined the vascularization in the decidua of the uterine horns. Few neovasculatures were seen in the AA98-treated lateral horns (Fig. 3G,J), whereas numerous neovasculature structures, including a well-developed vascular zone and endothelial lining blood sinusoids, were observed in the mIgG-treated lateral horns (Fig. 3H,J). These observations indicate that excessive apoptosis and loss of angiogenesis in the decidua accompany the failure of embryo implantation induced by AA98.

Discussion

All human conceptions (40–50%) are lost before 20 weeks of gestation due to spontaneous abortion and recurrent miscarriage (Wilcox et al., 1988; Zinaman et al., 1996). Although the underlying mechanism of the pregnancy failure is poorly understood, defects of uterine receptivity and trophoblast invasion have been considered as important causes (Khong et al., 1987; Hustin et al., 1990; Tabibzadeh, 1998; Tabibzadeh et al., 1999).

Adhesion molecules play important roles in mediating embryo attachment and trophoblasts invasion during the process of implantation. Recently, soluble CD146, an adhesion molecule, has been reported to be dramatically increased in the plasma of women with unexplained pregnancy loss and recurrent miscarriage (Pasquier et al., 2005). This correlation suggested that CD146 dysfunction might underlie the development of these types of pregnancy diseases. In this study, we tested this possibility by examining the expression of CD146 during early pregnancy, and the effect of blocking CD146 with a functional-perturbation antibody during blastocyst attachment and trophoblast invasion, both in vitro and in vivo.

We observed a specific and dynamic pattern of CD146 expression during the early stages of pregnancy. CD146 was selectively and strongly expressed on both the receptive uterine luminal epithelium and implantation-competent embryos during the implantation window. CD146 expression, which was absent in the uteri of non-pregnant mice, was dramatically upregulated in the uterine epithelium during the implantation window. Strikingly, at the end of the implantation window, CD146 expression also disappeared from the uterine luminal epithelium. The possibility for this temporal specific expression is that the expression of CD146 in the uterine epithelium may be regulated by ovarian steroid hormones during pregnancy. Indeed, progesterone and estrogen can regulate the expression of many genes that are required for embryo implantation [reviewed in Dey et al. (2004)]. Future studies that examine the CD146 expression changes in mice with abnormal ovarian steroid hormone functions will help to test this possibility.

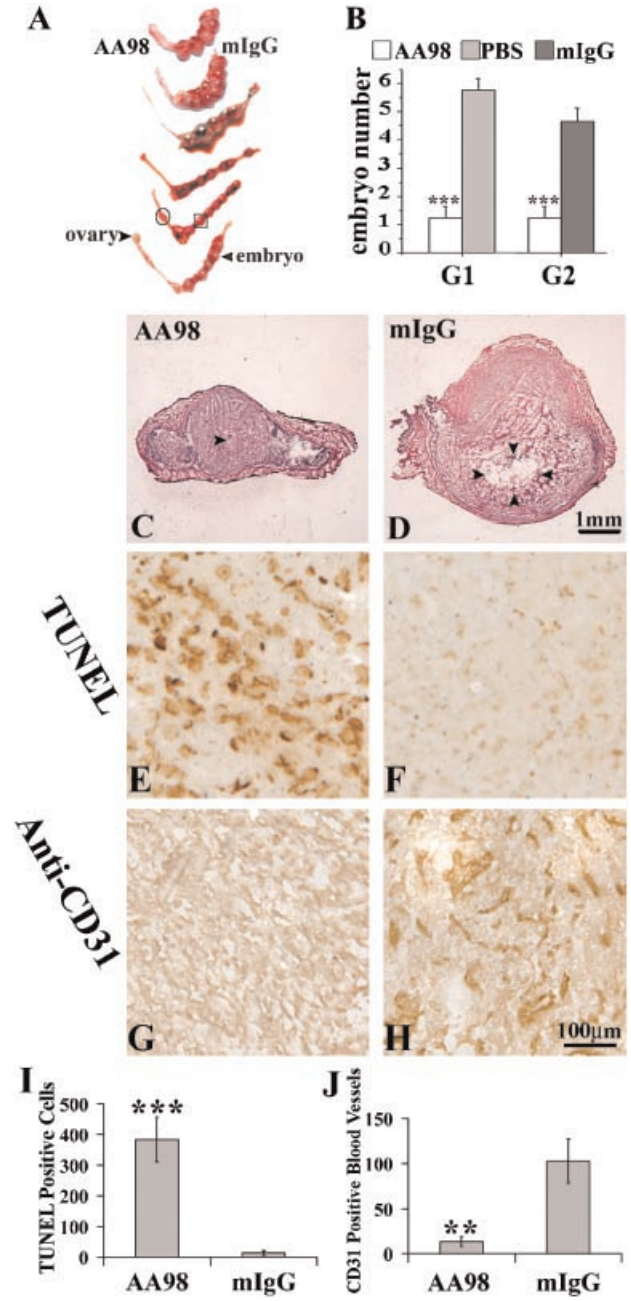

Fig. 3. Perturbation of CD146 with Ab AA98 causes pregnancy failure in mice. On 3.5 dpc, the left uterine horns received 2 μg/10 μl AA98 and the right horns received either PBS (Group 1) or the same amount of mIgG (Group 2). On 7.5–9.5 dpc, the uteri were removed from the mice (A) and the implanted embryos were counted (B). The data in (B) represent the mean values ± s.e.m. from 9 mice in each group; ($P < 0.001$). The hematoxylin and eosin staining shows that the AA98-treated uterine horn (black circle in A) contained small and disintegrated embryos (C; black arrowhead), while the mIgG-treated uterine horns (black box in A) contained well-developed embryos (D; black arrowheads). The TUNEL assay shows that much more apoptotic decidual cells were detected in the AA98-treated uterine horns (E) than in the mIgG-treated horns (F). Immunohistochemistry using anti-CD31 antibodies shows that the number of blood vessels in the decidua was remarkably reduced in the AA98-treated uterine horns (G), compared with the mIgG-treated uterine horns (H). The data in (I,J) represent the mean values ± s.e.m. from five images taken with 20× lens in TUNEL assay and anti-CD31 staining, respectively (**$P < 0.01$; ***$P < 0.001$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

After attachment, the embryonic trophoblast cells migrate through the uterine epithelium and invade the maternal endometrium. Insufficient trophoblast invasion leads to a spectrum of pregnancy complications, including spontaneous abortion (Khong et al., 1987; Hustin et al., 1990) and pre-eclampsia (Goldman-Wohl and Yagel, 2002). Invasion is accompanied by marked changes in the production of proteinases and in the expression of cell adhesion molecules by the invasive trophoblasts. However, although CD146 has been considered as a specific marker for human invasive trophoblasts (Shih and Kurman, 1996), its function in the trophoblasts is poorly understood. Here, we show that perturbation of CD146 function inhibits the migration of trophoblasts, as well as trophoblastic MMP secretion. This finding is consistent with our previous observation that CD146 fails to express on trophoblasts in pre-eclampsia, a pregnancy disease associated with insufficient trophoblastic invasion (Liu et al., 2004a). Similar findings on CD146 have been reported in the case of tumor cells, where enforced expression of CD146 in melanoma cells results in a significant increase in MMP2 activity and metastasis (Xie et al., 1997), while CD146-specific antibodies downregulate MMP2 expression and secretion by melanoma cells and inhibit their invasion (Mills et al., 2002). These results indicate that CD146 molecule is involved in trophoblastic cell invasion, which is crucial for early pregnancy establishment.

In animal experiments, the number of implanted embryos was reduced by more than 80% in the uterine horns that received AA98 on 3.5 dpc, compared with the contralateral horns that received either vehicle or control mIgG. Given the fact that blockade of CD146 inhibits embryo attachment onto the receptive uterine luminal epithelial cells and trophoblastic outgrowth in vitro, it is most likely that blocking CD146 interferes with the processes of embryo attachment and subsequently trophoblasts invasion in vivo, leading to implantation failure. Interestingly, intensified apoptosis and depressed angiogenesis were observed in the maternal uterus, especially in the decidua. It has been reported previously that in cases of spontaneous abortion, apoptosis is greatly intensified in the decidual tissue (Kokawa et al., 1998). We speculate that the maternal uterus did undergo decidual changes during the implantation window. However, in the mIgG AA98 treated uterine horn, the embryo failed to implant into the maternal decidua therefore the decidua degenerated. It will be of importance to investigate the downstream events in the maternal uterus after implantation failure.

In summary, our studies show that CD146 is a marker for the mouse receptive uterine endometrium and invasive trophoblasts, and that blocking CD146 efficiently inhibits embryo implantation, trophoblast invasion and causes pregnancy failure. Our data provide new insights into the biological function of CD146 and molecular mechanisms underlying unexplained pregnancy loss and recurrent miscarriage, suggesting that CD146 has potential as a diagnostic and therapeutic target in reproductive medicine.

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