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Angiogenic Activity of Human Villi and Decidua in the First Trimester of Pregnancy

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Abstract Angiogenic activity in the extracts of human villi and decidua in the first trimester of pregnancy was examined using chorioallantoic membrane assay. Blood vessel density on the chorioallantoic membrane increased greatly with the addition of villi extract, and slightly by decidua extract. Neovascularization with a spoke wheel appearance was induced by both villi and decidua, and this response pattern by the villi was stronger than that by the decidua. Maternal serum did not induce any vascular response. These results suggested that angiogenic activity was present both in the villi and decidua in the first trimester of pregnancy and that the villi activity was stronger than the decidua.

Introduction

The first trimester of human pregnancy is an important period for establishing fetoplacental circulation. The formation of the heart and great vessels in a fetus along with cord vessels are explained by the differentiation of germ cells in the embryo. There has been little information, however, concerning neovascularization in the intervillous tissue and the decidua, which are important sites for exchanging gases, nutrients and metabolites between fetus and mother.

Recently angiogenesis has been found to be closely related to the action of angiogenic factors in various physiologic and pathologic conditions, such as wound healing, chronic inflammation, rheumatoid arthritis and neoplasia³⁾⁶⁾⁷⁾. Angiogenic activity has been previously observed in various tissues of the human pregnant uterus⁴⁾⁸⁾⁹⁾. Burgos first reported the presence of angiogenic activity in the term placenta¹⁾ and several investigators reported that angiogenic

factors were found to be partially purified in the term placenta²⁾¹¹⁾¹²⁾¹³⁾. Presta et al observed the stimulatory effect of human placental tissue to the capillary endothelial cell growth and its function¹¹⁾¹³⁾. These facts suggest that in early human pregnancy angiogenic activity may also play an important role in establishing fetoplacental circulation. In the present study we examined the angiogenic activity of extracts obtained from the villi and the decidua in the first trimester of pregnancy.

Materials and methods

1. Preparation of the extracts of decidua and villi

Decidua and villi specimens were obtained from noncomplicated legally induced abortions performed in the first trimester of pregnancy. The number of specimens were six for the decidua and nine for the villi, and gestational ages ranged from 6 to 11 weeks of gestation. The extraction of villi and decidua was performed according to the method of Burgos with

slight modification²⁾. The decidua and villi were separated and washed with CMF-PBS solution (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4) and blood was removed. They were homogenized at 10,000 r. p. m. for 2.5 min twice each with Ace homogenizer. To this homogenate 3 volumes of the following solution were added: 10% propan-2-ol, 50 mM Tris/HCl (pH 7.2), 75 mM NaCl, 2 mM phenylmethylsulfonylfluoride, 2 mM ethylenediaminetetraacetic acid disodium salt, and 2.5 mM iodoacetamide. Extraction was performed under constant shaking for 20 hours. The extracted solution was centrifuged at 900 G for 30 min, and the supernatant was further centrifuged at 50,000 G for 90 min. After protein concentration of the extract was measured by Lowry's method, the extract was concentrated with ultrafiltration with a diaflo membrane having a molecular weight cut off of 10,000 Daltons (Advantec Toyo). It was then dialyzed against 0.15 M NaCl and 1 mM NaHCO_3 solution. After the pellet was removed with centrifugation at 900 G for 20 min, the supernatant was passed through a millipore filter (Cellulose acetate, 0.2 μm) and freeze-dried. All the above procedures were performed at 0 to 4°C.

2. Preparation of maternal serum

Maternal serum was prepared from five non-complicated pregnant women in the first trimester of pregnancy and was passed through a millipore filter and freeze-dried.

3. Chorioallantoic membrane (CAM) assay

On the eleventh day of an incubated fertilized hen egg, a 1×1 cm window was cut and the egg shell and shell membrane were removed. A three minute perforation was made in the exposed CAM with a 30 G needle to assist the absorption of the sample through the extodermal layer of the CAM. Freeze-dried samples of villi and decidua extracts were dissolved in 0.15 M NaCl and 20 mM Tris-HCl (pH 7.4) solution and were adjusted to the protein concentration of

2.0 mg/10 μl , and 10 μl of the solution was applied to a paper disk of 5 mm in diameter. The disk was then put in the center of the exposed CAM.

The maternal serum sample was applied to the CAM in the same way. The above mentioned dissolving solution (0.15 M NaCl, 20 mM Tris-HCl, pH 7.4) was used as a control. The sample charged eggs were closed with tape and were further incubated for an additional 4 days at 37°C under humidified conditions. The egg shell was then removed and a photograph was taken. Six eggs were used for each sample.

4. Quantitative and qualitative evaluation of angiogenic activity on CAM

The eggs with thickness or turbidity of CAM around the disk due to local inflammation were excluded from data analysis. The egg showing the strongest neovascularization in each sample was chosen as the representative of the responses to the sample. Vascular response on the CAM was analysed qualitatively and also quantitatively. Angiogenesis was characterized by an increase in the number of vessels on the CAM. Quantitative analysis was performed according to the method of Matsuno¹⁰⁾ with modification. The sample charged disk was centered in the circle, and concentric circles with radii of 5, 7.5, 10, 12.5 and 15 mm were drawn on the photograph. Vessels crossing the circles were counted irrespective of the blood vessel patterns and blood vessel density was calculated by dividing the number of blood vessels crossing the various circles by the circumference. For qualitative analysis, typical vascular response was characterized by the presence of a "spoke wheel appearance" around the disk⁶⁾. For the evaluation of the strength of this vascular response, the number of blood vessels showing spoke wheel patterns originating directly from the disk was counted at different distances from the disk. For statistical analysis, the Student t-test was used.

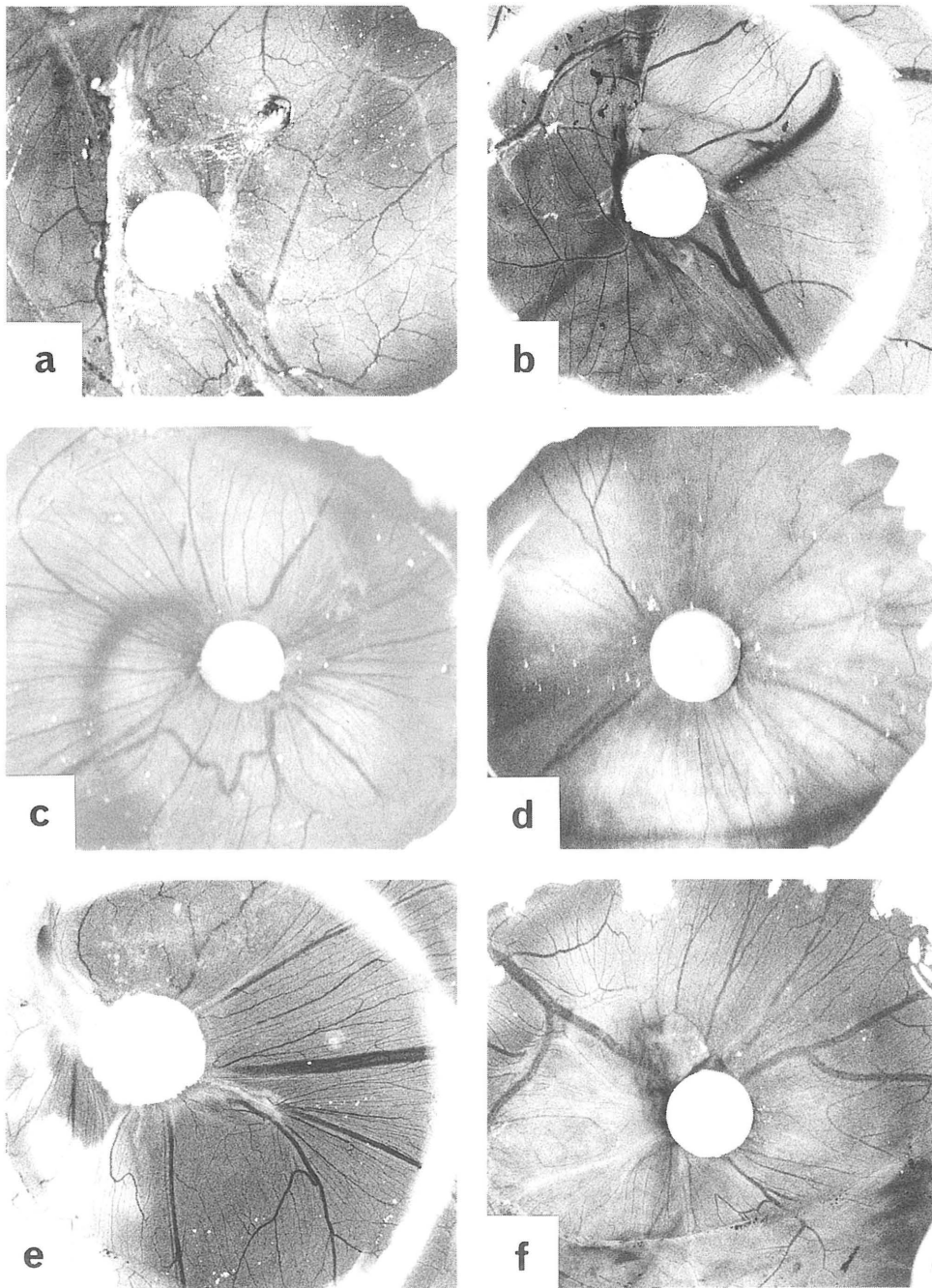


Fig. 1 Vascular response patterns on the chorioallantoic membrane.

Test samples adjusted to the protein concentration of 2 mg/10 μ l were applied to the disk and the disk was put at the center of the CAM and vascular responses were examined as described in the materials and methods section.

a) control: 10 μ l of 0.15M NaCl and 20 mM Tris-HCl (pH 7.4) solution, b) 2 mg of maternal serum at 16 weeks of gestation, c) villi extract at 8 weeks of gestation, d) villi extract at 10 weeks of gestation. e) decidua extract at 7 weeks of gestation, f) decidua extract at 9 weeks of gestation.

Results

Vascular responses on the CAM were shown in Fig. 1. In the control, neither an increase in blood vessel density nor any change in the vascular pattern was observed around the disk (Fig. 1a). Nor did the maternal serum induced any vascular response (Fig. 1b). Villi extracts at 8 and 10 weeks of gestation increased blood vessel density around the disk and its vasculature revealed a spoke wheel appearance (Fig. 1c, d). Decidua extracts at both 7 and 9 weeks of gestation also increased vasculature and induced a spoke wheel appearance (Fig. 1e, f).

To evaluate the intensity of neovascularization on the CAM, blood vessel density was measured at each of the previously mentioned distances from the disk (Fig. 2). In the control case, blood vessel density did not change in relation to the distance from the disk. Even by treatment with maternal serum, blood vessel density was not different from that of the control and no vascular response was induced. In the CAM charged with villi extracts from 8 to 11 weeks of gestation, however, blood vessel density ($1/\pi\text{cm}$) at a distance of 5 mm from the disk (24.4 ± 3.8) was significantly higher, compared to that at a distance of 15 mm from the disk (11.4 ± 5.0 ; $p < 0.001$). By treatment with decidua extracts from 6 to 10 weeks of gestation, the blood vessel density at a distance of 5 mm from the disk (19.8 ± 7.3) was significantly higher, compared to that at a distance of 15 mm from the disk (10.8 ± 4.2 ; $p < 0.05$). These findings indicate that extracts of villi and decidua induce angiogenesis in the CAM. Blood vessel density by the treatment with the villi was higher than that with the decidua.

As the induction of spoke wheel type blood vessels is considered to be characteristic of the angiogenic activity, both presence and / or absence of vasculature with a spoke wheel appearance was then examined (Table 1). Both the villi and decidua extracts induced neovas-

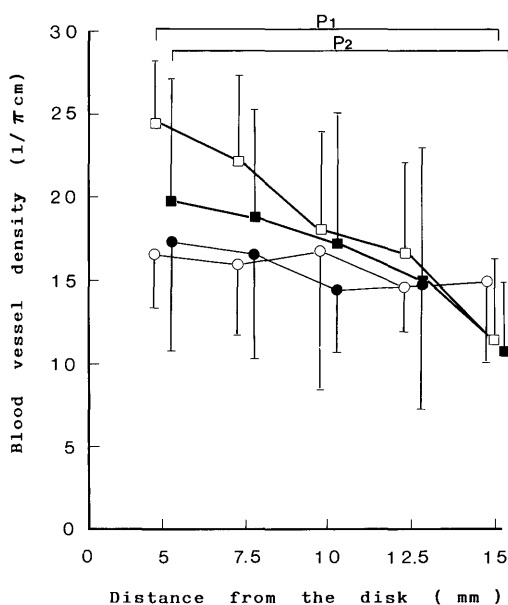


Fig. 2 Changes in blood vessel densities on the chorioallantoic membrane.

Blood vessel density around the sample charged disk was measured at given distances from the disk as described in the materials and methods section. Density was shown as the blood vessel number crossing the constant length (πcm) of circles irrespective of presence or absence of a spoke wheel appearance. Distance from the disk was that from the center of the disk. The results were shown as mean $\pm$ S. D.

○, control: 0.15M NaCl and 20 mM Tris-HCl (pH7.4) solution;

●, maternal serum at 16 weeks of gestation;

□, villi extracts at 8 to 11 weeks of gestation ($n=9$);

■, decidua extracts at 6 to 10 weeks of gestation ($n=6$).

P_1 , P_2 : statistically significant between 5 mm and 15 mm in villi and decidua respectively. $P_1 < 0.001$ (villi), $P_2 < 0.05$ (decidua).

cularization with a spoke wheel appearance on the CAM, in contrast to nonspecific vasculature on the control CAM and the maternal serum charged CAM. The intensity of spoke wheel appearance was then compared between villi and decidua by the number of blood vessels

which showed spoke wheel appearance on the CAM (Fig. 3). Stronger spoke wheel vasculature was provoked by villi extracts compared to those of the decidua at both 7.5 mm (5/9 versus 0/6, in cases with 16 or more blood vessels) and 12.5 mm (5/9 versus 0/6, in cases with 12 or more blood vessels) from the disk.

Discussion

The neovascularization of the vili and decidua is important for the formation and growth of the placenta. Burgos found angiogenic activity in the conditioned media of human chorionic- and decidua-membranes and in the term placenta¹⁾, and isolated angiogenic peptide from these tissues²⁾. Moscatelli et al

also prepared a protein from the human term placenta with angiogenic activity¹¹⁾¹²⁾¹³⁾. Little is known, however, about angiogenic activity in the villi and decidua in the first trimester of pregnancy which is an important period for the development of the placenta.

Fuchs examined angiogenic activity of the decidua during pregnancy in the CAM assay and observed angiogenic activity in the decidua in the early stage of pregnancy, but he charged the tissue itself to the CAM without extraction procedures, and the nature of villi was not examined⁸⁾. In the present study we examined angiogenic activity using villi and decidua extracts and angiogenic activity was observed in both cases. Regarding angiogenesis of the decidua, angiogenic activity has been reported in the endometrium⁴⁾⁵⁾ and in the decidua in early pregnancy⁸⁾. Our results correspond to these reports. As no angiogenic activity was observed in the maternal serum in the present examination, angiogenesis in the decidua is considered to be induced by substances which are produced in the decidua.

In conclusion, extracts of villi and decidua in the first trimester of pregnancy revealed angiogenic activity and the activity in the villi was stronger than that in decidua. These

Table 1 Vascular responses with spoke wheel appearance after treatment with villi and decidua extracts.

	total cases	spoke wheel appearance	
		presence	absence
villi	9	7	2
decidua	6	4	2
maternal serum	5	0	5
control	6	0	6

(Digits: number of cases)

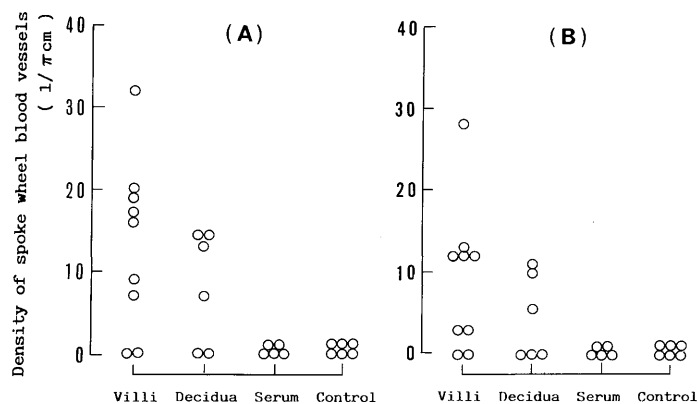


Fig. 3 Intensity of vascular responses with spoke wheel appearance.

(A) 7.5mm from disk; (B) 12.5mm from disk.

Blood vessel density around disk was measured as in Fig. 2, but only blood vessels which showed a spoke wheel appearance were counted.

results suggest that angiogenic substance (s) produced in villi and decidua may play an important role for establishing the feto-and-utero-placental circulation in early pregnancy.

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(和文抄録)

ヒト妊娠初期絨毛および脱落膜の血管増殖作用

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本田直利・中野仁雄

胎盤での血管増殖の機序を明らかにする目的で、ヒト妊娠初期の絨毛および脱落膜中での、血管増殖に対する活性物質の有無について検討を加えた。

合併症のないヒト正常妊娠 6-11 週の初期中絶例から、絨毛および脱落膜を分取し、CMF-PBS 溶液にて洗浄、血液除去後、ホモジェナイズし、これを Burgos の変法により 75 mM NaCl, 50 mM Tris-HCl (pH 7.2), 2 mM EDTA-2 Na, 2 mM PMSF, 10 % Propan-2-ol, 2.5 mM Indoacetamide 溶液で 20 時間抽出した。この抽出液を 900 G で 30 分、さらに、50,000 G で 90 分遠心後、上清を限外濾過にて濃縮、透析し、ミリポアフィルターで滅菌後凍結乾燥したものを試料とした。血管増殖作用は、胎生 11 日目のニワトリ受精卵の漿尿膜上に試料 2 mg を添加し、37°C で 4 日間インキュベート後、漿尿膜上の新生血管の形態お

よび分布密度から判定した。

妊娠初期の母体血清では、血管増殖作用は認められなかった。すべての新生血管の分布密度 (血管数/ π cm) は、絨毛ならびに脱落膜抽出液のいずれの添加によっても、試料から近位 (5 mm) (絨毛 24.4 ± 3.8 , 脱落膜 19.3 ± 7.3) では、遠位 (15 mm) (絨毛 11.4 ± 5.0 , 脱落膜 10.4 ± 4.2) に比べて有意に高値を示した。また試料を中心として車軸状の血管新生が、絨毛で 7/9 例、脱落膜で 4/6 例に認められた。その程度は試料の近位部で 16 以上の車軸状の血管数を有するものは、絨毛で 5/7 例、脱落膜では 0/4 例であり、絨毛は脱落膜より高度の血管増殖作用を示した。

以上ヒト妊娠初期絨毛および脱落膜は、いずれもニワトリ受精卵漿尿膜法において血管増殖作用を示し、かつ絨毛は脱落膜に比べてより強い作用を有している。