血管內皮生長因子（VEGF）對妊娠毒血症孕婦胎盤生長的影響

計畫類別：▼個別型計畫

計畫編號：NSC 90 - 2314 - B - 006 - 170 -

執行期間：90年08月01日至91年07月31日

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中華民國91年10月30日
中文摘要：

Vascular endothelial-cadherin (VE-cadherin) 是一種有關於細胞増生，通透性，及微血管形成的黏連分子。在胎盤形成的過程當中，滋养層細胞行成一管狀的構造時，可以發現到 VE-cadherin 表現在中間及終端的絨毛上面。血管內皮生長因子 (VEGF) 是一種強力的血管生成因子，它能導至內皮的増生及微血管的生成。本研究的目的在於探討 VEGF 誘導胎盤內 VE-cadherin 生成及表現的可能機制。我們收集第 14，18，21 天鼠胚的胎盤作實驗，第十八天的鼠胎盤抽取物經處理並置於 37℃ 含 5% CO2 的培養箱中培養 24 小時，並利用 Western blot 分析法來測量其中的 VE-cadherin，VEGFR-1，VEGFR-2 的含量。並利用免疫沉澱法 (immunoprecipitation) 來證實 Western blot analysis 的正確性。我們的目的有二；1：胎盤組織中 VE-cadherin，eNOS，VEGFR-1，及 VEGFR-2 的濃度和懷孕的關係；2：VEGF 誘導胎盤中 VE-cadherin 產生的過程中一氧化氮 (NO) 所扮演的角色。結果我們發現；1：VE-cadherin 的濃度和 eNOS；2：第十八天的鼠胎盤表現出最多的 VEGFR-1 但是最少的 VEGFR-2；3：VEGF 誘導 VE-cadherin 產生的過程可以被 L-argamine 增強。結論是 VEGF 和 VEGFR-1 的結合可以增加 VE-cadherin 的產生長經由 (NO-dependent 途徑)
Abstract:

Vascular endothelial-cadherin (VE-cadhein), a calcium-dependent homotypic adhesion molecule, is related to permeability, cell proliferation, and capillary formation. During placental development, the differentiation of trophoblasts forms vascular-like structures. VE-cadherin expresses in the intermediate and terminal villi. Vascular endothelial growth factor (VEGF), a potent vascular permeability factor or angiogenic factor, induces vascular permeability, endothelial proliferation, and capillary formation. The purpose of this study was to examine the possible mechanisms by which VEGF induced the expression of VE-cadherin in rat placenta. The placenta from rats on gestation day 14 (G14), 18 (G18) and 21 (G21) were used. Placental explants from G18 rats were pretreated with various compounds in vitro for 24 hours in a 5% CO₂ incubator at 37 °C. Western blot analysis measured the protein abundance of eNOS, VE-cadherin, VEGFR-1 and VEGFR-2. Immunoprecipitation assay was used to confirm the results of Western Blot analysis. According to the purpose, our aims were to examine: 1) the effect of pregnancy on the protein abundance of VE-cadherin, eNOS, VEGFR-1, and VEGFR-2 in placental tissues and 2) the involvement of NO in the induction of placental VE-cadherin by VEGF. Our data indicate that 1) the protein profile of VE-cadherin is related to that of eNOS over gestation, 2) G18 placenta expressed the greatest amount of VEGFR-1 but the lowest amount of VEGFR-2, 3) the induction of VE-cadherin protein by VEGF can be enhanced by L-arginine. In conclusion, the binding of VEGF to VEGFR-1 may increase the protein abundance through a NO-dependent pathway.

<Introduction>

Vascular endothelial-cadherin (VE-cadhein) mainly exists in all types of vascular endothelial cells. During embryo development, VE-cadherin appears when mesodermal precursors are differentiated to endothelial cells (Breier et al., 1996). Hordijk et al (1999) showed that in vitro inhibition of VE-cadherin by a VE-cadherin antibody (c175) increases the permeability of FITC-dextran through the monolayer of cultured HUVECs. Transfection of VE-cadherin cDNA in Chinese hamster ovary (CHO) cells inhibites cell growth (Caveda et al., 1996). Confluent HUVEC cells are incubated for 2 or 24 hours on fibrin gel, the formation of capillary is inhibited after adding VE-cadherin antibody (Bach et al., 1998). It is thought that the promotion of the intercellular interaction by VE-cadherin facilitates endothelial cell recognition, cell-to-cell adhesion, vascular integrity, and essential for the assembly.

Immunohistochemistry studies show that VE-cadherin also exists in non-endothelial cells from the placenta. As the embryo grows to the blastcyst, the surrounding cells become trophoblasts. After invading to endometrium, trophoblasts
differentiate to cytotrophoblasts. The cytotrophoblasts further differentiate into: syncytiotrophoblasts, invasive intermediate trophoblasts, and extravillous trophoblasts. Syncytiotrophoblasts with cytotrophoblasts form villi, a vascular-like structure. Extravillous trophoblasts become endovascular trophoblasts, which replace the endothelial cells in spiral arteries (Bamberger et al., 2000). VE-cadherin expresses in the intermediate and terminal human villi (Leach et al., 1993). In addition, VE-cadherin exists in cytotrophoblasts that invade to spiral arterioles (Zhou et al., 1997). Deficiency of VE-cadherin prevents organization of endothelial cells in vessel-like patterns in embryoid bodies and embryo die at early developmental stage (Vittet et al., 1997). The lack of a functional VE-cadherin gene in mice, the embryo dies at day 9.5 due to vascular insufficiency (Carmeliet et al., 1999). All data demonstrate that the presence of VE-cadherin is required for placental development.

Vascular endothelial growth factor (VEGF), a potent vascular permeability factor or angiogenic factor, is composed of two identical subunits covalently linked by disulfide bonds. It is known that the binding of VEGF to its receptors induces endothelial proliferation and capillary formation with the production of NO. VEGF increases the release of calcium from intracellular stores and then activates endothelial nitric oxide synthase (eNOS) (Petrova et al., 1999). The induction of nitric oxide (NO) by VEGF can activate MAP kinase and increases endothelial proliferation (Shizukuda et al., 1999). In the presence of VEGF, the suppression of NO/cGMP production reduces endothelial proliferation and capillary formation (Papapetropopulos et al., 1997). Therefore, the NO/cGMP pathway is involved in VEGF-induced capillary formation and vessel remodeling in endothelial cells. In the placenta, VEGF and VEGFR-1 can be detected in trophoblast cells by immunolocalization and VEGFR-2 mRNA can be detected in trophoblasts by RT-PCR (Ahmed et al., 1997). VEGF<sup>−/−</sup> embryos dies in utero between day 11 and day 12 (Carmeliet et al., 1996). Mouse embryos homozygous from a targeted mutation in the VEGFR-1 locus dies in utero between day 8.5 and day 9.5 (Fong et al., 1995). Embryos in lack of VEGFR-2 gene become lethality on day 8.5 and day 9.5 (Shalaby et al., 1995). These evidences suggest that VEGFR-1 and VEGFR-2 play an important role in vasculogenesis, angiogenesis, and placentation. However, it has not been reported regarding the regulation of VE-cadherin by VEGF in placental tissues. Therefore, the purpose of this study was to examine whether VEGF increased the expression of VE-cadherin through a NO-dependent pathway.

<Results>

Objective 1 to characterize the expression profile of VE-cadherin during pregnancy. Fresh placental tissues were taken from pregnant rats on G14, G18, and
G21. Upper panel in Fig 1 shows Western blotting data of VE-cadherin (130 kD) and β-actin (43 kD) during pregnancy. Lower panel in Fig 1 shows the quantitative graph of VE-cadherin during gestation. The relative expression of VE-cadherin/ β-actin in the G14 placenta was expressed as 100 %. When normalized with the G14 placenta, the relative expression in G18 placenta was 57.2±16.3 %, and that in the G21 placenta was 23.9±8.9 %. When compared with the G14 placenta, the G18 and G21 placenta exhibited significantly lower level of VE-cadherin protein (p<0.05, n=6), and the G21 placenta exhibited the lowest level of VE-cadherin. In order to confirm the result of Western blot, we use immunoprecipitation (IP) analysis to examine the protein abundance of VE-cadherin again in Fig 2. (A) Shows a result of Western blot after IP. The band at 130 kD is VE-cadherin protein and lower band at 50 kD is non-specific protein for IgG. (B) Shows the relative expression of VE-cadherin/ non-specific protein during pregnancy. The expression of VE-cadherin in the G14 placenta was expressed as 100 %. When normalized with the G14 placenta, the G18 placenta was 70.38 % and the G21 placenta was 28.55 %.

**Objective 2 to characterize the expression profile of eNOS during pregnancy.**

Fresh placental tissues were taken from pregnant rats on G14, G18, and G21. Upper panel in Fig 2 shows Western blotting data of eNOS (140 kD) and β-actin (43 kD) during pregnancy. Lower panel in Fig 2 shows the relative quantitative graph of eNOS during gestation. The relative expression of eNOS/ β-actin in the G14 placenta was expressed as 100 %. When normalized with the G14 placenta, the relative expression in the G18 placenta was 72±7.5 %, and that in the G21 placenta was 55.7±7.8 %. When compared with the G14 placenta, the G18 and G21 placenta exhibited significantly lower level of eNOS protein (p<0.05, n=6).

**Objective 3 to study the dose-effect of VEGF on VE-cadherin expression by Western blot and immunoprecipitation analysis.**

Placental explants taken from G18 rats were treated with various doses of VEGF: 0, 0.01, 0.1 and 1 ng/ml. Upper panel in Fig 3 showed Western blotting data, VE-cadherin (130 kD protein) and β-actin (43 kD protein). Lower panel in Fig 3 showed VEGF at 0.01 ng/ml increased the expression of VE-cadherin. When VE-cadherin in the vehicle-group was expressed as 100%, that in the 0.01 ng/ml -group was 124.3±5.91%, that in the 0.1 ng/ml -group was 113.63±5.26%, and that in the 1.0 ng/ml -group was 114.75±8.8% (*p<0.05, n=6). In order to confirm the result of Western blot, we use immunoprecipitation (IP) analysis to examine the protein abundance of VE-cadherin again in Fig 4. (A) Shows a result of Western blot after IP. Upper land is VE-cadherin protein and lower land is non-specific protein. (B) Shows the relative expression of VE-cadherin/ non-specific protein. When VE-cadherin in
the vehicle-group was expressed as 100%, that in the 0.01 ng/ml -group was 108%, that in the 0.1 ng/ml -group was 105%, and that in the 1.0 ng/ml -group was 148%.

Objective 4 to examine the effect of NO modulators on VE-cadherin expression. Placental explants from G18 rats were treated with sodium nitroprusside (SNP, NO donor, $10^{-5}$ M) and L-arginine (NOS substrate, $10^{-4}$ M), respectively. Upper panel in Fig 5 showed Western blotting data of VE-cadherin (130 kD protein) and β-actin (43 kD protein). Lower panel in Fig 8 showed SNP and L-arginine increased the expression of VE-cadherin. When VE-cadherin in the vehicle-group was expressed as 100%, that in the SNP-group was 280 ± 41.62%, and that in the L-arginine-group was 231 ± 28.77% (*p<0.05, n=5). In order to confirm the result of Western blot, we use immunoprecipitation (IP) analysis to examine the protein abundance of VE-cadherin again in Fig 6. (A) Shows a result of Western blot after IP. The upper band at 130 kD is VE-cadherin protein and the lower band at 50 kD is non-specific protein for IgG. (B) Shows the relative expression of VE-cadherin/ non-specific protein. The expression of VE-cadherin in the vehicle-group was expressed as 100%. When normalized with the vehicle-group, the SNP-group was 335%, the L-NAME group was 113%, and L-Arg-group was 138%. After cultured for 24 hours in vitro, the medium were collected for measuring NO production by a NOA 280 model. Fig 10 showed SNP and L-arginine increased NO production. When the NO production in the vehicle-group was expressed as 100%, in the SNP-group was 117 ± 6.11%, and in the L-arginine-group was 118 ± 4.77% (*p<0.05, n=5).

Objective 5 to examine the reversal effect of L-NAME on the induction of VE-cadherin by VEGF. Placenta explants were taken from G18 rat. VEGF (0.1 ng/ml), L-NAME (NO inhibitor, $10^{-3}$ M), and L-arginine (NOS substrate, $10^{-4}$ M) were used. Upper panel in Fig 7 showed Western blotting data of VE-cadherin (130 kD protein) and β-actin (43 kD protein). Lower panel in Fig 7 showed VEGF increased the expression of VE-cadherin and L-NAME decreased the increase of VE-cadherin by VEGF. In addition, L-arginine reversed the effect of L-NAME on the expression of VE-cadherin. When VE-cadherin in the vehicle group was expressed as 100%, that in VEGF-group was 148.25 ± 20.26%, that in the VEGF/L-NAME-group was 66 ± 13.69%, and that in the VEGF/L-NAME/L-arginine-group was 117.5 ± 8.07% (p<0.05, n=4). In order to confirm the result of Western blot, we use immunoprecipitation (IP) analysis to examine the protein abundance of VE-cadherin again in Fig 8. (A) Shows a result of Western blot after IP. The upper band at 130 kD is VE-cadherin protein and the lower band at 50 kD is non-specific protein for IgG. (B) Shows the relative expression of VE-cadherin/ non-specific protein. When
VE-cadherin in the vehicle-group was expressed as 100%, that in VEGF-group was 125%, that in the VEGF/L-NAME-group was 113%, and that in the VEGF/L-NAME/L-arginine-group was 138%.

<Discussion>

Even though the mechanism for villous formation in the placenta is not well understood, like capillaries, the functional role of placental villi is to exchange nutrients and transport metabolites. During embryogenesis, capillary formation is divided to two stages: vasculogenesis and angiogenesis. Vasculogenesis occurs in the early stage when mesenchymal cells differentiate to endothelium-like cells (angioblasts) and then form primitive capillaries. Angiogenesis occurs in the late stage when new capillaries outgrow from pre-existing capillaries. Since the villous wall is consisted of cytotrophoblasts surrounded by syncytiotrophoblasts, villous formation in the placenta requires the interaction between cytotrophoblasts and syncytiotrophoblasts. After implantation, cytotrophoblasts become syncytiotrophoblasts, invasive intermediate trophoblasts, and extravillous trophoblasts. Syncytiotrophoblasts with cytotrophoblasts form terminal villi, a vascular-like structure. Extravillous trophoblasts become endovascular trophoblasts, which replace the endothelial cells in spiral arteries (Benirschke et al., 1990). VE-cadherin expresses in the intermediate and terminal human villi (Leach et al., 1993). In addition, VE-cadherin exists in cytotrophoblasts that invade to spiral arterioles (Zhou et al., 1997). The current working model of angiogenesis includes three phases: initiation, progression, and maturation-termination. VEGF induces angiogenesis through various mechanisms. In the initiation phase, VEGF alters morphology of the quiescent endothelial cells by inducing the expression of $\alpha_v\beta_3$-integrin, loosens their intercellular junctions by phosphorylating VE-cadherin, and modifies the arrangement of extracellular matrix by increasing the release of matrix metalloproteinases (MMP). Thus, VEGF can activate endothelial cells by changing their proteolytic activity (Pepper et al., 1998) and stability with adjacent cells (Suarez et al., 2001). In the progression phase, VEGF triggers proliferation, migration, and assembly of ECs (Takahashi et al., 1998; Ratajska et al., 1995). In the maturation-termination phase, ECs no longer proliferate.

The appearance of ve-cadherin on the cleft of inter-endothelial junctions is related to VEGF-mediated survival and capillary formation (Carmeliet et al., 1999). This study was to further examine the action mechanism of VEGF on placental VE-cadherin. Our data in Fig. 3 and 4 indicate that in vitro treatment with VEGF (1 ng/ml) induced the expression of placental VE-cadherin protein. Even though L-NAME did not suppress the VEGF-induced expression of VE-cadherin, L-arginine further increased the VEGF-induced expression of VE-cadherin (Fig. 7 and 8). The data suggest that the induction of VE-cadherin by VEGF may be related to NO-mediated pathways. Because the increase of NO by L-arginine and SNP increased the expression of VE-cadherin, the NO-mediated signaling pathway may influence the expression of VE-cadherin.

Fig. 1 shows the decrease of VE-cadherin protein over gestation. Fujimoto et al. (1998) reported that estrogen transiently decreases in VE-cadherin protein and its mRNA leading to increase vessel permeability in HUVEC cells. As we know, the growing fetus requires more metabolite exchange with the progression of pregnancy. To fulfill the fetus need, the placenta undergoes morphological changes including the increased number of terminal villi and the decreased thickness of villous wall. Relative to the increase in the size of the placenta, the decrease in VE-cadherin protein abundance may be related to the increase in villous permeability for promoting placental materials exchange. However, it is not clear how to down-regulate the expression of VE-cadherin protein in late-gestation when the expression of VEGFR-1 on G18 is relative higher than that on G14. Thus, our study focuses on the possible mechanism for the regulation of VE-cadherin protein by
VEGF. The data in Fig 3 show the expression profile of placental eNOS during pregnancy is similar to that of VE-cadherin. Kroll et al. (1998) and Shen et al. (1999) reported that the increase of eNOS protein due to the activation of VEGFR-2 by VEGF in porcine aortic endothelial cells overexpressing VEGFR-2 (PAE/VEGFR-2). Our data indicates that the protein abundance of VEGFR-2 in the G18 placenta is significantly lower than that in the G14 placenta. Torry et al. (1997) also reported that only the first trimester invasive extravillous cytotrophoblasts expressed the mRNA and protein for VEGFR-2. We found that the G18 placenta expresses lower levels of VEGF-2 and eNOS when compared with the G14 placenta. Since our data indicate that PIGF NO involves the production of in the increase of VE-cadherin, we postulate that the decrease of VE-cadherin in the G18 placenta might be related to the down-regulation of the NO-mediated signaling pathway.

Since VEGFR-1 and VEGFR-2 exist in the placenta, we may ask how VEGFR-1 and VEGFR-2 contribute to villous formation. Bussolati et al. (2001) reported that the transfection of human VEGFR-1 or VEGFR-2 into porcine aortic endothelial (PAE), PAEV-VEGFR-1 and PAEV-VEGFR-2 cells, plated on a Matrigel. When PAEV-VEGFR-1 cells were stimulated with VEGF (25 ng/ml), cells organized into complete tubular structure. VEGF-mediated tubular network was inhibited with anti-VEGFR-1 antibody. Low concentrations of VEGF (1-10 ng/ml) significantly increase NO release in PAEV-VEGFR-1 cells, but no in PAEV-VEGFR-2 cells. In contrast PAEV-VEGFR-1 cells, PAEV-VEGFR-2 cells were unable to establish a network of tubular-like structures on Matrigel when stimulated with VEGF. The induction of proliferative activity by VEGF in HUVECs was inhibited by anti-VEGFR-2 antibody, but not by anti-VEGFR-1 antibody. In addition, the increase of endothelial proliferation by VEGF (10-50 ng/ml) can be further enhanced by anti-VEGFR-1 antibody. These data suggest that the activation of VEGFR-1 induces the network of tubular-like structures and NO production but decreases VEGFR-2 induced endothelial proliferation. In other words, the higher concentrations of VEGF (>10 ng/ml) through a VEGFR-2-mediated pathway influence cell proliferation but the lower concentrations of VEGF (< 10 ng/ml) through a VEGFR-1-mediated pathway contributes to tube formation. Torry et al. (1997) also reported that only the first trimester invasive extravillous cytotrophoblasts expressed the mRNA and protein for VEGFR-2. Current findings suggest that the presence of VEGFR-2 in the first half of pregnancy is associated with cytotrophoblast proliferation and the presence of VEGFR-1 in all stages of pregnancy is associated with villous formation.

References


Fig 1. The effect of pregnancy on the expression of VE-cadherin. Placental tissues were taken from rats on G14, G18 and G21. Upper panel shows the result of Western blot and lower panel shows the quantitative result. When compared with the G14 placenta, the G18 and G21 placenta exhibited significantly lower level of VE-cadherin. The different symbol between two groups indicates significant difference. (p<0.05, n=6)
Fig 2. The effect of pregnancy on the expression of eNOS. Placental tissues were taken from rats on G14, G18 and G21. Upper panel shows the result of Western blot and lower panel shows the quantitative result. When compared with the G14 placenta, the G18 and G21 placenta exhibited significantly lower level of eNOS. The different symbol between two groups indicates significant difference (p<0.05, n=6)

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<th>Gestation days</th>
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Relative expression (% of G14)
Fig 3. The dose-effect of VEGF on the expression of VE-cadherin. Placental explants were taken from G18 rats and were treated with various doses of VEGF: 0, 0.01, 0.1 and 1 ng/ml. Upper panel shows the result of Western blot and lower panel shows the quantitative result. When compared with the vehicle (0 ng/ml), VEGF at 0.01 ng/ml increased VE-cadherin expression (p<0.05, n=6).
Fig 4. The dose-effect of VEGF on VE-cadherin expression by immunoprecipitation (IP). Placental explants were taken from G18 rats and were treated with various doses of VEGF: 0, 0.01, 0.1 and 1 ng/ml. (A) shows a result of Western blot after IP. (B) shows the quantitative result. C as a negative control did not contain VE-cadherin antibody precipitate.
Fig 5. The effect of NO modulators on the expression of VE-cadherin. Placental explants from G18 rats were treated with SNP (NO donor, 10^{-5} M) and L-arginine (NOS substrate, 10^{-4} M), respectively. Upper panel shows the result of Western blot and lower panel shows the quantitative result. When compared with the vehicle, L-Arg and SNP induced greater amount of VE-cadherin. (*p<0.05, n=5)
Fig 6. The effect of NO modulators on the expression of VE-cadherin by immunoprecipitation (IP). Placental explants from G18 rats were treated with SNP (NO donor, 10^{-5} M), L-arginine (NOS substrate, 10^{-4} M), and L-NAME (NO inhibitor, 10^{-3} M), respectively. (a) showed a result of IP. (B) showed the quantitative result. SNP and L-arginine induced greater amount of VE-cadherin. C as a negative control did not contain VE-cadherin antibody precipitate.
Fig 7. Reversal effect of L-NAME on the induction of VE-cadherin by VEGF. Placental explants were taken from pregnant rats on G18. L-NAME (NO inhibitor, 10^(-3)M) was used. Upper panel shows the result of Western blot and lower panel shows the quantitative result. When compared with the vehicle, VEGF at 0.1 ng/ml significantly increased the amount of VE-cadherin. L-Arg reversed the effect of L-NAME on the expression of VE-cadherin. The different symbol between two groups indicates significant difference. (p<0.05, n=4)
Fig 8. Reversal effect of L-NAME on the induction of VE-cadherin by VEGF (0.1 ng/ml) by IP. Placental explants were taken from G18 rats. L-NAME (NO inhibitor, 10^{-3}M) was used. (A) shows a result of Western blot after IP. (B) shows the quantitative result. C as a negative control did not contain VE-cadherin antibody precipitate. C2 as a negative control in the absence of sample.