行政院國家科學委員會補助專題研究計畫成果報告

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黏合蛋白質與胎盤血管功能的相關及與子癲前症發生機轉之探討

Integrins and placental vascular function: implications for the pathogenesis of preeclampsia

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計畫編號：NSC－90－2314－B－002－450
執行期間：90　年　8 月　1 日 至　91　年　7 月　31 日

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中　華　民　國　91 年　10　月　15　日

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一、中文摘要

子癲前症為人類妊娠之嚴重合併症，約發生於 4-5%的所有懷孕, 目前仍為母親與胎兒致死與罹病率之首要原因。目前致病機轉仍不完全清楚，只知大略可分為兩個致病階段：懷孕初期的胎盤的不完美的發育，以及懷孕後期全身血液循環的不良適應；但目前認為最主要機轉仍在於滋養層細胞侵襲能力受阻以及母體子宮螺旋動脈的血管轉型重塑出了問題。

在人類排卵後三週，增殖中的滋養層細胞由絨毛端侵入融合滋養層細胞層，而形成滋養層細胞殼並與母體組織相接觸。在胎兒母親介面處的滋養層細胞亦開始侵入母體子宮螺旋動脈的血管管腔內，隨後子宮螺旋動脈血管內的滋養層細胞逐漸破壞並取代血管的內皮細胞，同時血管週圍的平滑肌細胞亦逐漸被瓦解，使得被轉型重塑的血管不受血液中血管收縮因子的影響，而允許更多的充氧血可進入絨毛間隙以營養胎兒。目前關於如何誘發子宮螺旋動脈血管內的滋養層細胞逆流而上，以及這些細胞如何達成血管轉型重塑，仍充滿許多未知的挑戰，事實上我們只知道如果血管轉型重塑不成功，往往與子癲前症與胎兒生長遲緩的發生有相關。

細胞黏合蛋白為細胞表面掌控細胞與細胞及細胞與細胞外間質黏合的一組分子，在廣泛生理層面扮演重要角色，如器官發育，組織重塑，血栓形成及白血球移動等。因細胞黏合蛋白的訊息傳導與胞內微小纖維有關，因此咸信與掌控細胞的動態頗有重大關連。

近年來由於對滋養層細胞所表現的細胞黏合蛋白的研究，對調控滋養層細胞的侵襲與血管內滋養層細胞的逆流而上的機轉露出一線曙光。在第一週產期的人類胎盤，α_6的細胞黏合蛋白主要分佈滋養層細胞的幹細胞，而分化為侵襲性滋養層細胞時，α_6的細胞黏合蛋白顯著向下調控，而$\alpha_5\beta_1$及$\alpha_1\beta_1$反之則明顯向上調控；這些資料顯示人類滋養層細胞在表現"受控制"侵襲的路徑時，明顯地與胞內黏合蛋白的互轉調控有極大關聯，不當的胞內黏合蛋白的互轉調控則推測與不正常的滋養層細胞侵襲能力有關，繼而導致子癲前症(侵襲能力低下)或絨毛膜癌(侵襲能力過多)。

雖然細胞黏合蛋白的研究透露了滋養層細胞侵襲母體組織的奧秘，然而，母體子宮螺旋動脈血管內的滋養層細胞逐漸破壞並取代血管的內皮細胞的真正機轉卻較少被研究。另外，究竟何種因子啟動滋養層細胞所表現的細胞黏合蛋白胞內的互轉調控目前亦沒有答案。我們將收集第一週產期、第三週產期正常與不正常的懷孕，尤其是子癲前症的患者，採集胎盤底部與絨毛，以免疫螢光化學反應、掃瞄式電顯、穿透式電顯、免疫電顯、以及共軛焦距螢光顯微，去研究血管內的滋養層細胞及其相關組織超微構造，去釐清黏合蛋白在滋養層細胞逐漸破壞並取代血管的內皮細胞時所扮演的角色；黏合蛋白在血管內滋養層細胞逆流而上時，會如何改變分佈型態；以及在正常和不正常的妊娠中，當黏合蛋白有表現時，其相對應的超微結構如何變化。另外過去研究顯示，在子癲前症的患者，其血管內滋養層細胞的形態會顯著縮小，在本實驗亦將檢驗是否其超微

結構、尤其是細胞極性，是否產生變化。最後，在白血球當宿主遇外在感染時，其化學驅動受一些發炎因子，如干擾素或腫瘤壞死因子α的調控，而目前已知胚胎在著床時，滋養層細胞會有腫瘤壞死因子α的表達，我們亦將檢驗腫瘤壞死因子α與血管內滋養層細胞的逆流而上與血管重塑是否有所關聯。

本計劃牽涉發育生物學、生殖生理學、細胞生物學中非常重要的課題，研究的方向與目前人類懷孕的最嚴重合併症—子癲前症又息息相關，透過此基礎研究去探索臨床醫學，相信能更進一步去探究生命之奧秘。

關鍵詞：黏合蛋白，滋養層細胞，血管功能，人類，子癲前症。

Abstract

Preeclampsia is a severe complication of human pregnancy. It occurs in 4-5% of all pregnancies and remains a leading cause of maternal and neonatal mortality and morbidity. The pathophysiology of this syndrome is not fully understood. Two stages of vascular dysfunction can be attributed. In the early pregnancy suboptimal development of the placenta and a hymodynamic maladaptation to pregnancy exist. However, the key event of this syndrome should still focus on the upstream event- defective trophoblast invasion and poor transformation of maternal spiral arteries.

During the third post-ovaulatory week, proliferating cytotrophoblast at the tips of the villi penetrates the syncytium to form a trophoblast shell and make contact with the underlying maternal tissue. Cytotrophoblast also migrates up from the trophoblast shell along the inner wall of maternal spiral arteries. The endothelium of the spiral arteries is gradually displaced by the endovascular trophoblast, and the smooth muscle layer of the vessel is also disrupted, and consequently unresponsing to the serum vasoactive factors. Although the spiral artery remodeling is crucial for the embryo survival and devlopment, its actual machenism is poorly understood. Thus far, we only knew the fact that poorly spiral artery remodeling usually result into preeclampsia and fetal growth restriction.

Integrins are a set of cell surface adhesion molecules that regulate cell-cell and cell-extracellular matrix protein interactions. They play a critical role in a wide fields of biologic processes, including organogenesis, tissue remodeling, thrombosis, and leukocyte migration. Because the integrins signaling involves the activation of actin dynamics, they are regard as the key to switch on the cell motility.

Rcently the investigation of integrins expressed by the trophoblast shed a light in the regulation of trophoblast invasion and migration. In the first trimester human placenta, α_6 integrins is mainly restricted in cytotrophoblast stem cell, and downregulated in the invasive cytotrophoblast. Whereas $\alpha_5\beta_1$ and $\alpha_1\beta_1$ integrins are upregulated in differentiating and invasive cytotrophoblasts. These data suggests human trophoblasts gain their “controlled” invasive phenotype via the pathway associated with integrins switching in their cell inegrins repertoire. Inadequet switching may lead to abnormal trophoblast invasiveness, such inadquet in preeclampsia or excessive in choriocarcinoma.

Although the investigation of integrins expression gives us a light in the study of the extravillous trophoblast invasion, the study of endovascular trophoblast is lessly understood. Besides, how and what initiates the integrins switching along the invasive pathway is also unknown. We collect the placental bed biopsy from first trimester pregnancy, uncomplicated term pregnancy and also term preeclampsia cases. We use immonohistocytochemistry to identify endovacular trophoblast, transformed spiral arteries, and the related anatomy. Efforts will be directed to integrins distribution pattern among the normal and abnormal pregnancy (esp. preeclampsia). We also use detailed electromicroscopy techniques (TEM, SEM, and IEM) to clarify the ultrastructure changes associated with the integrins expression (either normal or abnormal pregnancies). Previous investigation also indicated the endovascular trophoblst of preeclampsia pregnancy is smaller than that of normal control. We will check by TEM and IEM to check whether the epithelial polarity and the ultrastructure change or not. Finally, the expression of integrin ligands is modulated by inflammatory signals, such as interferon or tumor necrosis factor α. Current knowledge has noted that TNFα expresses in the blastocyst and also the trophectoderm. By using immunohistocytochemistry, we will also check the distribution of TNFα or other inflammatory signals and their expression of specific receptors in the microenvironment of implantation.

This project deals the important subjects of developmental biology, reproductive physiology, cell

biology, and the final goal will be directed on the severe human pregnancy complication – preeclampsia. We believe using basic researches technique to investigate the clinical disease will have important implications for the mystery of life science.

Keywords: integrins, trophoblast, vascular function, human, preeclampsia.

二、緣由與目的

The adhesive interactions between cells and the interactions of cells with extracellular matrix proteins play a role in embryonic and organ development, in host defense, and in the maintenance of vascular and epithelial integrity. The loss of adhesive interactions as well as a stimulation of adhesion may result in various disease statuses.

The integrins are a set of cell surface adhesion molecules that regulate cell-cell and cell-extracellular matrix protein interactions. They play a critical role in a wide fields of biologic processes, including organogenesis, tissue remodeling, thrombosis, and leukocyte migration. These family members are composed of two subunits, alpha (α) and beta (β), that traverse the cell membrane and are characterized by noncovalent interactions. The association of the α subunits (120 to 180 kd) and β subunits (90 to 110 kd) and the presence of divalent cations are essential for ligand binding. The integrins, now numbering more than 20, are further divided into subgroups based on their beta subunit.

Integrin-dependent adhesive interactions involve a complex set of events that include activation, ligand binding, reorganization of the cytoskeleton, and adhesion. The expression of integrin ligands is modulated by inflammatory signals, such as interferon or tumor necrosis factor TNFα. The role of integrins involving in leukocyte migration has been investigated. The chemokines released from the underlying, inflamed endothelial layer activate an intracellular adhesion cascade through the interaction with their specific receptors on the neutrophil. This intracellular signaling cascade is a complex event that results not only in activation of the integrin but also affects the cytoskeleton, so that the cell extends along the surface to strengthen the adhesion to the endothelial cell. Subsequently, the neutrophil traverses the endothelial cell wall and enters into the subendothelial space to provide the required response.

The above-mentioned steps in the activating chemotaxis of neutrophil is somewhat similar to the initiation of invasive trophoblast migrate up in a retrograde fasion to remodel the spiral artery.

三、研究方法

I. SAMPLE COLLECTION: Chorionic villi and deciduas of the first-trimester pregnancy will be obtained from women undergoing elective termination due to blighted ovum or other psycosocial problems. Third-trimester specimen will be obtained during cesarean section at term. Patients with preeclampsia were diagnosed and enrolled according to the following criteria:

(1) No history of hypertension prior to pregnancy; increase in diastolic pressure of 15 mmHg or systolic pressure of 30 mmHg compared with blood pressure obtained before 20 wk of gestation

(2) Proteinuria ≥ 1g/24 h or ≥ 30 mg/dl (or 3+ on urine dipstick) in 6 hour apart urine specimen;

(3) Presence of pathological edema (edema in the non-depedent body part, such as face, hand).

II. IMMUNOHISTOCYTOCHEMISTRY

Tissue fixation, and immunocytochemistry

The tissues are fixed/rinsed/blocking according to the standard condition described *(Cell, Volume II: Subcellular localization of genes and their products, Spector DL et al., 1998 Cold Spring Harbor Laboratory Express).*

1. Tissues sections (5μm) are dewaxed and rehydrated conventionally.
2. Quenching of endogenous peroxidase is achieved by incubation with 0.3% hydrogen peroxide in methanol for 30 mins at room temperature.
3. All tissue sections are exposed to a non-immuned block with normal rabbit serum for 30 minutes at room temperature.
4. Incubation with the primary antibodies is carried out at 4°C overnight with various dilutions for the specific antibodies.
5. Thereafter tissues sections are labeled with an avidin-biotin-peroxidase detection system Vectastain

(Vector Lab, Burlington VT, USA).

6. Each step is followed by a meticulous washing with PBS.
7. Finally, 3,3'-diaminobenzidine is used as chromogen.
8. Counterstaining was performed with hematoxylin.

For cryostat section (8-10μm), the steps are the same except skip the procedures of dewaxing and dehydration.

Antibodies

Markers for cytotrophoblasts

In chorionic villi and column portion of anchoring villi, cytokeratins are the most useful markers. Anti-pancytokeratins can stain syncytiotrophoblast, cytptrophoblast (stem cell), and also the invasive interstitial trophoblast. Nonetheless, the glandular epithelium, which thereotically regresses after 10 weeks' gestation, will be stained positive for anti-pancytokeratin. The architecture of glandular epithelium loos like transformed spiral artery with endovascular trophoblast in the lumen. We use CD56 (NCAM) as an immunomarker for the differentiation between endovascular trophoblast ($CD56^+$) and glandular epithelium ($CD56^-$).

Markers for smooth muscles component of the decidual vessels

We use phalloidin to recognize all the F-actin components in the placental bed biopsy. Phalloidin is a non-specific actin-binding toxin derived from mushroom. Either α, β, and γ forms of actin will be recognized by phalloidin. This will help to localize the decidual vessels (either vein or artery). We also use CD31 (PECAM) as a marker to localize the endothelial cells in the lumen of spiral artery.

Markers for cytokines that are putatively essential for implantation

We use a panel of antibodies to cytokines and their receptors that we intend to test the roles in the embryonic implantation.

Antibody for integrins staining

α_1	α_2	α_3	α_4	α_5	α_6	α_v
1:100	1::100	1:600	1::300	1:100	1:600	1:100

β_1	β_2	β_3	β_4	β_5	β_6	
1::200	1::100	1::100	1::400	1:100	1:100	

Immunostaining

We use fluorescein-conjugated anti-mouse IgG as secondary antibodies to label the monoclonal antibodies, and rhodamine-antimouse IgG as seconday antibodies to label the polyclonal antibodies. Other fixation, washing, incubation techiniques are followed the standard protocols (see *Cells: a laboratory manual, by Spector DL, et al., Volume 3 Subcellular location of genes and their products*).

III. Transmission electromicroscopy (TEM)

IV. Scanning electromicroscopy (SEM)

V. CONFOCAL MICROSCOPY

Tissues sections (30μm) are fixed and stained in the standard processes of immunohistocytochemistry. The post-stained slides will be examined using Zeiss confocal microscope (LSM 510). Optical sections will be collected at 0.5 micrometer. Steps through individual cell nuclei for analysis. Distribution of EGFP will be localized on the different optical sections.

四、結果與討論

The followings are illustrations of our preliminary result for the first year project.

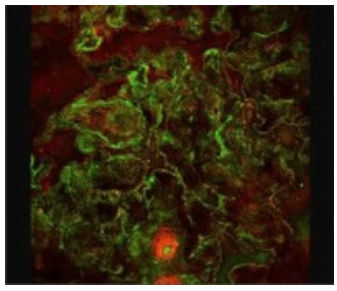


Fig.1 Confocal laser microscopy of placental bed biopsy of term pregnancy demonstrated the basal polarity of α6 integrins distribution (FITC-decorated). Rhodamine decorates the staining of actin filament.

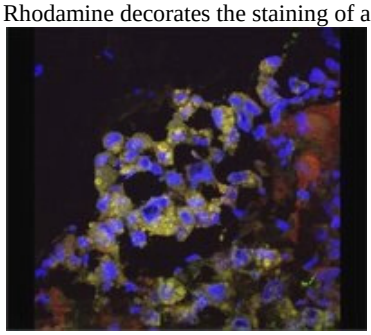


Fig.2 Confocal laser microscopy of endovascular trophoblast. It demonstrated NCAM (CD 56) positive staining of the endovascular trophoblast (FITC-NCAM; Rhodamine-actin, and DAPI triple staing).

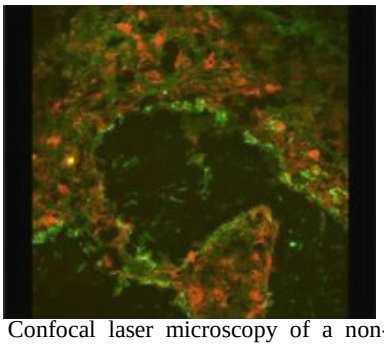


Fig.3 Confocal laser microscopy of a non-transformed spiral artery of a placental bed biopsy. (PECAM-FITC; Rhodamine-actin).

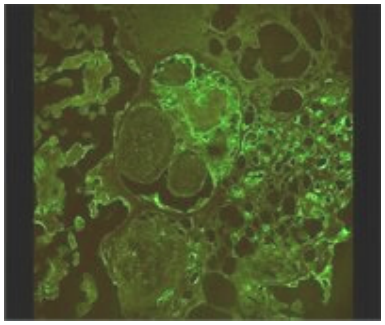


Fig. 4 In case of preeclampsia, extensive nodules formation was noted in the interface of free-floating villi and deciduas. (CK7-FITC)

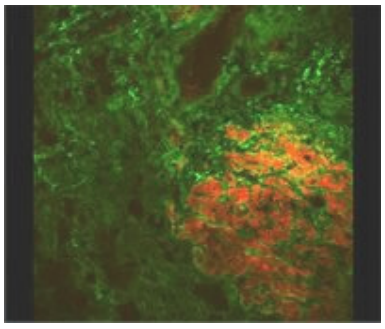


Fig. 5 In case of placenta accreta, aggregate of invasive trophoblasts was noted in the muscular layer. These extensive infiltration of trophoblast manifested strong positive staining of α6-integrin, which should never appear for the trophobalst in this area. (α6-FITC; actin-Rhodamine)

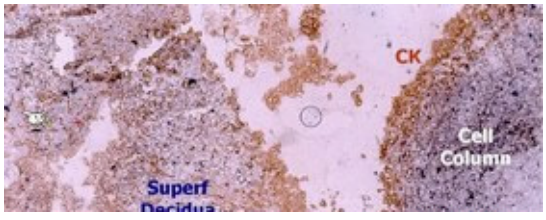
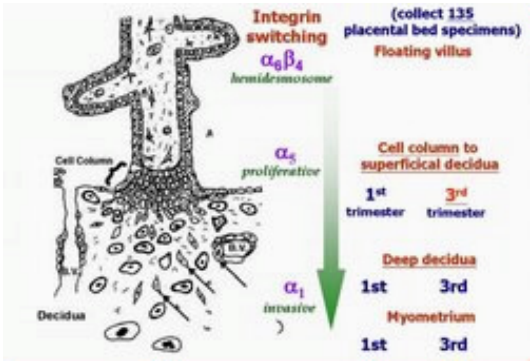


Fig. 5 Alkaline phasphotase conjugated with transforming growth factor β receptor type I (upper) and DAB-decorated CK7 staining (represents trophoblast in this area). The section was located in the interface of superficial deciduas and deeper portion of cell column. This area is remarkable for the interstitial trophoblast beginning to invade maternal stroma. This section demonstrate the downregulation of TβR1 is coexistent with integrins switching from α5 to α1.



We found the invasion of extravillous trophoblast is associated with downregulation of the receptor (both type I and II) of transforming growth factor β (TβRI and II) when these trophoblasts detach cell columns. The trophoblast invasion into deep decidua is also associated with the upregulation of TβRs of implntation environment- deep decidual tissue and myometrium. These changes occur with switching of integrins repertories. Moreover, the physiological adaptation of spiral artery are also paradoxically associated with the upregulation of TβRs of extravillous trophoblasts around the perivascular space, indicating the TGFβ is one of the key regulator of trophopblast invasion. The switching of TGFβ receptors of trophoblast is also associated the phenotype change: from unipolar motile form to multipolar, stellate shape trophoblast. It also indicates TGFβ might affect the morphology and function in the process of spiral artery remodeling.

Zhou Y et al. first indicates the integrin switching from α5 to α1 for the invasive trophoblast along their invasive pathway. Caniggia I also indicates

restoring the invasive capacity of trophoblast for pre-eclampsia by adding anti-TGFβ3 antibody. Thus integrins and TGFβ are delicately controlled in the trophoblast invasion. Our study further illustrate the integrins switching is associated with downregulation of TGFβ receptors along the invasive pathways of interstitial trophoblast. It is a novel finding that was never described in previous literature.

五、計畫成果自評

The results obtained from current project were exciting. Not only we found the role of TGFβ in implantation and associated with integrins repertoire switching, but also identified the important event in the process of spiral remodeling.

Besides, the establishment of first-trimester trophoblast culture was already successful. It will provide the materials for the sophisticated culture system that was designed for confirmation of our novel findings in the first year project.

However, the case enrollment of pre-eclampsia was not satisfied. Although we performed several placenta bed biopsy in cases of pre-eclampsia. However, negative biopsy was seen in several cases thus adequet study was not performed in the abnormal group. In the future, we will still try our best to enroll more cases of preeclampsia to understand the basic change of integrins and TGFβ in such abnormal placentation.

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