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Expression and function of a new angiogenic factor AA98 target molecule at the maternal-embryonic boundary of rhesus monkey

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Abtract The target molecule of monoclonal antibody AA98 (AA for short) is a new vascular endothelial cell related factor and plays a role in angiogenesis as indicated by the previous data. To investigate its role in angiogenesis and placentation in primate, we examined its expression in the implantation sites on D17, 19, 28 and 34 of gestation in rhesus monkey by immunohistochemistry and Western immunoblot. Western blot analysis showed that the primary antibody used in this study was specific for its epitope. AA protein was mainly expressed in small blood vessels and in some cytotrophoblast cells. The AA staining was found mainly in the endothelial cells and vascular small muscle. This observation supported the AA's role in angiogenesis. AA was spatio-temporarily expressed in cytotrophoblasts: weak in proliferating trophoblast within cell column and endovascular trophoblast, strong in trophoblastic subpopulation within the basal plate and vascular trophoblast; AA staining within the basal plate was down-regulated during early placentation. The shift of AA98 expression in extravillous trophoblasts suggestes a role of this new factor during the course of cytotrophoblast metastasis and spiral artery remodeling. The spatio-temporarily expression indicats that AA98 could be also used as a trophoblast cellular marker to characterize the acquisition of a vascular endothelial and invasive phenotype.

Keywords: AA98, placentation, angiogenesis, invasion, rhesus monkey.

Primate placentation involves a series of cellular interaction, proliferation, adhesion and invasion account for the progressive tissue remodelling at the implantation site[1]. Growth and development of placenta and its subsequent invasion into the deciduas is likened to the invasion of healthy tissue by malignancy[2], and neovascularization is an integral event in the process of placental development. Blood vessel formation can be divided into two main processes, vasculogenesis and angiogenesis. In the former, the primitive vascular network is established; the

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latter is remodelling of an existing vascular network. Extensive angiogenesis occurs in both the fetal villi and the maternal deciduas, for establishing vascular structures required for the fetal-maternal substance exchange.

A lot of factors have been found to be involved in the angiogenesis, including vascular endothelial growth factor, placenta growth factor, basic fibroblast growth factor[3] and angiopoietins[4]. Angiogenetic antibody (AA) is a newly found angiogenic factor with molecular weight of 100 kD[1]. This new factor was identified in several kinds of tumors (unpublished data). Previous results indicate that AA plays a role in angiogenesis in tumor tissues, but the role of this factor in implantation at early stages is unknown. Because of the difficulty in obtaining human implantation site at the early stage, we have established a rhesus monkey model to define the time of implantation and obtain the tissue materials of implantation sites at various times to conduct such studies. It is well documented that blastocyst implantation in rhesus monkey is initiated on D 8–9 post-ovulation with the penetration of luminal epithelium by polar syncytiotrophoblast cells. The morphological changes of these different cell types and the timing of their appearance in the implantation site show close similarities between rhesus monkey and human being[5]. The aim of the present study is to analyze the localization and expression patterns of AA in different compartments at the early implantation site in rhesus monkey in relation to angiogenesis and placentation.

1 Materials and methods

（ⅰ）Animals. Healthy, adult male and female rhesus monkeys (*Macaca mulatta*) from the monkey colony of the Primate Research Center, Kunming Institute of Zoology, the Chinese Academy of Sciences were used. The animals were caged individually and evaluated daily by visual examination of the perineum for menses, with the onset of menses defined as D1 of the menstrual cycle. Adult female monkeys with regular menstrual cycles of approximately 28 d were chosen for this study. Male rhesus monkeys of proven fertility from previous matings were used for mating. Female monkeys on D11 of their menstrual cycle were caged with a male monkey for 3 d. Vaginal smears were examined the next morning for the presence of sperm. The day that a positive smear for sperm was found was designated D1 of pregnancy. The uteri were removed surgically from 12 animals at D17, D19, D28, D34 of pregnancy respectively (3 animals each group) and the specimens was quickly washed in cold phosphate-buffered saline (PBS) to remove adherent blood, then placed in cold 4% paraformaldehyde fixative for 24 h at 4℃ and further processed through graded dehydration, clearing and embedding in paraffin for immunohisto-

chemistry. Part of speciments were cryopreserved at –70℃ for Western blot.

（ⅱ）Reagents. Except AA980, the other primary antibodies were obtained from Santa-Cruz (USA); secondary antibodies, labeled avidin and Vector-red substrates were from Vector (USA); DAB substrates from DAKO (USA); CDP-Star™ from Roche (Germany); and levamisole from Sigma.

（ⅲ）Western blot. Immunoblot was done as described by Gao et al.[6] to verify the specificity of the mouse anti-human AA antibody used in this study with the monkey endometrium. The tissue was homogenized and the supernatant from ultracentrifugation was run on a 10% SDS-PAGE gel under reduced conditions. After being transferred to the PVDF membrane, individual lanes were cut and blocked with 5% nonfat milk/PBS for 1 h, followed by incubation at 20℃ for 1 h with either mouse anti-human AA antibody (IgG, 0.2 μg/mL) or normal mouse immunoglobulin G (IgG, 0.2 μg/mL, as a negative control) in 5% milk/PBS. The membranes were washed 3 times for 5 min each in 5% milk/PBS and incubated with AP-conjugated horse anti-mouse IgG (0.2 μg/mL) in 5% milk/PBS for 1 h respectively. The membranes were washed in PBS 3 times for 5 min each, followed by 10 min of incubation with CDP-Star™, then exposed on X-ray film.

（ⅳ）Immunohistochemistry. All primary antibodies used in this study were diluted in 10% horse serum-PBS (*v/v*), and the other antibodies were in 5% horse serum-PBS. All reactions were carried out at RT. The antibodies and concentration of each were as follows: mouse anti-human AA (1.5 μg/mL); mouse anti-human cytokeratin (1 μg/mL, to identify trophoblast cells); mouse anti-human PCNA (0.5 μg/mL, to identify proliferating cells); rabbit anti-human actin (1 μg/mL, to identify vascular smooth muscle cells); Bio-conjugated horse anti-mouse IgG (2 μg/mL); HRP conjugated avidin (2 μg/mL); AP-conjugated goat anti-rabbit IgG (2 μg/mL).

Several antigen retrieval protocols and two types of staining were used in the immunohistochemistry detection. The retrieval protocols included: no retrieval, trypsin (0.1%, 37℃, 15 min), citrate buffer (10 mmol/L, pH 6.0, 98℃ for 20 min) or in EDTA solution (0.1 mol/L, pH 8.5, 94℃ for 15 min). The results of this analysis revealed that the optimal procedure was EDTA solution retrieval.

HRP-DAB staing: As reported by Gao et al.[7], serial 4 μm sections of tissue were cut, deparaffinized, and rehydrated through degraded ethanol. $3\%H_2O_2/H_2O$ was

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used to quench the endogenous HRP. Antigen retrieval was performed in EDTA solution and cooling at room temperature for 20 min. Non-specific binding was blocked with 10% (*v*/*v*) normal horse serum in PBS for 1 h. The sections were incubated with primary antibody or the same concentration of unrelated immunoglobulins from the same species as negative control for 1 h. The sections were then incubated with biotinylated secondary antibody followed by an avidin-HRP complex and DAB substrates.

AP-red staining: the blocking and antibody reaction protocols were described as in HRP-DAB staining，the secondary antibody reaction was followed by an AP-avidin complex and Vector Red substrates, endogenous alkaline phosphatase activity was inhibited by supplementing 1 mmol levamisole into substrate solution.

A double staining technic using the antibodies to cytokeratin and actin was performed to localize the vascular trophoblast cells. After being labeled by mouse anti-human ctyokeratin with the HRP-DAB staining, the sections were blocked with 10% horse serum/PBS for 1 h, then reacted with rabbit anti-human actin for 2 h, followed by an AP-red staining, thus the trophoblast cells were labeled brown and the blood vessel wall red.

(ⅴ) Microscopic assessment. The mounted sections were examined using a Nikon microscope. For assessment of staining in cells of different compartments, semi-quantitative subjective scoring was done blinded by three investigators using a 4-scale system: "−" = nil; "+/−" = weak; "+" = moderate; and "++" = strong , as described by Yue et al.[8].

2 Results

(ⅰ) Western blot. As shown in Fig. 1, a band of about 100 kD was detected in monkey endometrium using mouse anti-hAA antibody in immunoblots, but not detected when the antibody being replaced by normal mouse IgG.
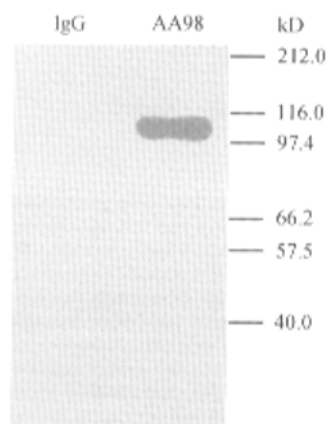

Fig. 1. Western blot analysis of AA protein in rhesus monkey endometrium. A band of about 100 kD was detected in monkey endometrium using mouse anti-hAA antibody in immunoblots, but not detected when the antibody was replaced by normal mouse IgG.

(ⅱ) Immunohistochemistry. The localization and median score with ranges of immunohistochemical staining for AA in the fetal and maternal cellular subpopulations in rhesus monkey implantation sites were summarized in Table 1. AA protein was mainly expressed in smooth muscle, blood vessels and some cytotrophoblast cells.

(1) Expression of AA in the villous placenta, anchoring villi and basal plate. Membrane-associated AA staining was observed in some cytotrophoblast cells (Fig. 2(d)). The polarized proliferating CT (PCNA-positive, Fig. 2(d)′, PT) at the proximal tip of cell columns was AA-negative (Fig. 2(d), PT); the CT in the middle of anchoring cell columns weakly expressed AA (Fig. 2(d), IT), and these cells also showed weak-proliferating cells as indicated by Fig. 2(d) (IT, PCNA-weakly positive); the non-proliferating CT within the basal plate (Fig. 2(d), BP) were strongly positive for AA staining (Fig. 2(d), BP).

In different stages of placentas, the AA staining pattern in cytotrophoblast at the basal plate varified markedly in a stage dependent manner (Fig. 2(i) —(l)), reaching the maximum expression on D17, and the lowest level on D34 of gestation. AA staining in villous CT and all the syncytiotrophoblast was negative.

(2) Expression of AA in trophoblast within the deciduas. A double immunohistochemical staining was performed in the present study to localize trophoblast cells with anti-cytokeratin antibody (brown) and vascular smooth muscle with anti-actin antibody (red) in the same section. A great number of cytokeratin-positive extravillous cytotrophoblasts (EVT) were detected in the endometrium on D28 and D34 of gestation. The relative degree of AA in the endovascular trophoblasts (extravillous cytotrophoblasts in the arterial lumen), vascular trophoblasts (extravillous cytotrophoblasts in the wall), perivascular trophoblasts (stromal extravillous cytotrophoblasts adjacent to the artery) and the trophoblasts that had replaced the vascular endothelial cells on the serial sections demonstrated a highly varying pattern: Strong staining was observed in the endovascular trophoblasts that did not contact with blood vessel (Fig. 2(a), arrow), and weak staining was detected in the trophoblast which attached to the endothelial cells or invaded the vascular walls (Fig. 2(a), arrow head). The spiral arteries near the basal plate were often destroyed severely by trophoblasts as shown in Fig. 2(b)′, showing the spiral artery being destroyed by trophoblast and losing its identity. A strong AA staining was found in these trophoblast cells (Fig. 2(b)). A lot of perivascular trophoblasts presenting as single cells were found in deciduas (Fig. 2(c)′), but no AA staining could be detected in these cells (Fig. 2(c)). Some endothelial trophoblasts which replaced the vascular endothelial cells were identified in the samples on D34 of gestation, and AA was strongly expressed in these trophoblasts (Fig. 2(g)).
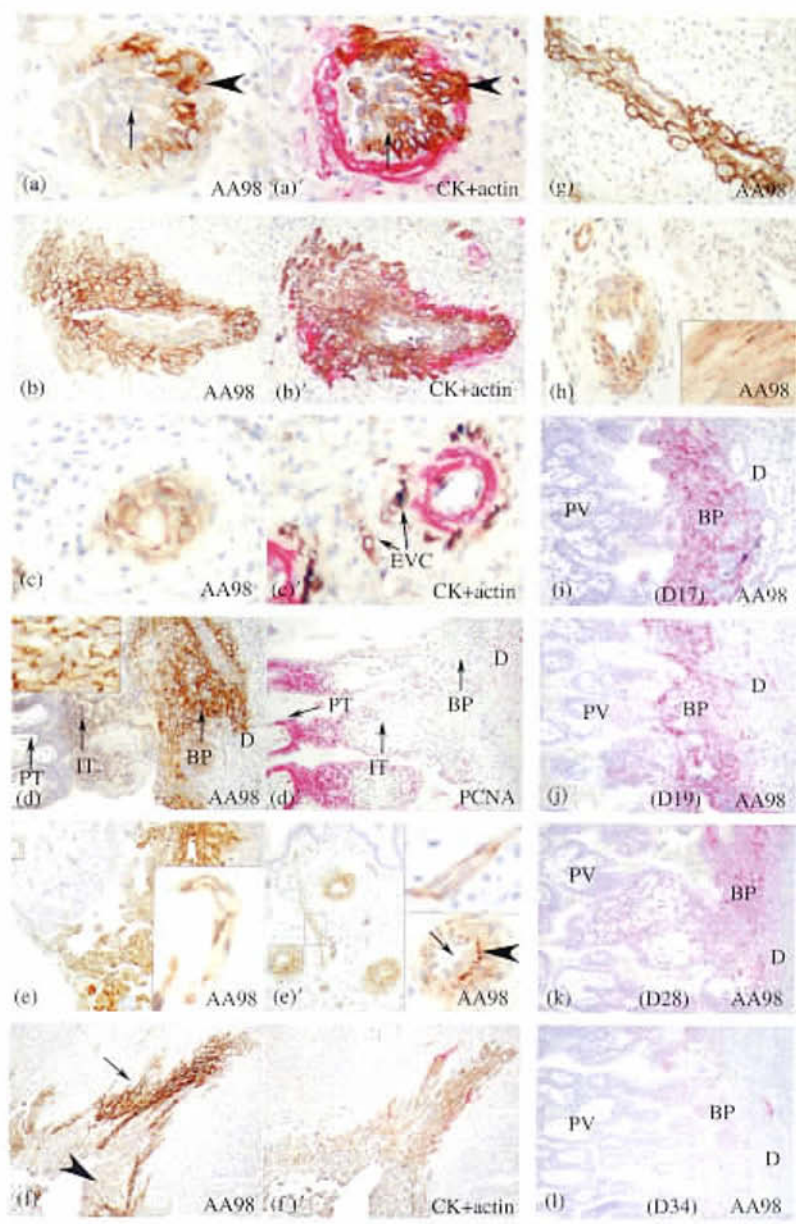
Fig. 2. Expression of AA protein in rhesus monkey implantation sites. (a) AA is weakly expressed in the endovascular trophoblasts that did not contact with blood vessel (arrow), and strongly expressed in the trophoblasts that attached to endothelial cells or had invaded the vascular walls (arrowhead). The localization of trophoblasts is detected by (a)′, a double immunohistochemical staining in the next section to localize trophoblast cells (with anti-cytokeratin antibody, brown) and vascular smooth muscle (with anti-actin antibody, red), ×400. (b) AA is strongly expressed in the endovascular trophoblasts that destroyed the spiral arteries near the basal plate severely; (b)′ the double staining shows the spiral artery that has been destroyed by trophoblast and lost its identity, ×200. (c) No AA staining could be detected in the perivascular trophoblasts presenting as single cells, but in (c)′, these cells can be detected in the next section, ×400. (d) Membrane-associated AA staining was observed in some cytotrophoblast cells. No AA expression can be found in the CT at the proximal tip of cell columns (PT), the CT throughout anchoring cell columns weakly expressed AA (IT), and the CT within the basal plate (BP) are strongly positive of AA staining; (d)′ the anchoring villi labeled with PCNA, and a reverse expression with AA can be found, ×100. (e) AA is strongly expressed in the endothelial cells of newly formed blood vessels in villi and (e)′, in the deciduas, but weakly expressed in the endothelial cells of big spiral arteries (arrow), at the same time, AA is also strongly expressed in vascular smooth muscle cells (arrowhead), ×100, ×400. (f) Some cuneiform EVT cell groups could be found in the basal plate, AA is expressed strongly in invading cells (arrow), and weakly in the following cells (arrowhead); (f)′ double staining in the next section to label the trophoblasts, ×100. (g) AA is expressed strongly in the endothelial trophoblast replacing the vascular endothelial cells, the section is from D34 of gestation samples, ×400. (h) AA is strongly expressed in vascular smooth muscle cells (arrowhead) within myometrium, and weakly expressed in myometrium, ×200. (i)—(l) The relative degree of AA staining in basal plate of D17 (i), D19 (j), D28 (k), and D34 (l) of gestation. A decreased expression can be seen, ×100.

Table 1 The localization and median score for AA in rhesus monkey implantation sites[a]

Trophoblast cells								Vascular endothelial cells		Smooth muscle cells		Other cells	
Fetal						Maternal							
VP		AV/BP				ETT	++	villous	+	Myometrium	+	Stromal cells	−
		D17	D19	D28	D34	VCT	++	stromal capillary	+	Vascular	++	Glandular epithelium	−
CT −	BPCT	++	++	+	+/−	ECT	+/−	spiral artery	+/−			Blood cells	−
ST −	IT	+	+	+	+/−	SCT	−	vein	+/−				
	ST	−	−	−	−	PCT	−	myometrial	+/−				

a) VP, Villous placenta; AV/BP, anchoring villi/basal plate; CT, cytotrophoblast; IT, intra-column trophoblast; ST, syncytiotrophoblast; ECT, endovascular trophoblasts (cytotrophoblasts in the arterial lumen); VCT, vascular trophoblasts (cytotrophoblasts in the wall); PCT, Perivascular trophoblasts (stromal cytotrophoblasts adjacent to the artery); SCT, stromal cytotrophoblasts (trophoblasts in the stroma but not adjacent to the artery); ETT, trophoblasts replacing the vascular endothelial cells.

(3) Expression of AA in vascular endothelial cells and smooth muscle cells. AA staining was positive in vascular endothelial cells. A strong expression was identified in the endothelial cells of newly formed blood vessels in villi (Fig. 2(e)) or deciduas (Fig. 2(e)′), and relatively weak in the endothelial cells of big vessels (Fig. 2(e)′, arrow). Smooth muscle cells expressed AA in a spotted-like feature (Fig. 2(h)). A stronger staining could be found in vascular smooth muscle cells (Fig. 2(e)′, arrowhead and Fig. 2(h)) when compared with mymetrium. Other endometrial cell types did not express AA at a notable level.

3 Discussion

Using Western blot and immunohistochemistry analysis, the temporal and cell lineage restricted expression pattern of the new angiogenetic factor AA in the early implantation sites of rhesus monkey was investigated. Western blot analysis showed that the AA98 antibody used specifically recognizes an epitope with the molecular weight of about 100 kD, indicating the specificity of the mouse anti-human AA antibody with the monkey tissue. Using the immunohistochemistry analysis, we found that AA was expressed in the EVT of the anchoring villi and trophoblast cells that are invading the maternal blood vessels or replacing the vascular endothelial cells in addition to the expected expression of AA in both fetal and maternal endothelial cells.

During implantation and placentation in primate, extensive angiogenesis occurs both in the fetal villi and in the maternal deciduas to establish the vascular structures required for the fetal-maternal blood exchange. In this study, we have demonstrated that AA was expressed in endothelial cells of both the fetal and maternal vessels of the implantation site. A stronger AA expression was identified in newly formed vascular endothelial cells of the villous and decidual vessels compared with the mature vessels such as arteries. This observation supports a role of AA in angiogenesis. AA staining was positive in all smooth muscle cells, a much higher expression was observed in vascular smooth muscle cells that was similar to the expression pattern of the angiogenetic factors bFGF and VEGF[9,10]. The expression of bFGF and VEGF in smooth muscle may be connected with a role of storage and recruitment to other cells. Whether such spatial expression pattern of AA may exert the same function remains to be investigated.

Remodeling of blood vessels including loss of the muscle and elastic tissue surrounding the vessels and replacement of the endothelial cell lining with fetal trophoblasts at the fetal-maternal interface is an essential determinant for establishing and maintaining a healthy pregnancy. In normal human pregnancy, the trophoblast differentiates from proximal cell column cytotrophoblasts into two lieages: a villous phenotype that results in cell fusion and formation of syncytium and an extravillous phenotype that adopts an invasive behavior. EVT cells migrate along spiral arteries and invade the vessel walls, producing physiological changes in both the dicidual and myometrial segments of arteries[11]. This process leads to the development of vessels with low resistance and high capacity, thereby enhancing blood flow to the intervillous space to meet the increasing fetal demands for nutrient and gas exchange[12]. The differentiation and invasion of CTC are regulated by a lot of factors, such as proteinases[13—15], growth factors, integrins, interleukins, etc.[11].

The first trimester invasive phenotype has recently been characterized, which demonstrated the acquisition of a vascular endothelial phenotype such as the up-regulation of VE-cadherin and PECAM-1[16,17]. These changes occur progressively, the cells migrate down the cytotrophoblast cell columns into the ceccidua and finally into the maternal endometrial vasculature ultimately to replace and remodel the arterioles of the endometrium. In the present study, we found that the AA expression in the cytotrophoblasts increased progressively as the cells migrate down the cytotrophoblast cell columns into the ceccidua: The villous CT and polarized proliferating (PCNA-posi- tive) CT at the proximal tip of cell columns were AA-negative; the weak-proliferating (PCNA-weakly positive) CT within anchoring cell columns slightly expressed AA, and the non-proliferating (PCNA-negative) CT within the basal

plate were strongly positive of AA staining. We also observed a spatio-temporal AA expression in endovascular invasive trophoblasts: Weak staining was observed in the endovascular trophoblasts that did not contact with blood vessel; strong staining was detected in the trophoblasts which had invaded into the blood vessels or replaced the vascular endothelial cells; but no expression could be detected in the perivascular trophoblasts and stromal cytotrophoblasts that presented as single cells. Some cuneiform EVT groups could be found in the basal plate (Fig. 2(f), (f)′), and the AA expression in invading cells (Fig. 2(f), arrow) was much stronger than the following cells (Fig. 2(f), arrowhead). The temporal- and lineage-specific pattern of expression of AA suggests that this protein functions during the critical phase of development of the fetal vasculature and reworking of the maternal vessels and may also be involved in the processes of trophoblast metastasis and invasion during normal placentation.

AA is specially expressed in endovascular but not in interstitial invasive trophoblasts supporting the earlier reports that the invasive trophoblasts were associated with a change in the expression of the repertoire of adhesion molecules and acquisition of endothelial cell markers. This is a good marker to distinguish trophoblasts that invade tissue from those that invade blood vessels.

In summary, the expression of AA by the fetal and maternal endothelial cells and endovascular invasive trophoblasts suggests that the AA may play a pivotal role in remodeling vessels at the fetal–maternal interface.

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