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行政院國家科學委員會專題研究計劃成果報告

氣球擴張術引起老鼠動脈血管內膜增生的研究：比較triflavin和抗$\alpha_v\beta_3$單源抗體的相對作用機轉及活性 (II) (2/2)

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主持人: 許準榕教授　　執行機構: 台北醫學大學醫學研究所

一、中文摘要

在病人施行冠狀動脈血管造形術 (percutaneous transluminal coronary angioplasty) 後，根據統計約有40-50 %的病患常會發生手術血管發生再窄化 (restenosis) 的現象；意即進行手術的部位其血管管腔減小，而使心肌的缺血現象產生最後導致心肌梗塞。一般而言，血管再窄化的發生約可分為3個主要步驟，依序為 (1) 血管內的彈性回縮作用 (elastic recoil)；(2) 血栓的形成(包括血小板的附著、凝集及釋放生長因子)與發炎細胞的侵入；(3) 血管中層平滑肌細胞的增生和移動以及細胞外基質蛋白 (extracellular matrix) 的擴張。目前有許多的動物實驗利用各種不同的藥物來試圖減少血管再窄化的發生；包括使用抗血小板凝集藥物 (如aspirin, ticlopidine), 抗凝血藥、鈣離子通道阻斷劑，降血脂藥物等等；但大多藥物經臨床試驗後發現效果卻不理想。

Triflavin為一種由出血性的蛇毒 (Trimeresurus flavoviridis) 中所分離的強效抗血小板凝集蛋白；它本身為單鍵含有70 amino acids; 在靠近C端位置含有Arg-Gly-Asp (RGD) 這三個amino acid, 在triflavin抑制血小板凝集過程中扮演了決定性的角色。Triflavin的作用機轉為競爭性的抑制fibrinogen和血小板$\alpha_{IIb}\beta_3$ integrin的結合作用，為一種專一性的$\alpha_{IIb}\beta_3$ integrin拮抗劑；因此在活體內亦能有效的防止血栓產生。在之前的研究中發現triflavin於手術一週及二週後可明顯抑制血栓的生成及血管內膜增生；同時，triflavin亦可抑制因氣球擴張術所引起的thromboxane B_2 的增加。

$\alpha_{IIb}\beta_3$ integrin本身為β_3 integrin的一員，屬於一種附著蛋白受體；它在體內參與了發育 (development)、發炎(inflammation)與血栓(thrombosis)等等。在血管再窄化的過程中，有關血管平滑肌的附著和移動；內皮細胞與細胞外基質蛋白的結合，此$\alpha_{IIb}\beta_3$ integrin都扮演了相當重要的角色。因此本計畫的主要目的，在探討abciximab (anti-$\alpha_{IIb}\beta_3$ integrin單源抗體)對於氣球擴張術後引起大白鼠頸動脈血管內膜增生的抑制作用以及其作用機轉的探討。

關鍵詞: 氣球擴張術，血管內膜增生，$\alpha_{IIb}\beta_3$，abciximab

Abstract

Recent large-scale trials of percutaneous transluminal coronary angioplasty (PTCA) have emphasized the frequency of stenosis recurrence about 40-50 % of patients with percutaneous treatment. Restenosis is the decrease of the vessel lumen at the site of the procedure, thereby leading to induce ischemia. The major steps in the development of restenosis are: (1) elastic recoil; (2) subclinical development of

thrombosis (platelet adhesion, aggregation, and release of growth factors) with inflammatory cell infiltration, and (3) medial smooth muscle cell proliferation and migration, followed by extracellular matrix expansion. There are numerous agents have been tested in animal models, such as antiplatelet drugs (aspirin, ticlopidine); antithrombotic drugs (hirudin, heparin); calcium-channel blockers; immunosuppresive agents, hypolipidemic agents; until recently none has translated into benefit in large-scale clinical trials.

Triflavin, a potent platelet aggregation inhibitor, was purified from the venom of *Trimeresurus flavoviridis*. Its sequence contains the Arg-Gly-Asp (RGD) in the carboxyl terminal domain. The RGD sequence of triflavin plays an important role in mediating the binding of triflavin towards glycoprotein IIb/IIIa complex ($\alpha_{IIb}\beta_3$ integrin). Triflavin inhibits platelet aggregation by interfering with the interaction of fibrinogen with the $\alpha_{IIb}\beta_3$ integrin. It is an effective agent in the prevention of thromboembolism. In our previously described, triflavin significantly inhibited neointimal hyperplasia and lowering the increased of thromboxane A_2 formation after balloon angioplasty in rat carotide arteries.

$\alpha_{IIb}\beta_3$ belongs a β_3 integrin family, involves in cell development, inflammation and thrombosis. The $\alpha_{IIb}\beta_3$ integrin is thought to play a major role in the adhesion and migration of smooth muscle cells and endothelial cells on extracellular matrices.

The present project was designed to determine the inhibitory mechanisms of abciximab (anti-$\alpha_{IIb}\beta_3$ integrin monoclonal antibody) in neointimal hyperplasia of balloon injured rat carotid arteries angioplasty.

Key words: balloon-induced injury, neointimal hyperplasia, $\alpha_{IIb}\beta_3$, abciximab

二、緣由與目的

冠狀動脈血管造形術(percutaneous transluminal coronary angioplasty, PTCA)自1977年來被廣泛地使用在治療缺血性心臟疾病上(Gruentzig et al., 1979)。心肌作功所需的氧氣，主要是由左右冠狀動脈所提供，若冠狀動脈發生栓塞或是其他原因造成血流供應不足的情況下，心肌就會發生短暫或永久性的缺血現象，稱為心肌缺血(myocardial ischemia)。若缺血的情況未改善，形成持續性的缺血，則會演變成心肌梗塞 (myocardial infarction)，進一步會促使心肌壞死而失去功能。 因此臨床上常施行冠狀動脈血管造形術，以疏通阻塞的血管，使心臟能恢復正常的循環血流。然而在施行冠狀動脈血管造形術後，根據統計約有40~50%的病患常會發生手術部位的血管再窄化(restenosis)的現象產生；即進行手術的部位血管管腔減少，使心肌的缺血現象再度發生，導致心肌梗塞的復發。因此，如何避免或預防在施行冠狀動脈血管造形術後的血管不再發生再窄化的現象，是目前許多學者專家共同關注的課題。

在施行冠狀動脈血管造形術時，由於導管前端的氣球受充氣而擴張，對造成阻塞的纖維血塊及血管壁產生一個牽扯(stretch)的力量，此牽扯的力量對血管壁會造成一個機械性的傷害，使血管壁內皮層(endothelial layer) 受損，內皮下層的基質蛋白會暴露出來；當導管前端的氣球消氣時，先前被擴張的血管壁則彈回，血管管腔反而較原先的管腔為小，稱為彈性回縮(elastic recoil) (Rensing et al., 1990)。接著當血管壁受傷後，含有膠原蛋白 (collagen)的內皮下層會暴露出來，提供一個血栓生成的表面 (thrombogenic surface)，接著使得血小板在此表面上附著及堆積。在血管受傷處由於內皮下層中特別是膠原蛋白的作用，一方面使血小板附著，另一方面則直接活化血小板，使血小板細胞膜上的醣蛋白 IIb/IIIa 受體 (glycoprotein IIb/IIIa receptor, $\alpha_{IIb}\beta_3$ integrin)暴露出來，接著血液中纖維蛋白原(fibrinogen)接合上去，而引起血小板的凝集及釋放反應 (Fukami et al.,

1977；Plow et al., 1981)。血小板的釋放反應會釋放出大量的促凝血物質(procoagulants)，血管收縮因子(vasoconstrictive factors)及細胞成長物質(mitogenic substances)；包括thrombin、血小板生長因子PDGF (platelet-derived growth factor), thromboxane A_2, serotonin, von Willebrand factor, ADP, fibronectin, Factor V及fibrinogen等；因此而促進血管管腔內血栓的形成 (Ip et al., 1991)。其中thrombin對於血管再窄化過程中的血栓形成及細胞增生扮演著相當重要的角色。Thrombin會促使纖維蛋白原轉變為纖維蛋白，並使血管平滑肌細胞產生plasminogen activator inhibitor (PAI) 及PDGF，以促使血管平滑肌細胞的增生、移動及細胞外基質蛋白的生成 (extracellular matrix production) (Okazaki et al., 1992)。Thrombin同時也會增加內皮細胞E- 及P-selectin的表現，使單核球 (monocytes) 及嗜中性白血球 (neutrophils) 結合到內皮細胞，使其移動並進入受傷的血管壁內；而活化的血小板會表現P-selectin, 藉此受體與白血球 (leukocytes)結合，而一起移入血塊中(Sugama and Malik, 1992)。

血管內膜受損後引起再窄化的最後一個步驟為新生血管內膜的增生作用(neointimal proliferation)，此步驟最明顯的作用為在受傷的血管處其平滑肌細胞的增生作用及細胞外基質蛋白的合成作用;因這二點而導致受傷血管的管腔窄化。首先血管內的血栓塊與一些生長因子，如basic fibroblast growth factor (bFGF) (目前已知最強的平滑肌細胞刺激因子)共同合作促使平滑肌細胞大量增生；同時亦會誘導平滑肌細胞從血管的中層 (media) 往內層(intima) 移動；共同催化此作用者尚有如insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF), transforming growth factor (TGF) 和其他的cytokines (Casscells, 1992)。其中IGF-I大量的儲存在血小板的α-granules內；同時血管的平滑肌細胞及內皮細胞當受到刺激時亦會被釋放出來 (Delafontaine et al., 1991)。由目前的許多動物實驗證實，在血管平滑肌細胞的移動過程中， PDGF扮演一個非常重要的角色(Ferns et al., 1991)。

目前有許多研究利用各種不同的藥物來減少血管內膜的增生，包括使用抗血小板凝集藥物，抗凝血藥物 (如heparin, hirudin)，鈣離子通道阻斷劑(calcium-channel blockers)，血管加壓素轉換蛋白抑制劑，免疫抑制劑，抑制細胞生長的藥物 (antimitogenic agents)，降血脂藥物，生長因子抑制劑及antisense molecular therapy等。但大多藥物效果都不理想。如經證實低分子量的heparin對於預防血管內膜的增生並無顯著的效果 (Serruys et al., 1994)。鈣離子阻斷劑diltiazem, nifedipine臨床顯示對於防止血管再窄化的現象並無正面的效果。再者，如corticosteroids可以抑制單核球與淋巴球之間的作用以及淋巴球的增生；因此可減少淋巴球生長因子(macrophage-derived growth factor)的表現(Berk et al., 1991)。然而，臨床試驗卻發現這類corticosteroids 無法有效減輕血管再窄化現象(Pepine et al., 1990)。此外，angiopepsin 雖可抑制IGF-I及 βFGF的釋放，但在臨床上並無減輕血管再窄化的效果 (Kent et al., 1993)。所以到目前為止並沒有任何一種抗細胞增生或免疫抑制劑在臨床上被證實可以減輕血管再窄化的現象。

由於冠狀動脈血管造形術會破壞血管內皮細胞的完整性，使血液接觸到內皮下層，進行一連串的反應如血小板附著、活化與凝集反應。當血小板附著到受傷的血管壁後，細胞內的granules會釋放出ADP, serotonin, thromboxane A_2, fibrinogen, fibrinonectin 及von Willebrand factor (vWF)等；這些物質會再進一步去活化鄰近的血小板，使血小板凝集，最後形成血栓(thrombus)。血小板的凝集主要是藉由其細胞膜表面上的醣蛋白IIb/IIIa ($\alpha_{II}\beta_3$ integrin)受體的作用而來 (Calvete, 1994)。當血小板受到活化時，血小板的醣蛋白IIb/IIIa受體的結構會改變，而使fibrinogen靠著其分子上的Arg-Gly-Asp (RGD) 序列接合上去而造成血小板凝集 (Plow et al., 1992); 而血

小板也會靠著其細胞膜上的 $\alpha_v\beta_3$-integrin 受體與細胞外基質蛋白如 vitronectin 結合，而使血小板附著到血管內膜，而進一步促使增生的反應發生。

由於血小板的附著、活化及凝集對於血管內膜的增生扮演一個相當重要的角色；而血小板的作用又必需藉由其細胞膜表面上的醣蛋白IIb/IIIa受體 ($\alpha_{IIb}\beta_3$ integrin) 來表現。 近年來的動物實驗顯示，經由抑制 $\alpha_{IIb}\beta_3$ 或 $\alpha_v\beta_3$ integrin受體，可達到減少血管內膜增生的作用(Matsuno et al., 1994)。最近，Coller (1985)發展出一種 $\alpha_{IIb}\beta_3$ integrin的拮抗劑，單源抗體7E3 (abciximab); 臨床上發現能有效的預防血栓及血管再窄化現象 (Topol et al., 1994)；但其詳細的作用機轉則上不清楚，因此本計劃擬進一步去探討其抑制血管再窄化的作用機轉。

三、結果與討論

研究結果顯示，將大白鼠頸動脈施行氣球擴張術後會造成血管內皮細胞明顯受損，進而引起血栓生成與血管新內膜的過度增生 (Figure 1)；將動物分別於手術後 7, 14 天經灌流固定，將受傷頸動脈取下，於光學顯微鏡下觀察，並經由電腦影像分析系統 (Image-Pro Plus)，進行組織分析。結果顯示，血管新內膜增生的程度會隨著時間的增加而有 time-dependent 的增加效應 (Figure 1)。經由電腦影像分析系統將受傷血管厚度及血管管腔的變化情形加以量化，以表示血管新內膜過度增生的情形 (Figure 2a) 以及血管管腔阻塞的程度 (Figure 2b)。血管經氣球擴張術破壞內皮細胞後，血管新內膜增生會隨時間的增加(0, 7, 14 天)而有逐漸增厚的趨勢 (ratio: 1.1-, 1.9-, 4.4-fold) (Figure 2a)。但在血管受傷後血管管腔受阻塞的程度，則在第一週就達到最高點，其後的三週 （第四週） 並未明顯有再增加的趨勢 (0, 7, 14 天) (4.3%, 82.1%, 91%) (Figure 2b)。由光學顯微鏡下觀察組織，得知在血管受傷初期 (一週)，阻塞血管的物質為大量的血栓形成 (Figure 1)；而受傷後期 (2 週以上) 造成阻塞血管的原因則被血管新內膜的過度增生所取代 (Figure 1)。

在進行氣球擴張術破壞血管內皮細胞後，利用內置式迷你滲透壓幫浦持續給與抗血小板凝集藥物abciximab (0.25 mg/kg/d) 7到 14 天，觀察abciximab對血栓生成、血管新內膜增生及血管管腔阻塞的影響。研究結果顯示在光學顯微鏡下觀察，發現在連續給與 abciximab一及二週後，血管受傷處不論是血小板凝集、血栓生成與新內膜增生的情形相對於未投藥的控制組有顯著的抑制作用 (Figure 2a)。經電腦影像分析系統加以量化後，發現在一週及二週後，在投與0.25 mg/kg/d的劑量下abciximab明顯抑制血管新內膜的過度增生；在血管管腔阻塞的情形方面，在投與abciximab一週及二週後相對於施行手術的控制組而言abciximab均能有效的預防血管的阻塞 (Figure 2b)。由本研究成果顯示，abciximab確實能明顯抑制血管新內膜增生反應。另一方面；進行氣球擴張術破壞血管內皮細胞後，會導致血液中nitrate及cyclic GMP的含量明顯的增加(Table 1,2)；在投與abciximab一週及二週後相對於施行手術的控制組而言abciximab並不能有效的抑制兩者的增加 (Table 1, 2)。反之，在進行氣球擴張術破壞血管內皮細胞後，我們發現，血漿中的thromboxane B_2 的合成明顯的增加 (Table 1)；在投與abciximab一週及二週後，我們發現，血漿中的thromboxane B_2 的合成明顯的被abciximab所抑制(Table 1)；另外，進行氣球擴張並不會使得血漿中的cyclic AMP產生明顯的變化(Table 2)。由本計畫研究發現，abciximab的確可抑制氣球擴張術所引起的新內膜增生反應，且其作用機轉可能與抑制血小板合成thromboxane B_2有關。

四、計畫成果自評

本研究計劃目前已達當初申請計劃時的內容；且本結果亦已投稿在國際著名學術期刊。因此，我們算是達到當初所設定

的目標。

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Table 1. Effects of Abciximab on Thromboxane B_2 and Nitrate Formation in Plasma after Balloon Angioplasty of Rats

Group	Nitrate (μM)	Thromboxane B_2 (ng/ml)
Sham	9.8 ± 1.2	0.27 ± 0.06
Angioplasty		
+ SAL (7 days)	15.0 ± 1.3**	2.56 ± 0.15***
+ SAL (14 days)	21.3 ± 1.9***	2.97 ± 0.43***
+ Abciximab (7 days)	16.5 ± 1.4**	0.84 ± 0.19#
+ Abciximab (14 days)	18.7 ± 1.6***	0.93 ± 0.17#

Rats were treated with abciximab (0.25 mg/kg/d) or isovolumeric normal saline (SAL) for 7 and 14 days after balloon angioplasty, then the plasma nitrate and thromboxane B_2 concentrations were determined as described in "Materials & Methods". Data are presented as means ± S.E.M. (n = 6). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with sham-operated rats; $^{\#}P < 0.001$ compared with normal saline-treated rats.

Table 2. Effects of Abciximab on Cyclic AMP and Cyclic GMP Formation in Plasma after Balloon Angioplasty of Rats

Group	Cyclic GMP	Cyclic AMP
	(pmol/ml)	
Sham	9.7 ± 1.3	48.5 ± 7.4
Angioplasty		
+ SAL (14 days)	16.5 ± 1.9*	41.8 ± 6.5
+ Abciximab (7 days)	15.9 ± 2.5*	54.1 ± 9.1
+ Abciximab (14 days)	20.4 ± 3.7*	59.5 ± 8.7

Rats were treated with abciximab (0.25 mg/kg/d) or isovolumeric normal saline (SAL) for 7 and 14 days after balloon angioplasty, then the plasma cyclic AMP and cyclic GMP concentrations were determined as described in "Materials & Methods". Data are presented as means ± S.E.M. (n = 6). *$P <$ 0.05 compared with sham-operated rats.

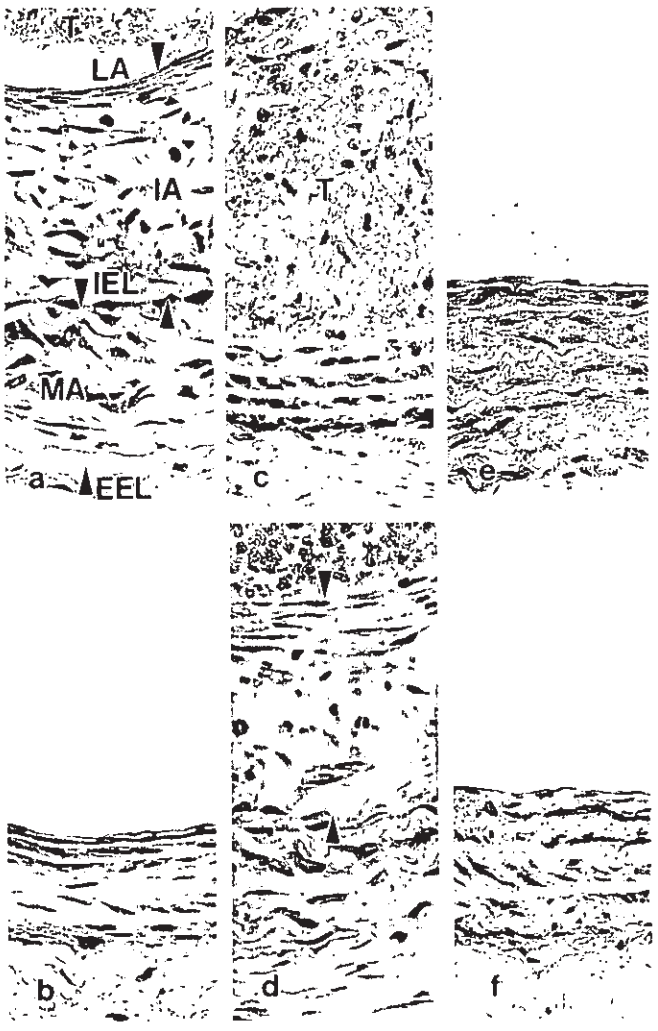

Fig. 1. Microphotographs showing a (a) cross-section of rat carotid arteries 14 days after balloon angioplasty. The intimal area (IA) is formed by the neointima. The medial area (MA) is encircled by the internal elastic lamina (IEL) and external elastic lamina (EEL). Note that the luminal area (LA) is filled with thrombus formation (T). (b) Sham-operated, (c) normal saline treatment for 7 days, (d) 14 days, and abciximab treatment for (e) 7 days and (f) 14 days after balloon angioplasty. Note that obvious neointimal hyperplasia (arrowheads) has developed 14 days after balloon injury.

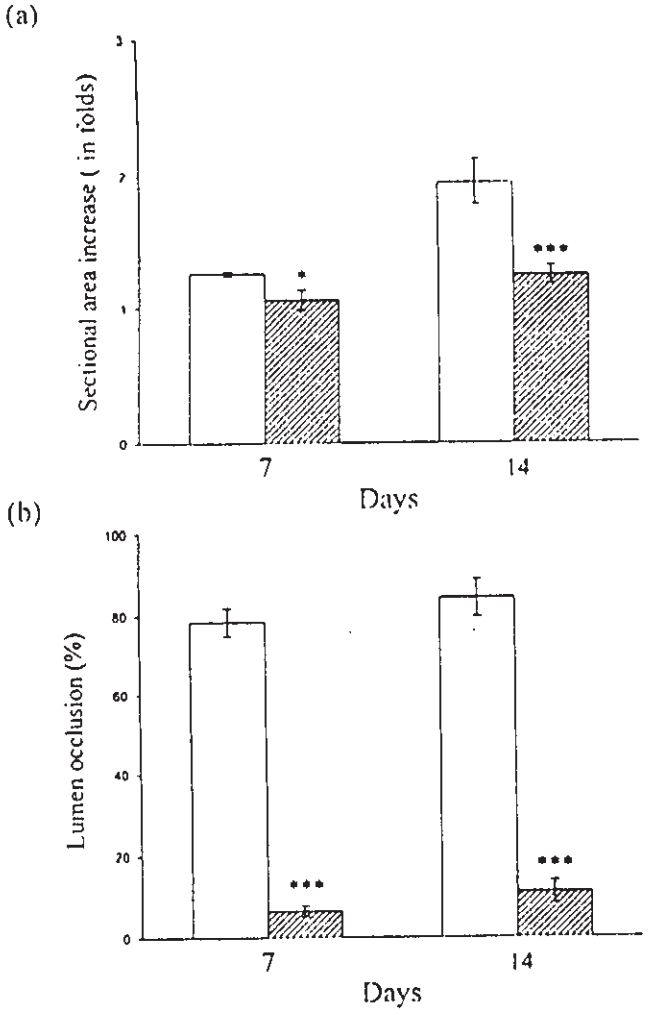

Fig. 2. Inhibitory effects of abciximab on (a) sectional area increase and (b) lumen occlusion after balloon angioplasty of rats. Normal saline (open bars) or abciximab (0.25 mg/kg/day, hatched bars) was infused intravenously for 7 and 14 days after angioplasty of the rat, then neointimal hyperplasia and lumen occlusion of rat carotid arteries were determined as described in Materials and Methods. Data are presented as means ± S.E.M. ($n = 8$). * $P < .05$ and * * * $P < .001$ compared with normal saline-treated rats.