行政院國家科學委員會專題研究計畫 成果報告

高血糖/糖尿病的 Stromal-derived factor-1a/CXCR4 的表現與動脈粥樣硬化之相關研究

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行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

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中文摘要及關鍵詞

台灣地區糖尿病的盛行率約在 5-10%之間，死亡率則高居十大死因第四位，位居第二、三位的腦血管疾病與冠心症，與糖尿病也密切相關。糖尿病患者之冠心症罹病率及死亡率與非糖尿病者相比約增加 2-4 倍，且一旦合併心血管疾病，糖尿病之死亡率將增為單獨罹患心血管疾病者的兩倍以上。此外，許多研究也證實，在糖尿病的前期如血糖耐受不良時，其動脈粥狀硬化之危險性已開始增加，由 DECODE 研究報告，更知高血糖包括飯前或飯後高血糖皆是糖尿病引起冠心症之獨立危險因子。

早期動脈粥狀硬化的特徵包括發炎細胞聚集，單核細胞藉助特殊接受體與血管壁之內皮細胞相結合轉形為巨噬細胞，血管內膜平滑肌細胞之增生和遷移，以及細胞間質的沉積等，而因此瞭解動脈粥狀硬化及糖尿病之間的關連，對早期動脈粥狀硬化的治療或預防，特別是糖尿病病人，提供了可能的治療標的。

趨化細胞激素就像是小型趨化性胜肽合成器，可以將白血球、單核白血球、原生細胞等，吸收到某個特定組織內。Stromal-derived-factor 1α (SDF-1α) 是許多趨化細胞激素其中之一。已證實在腫瘤細胞 SDF-1α的刺激能促使腫瘤細胞遷移，而早期動脈粥狀硬化的特徵包括發炎細胞聚集，單核細胞藉助特殊接受體與血管壁之內皮細胞相結合轉形為巨噬細胞，血管內膜平滑肌細胞之增生和遷移，以及細胞間質的沉積等作用引發。這些現象在糖尿病患者加上高血糖影響之後可能更加嚴重。其中為何高血糖影響會更加嚴重，有些仍待驗證的機制。趨化細胞激素 SDF-1α可能扮演其中角色。

我們的研究發現在高糖之下處理加入 SDF-1α，巨噬細胞 J774 有 SDF-1α的表現，但單高糖刺激似乎仍不足以使 SDF-1α分泌至細胞外。血管平滑肌細胞 A7r5 在高糖狀態能促進細胞增生跟 migration 的情形。血管平滑肌細胞 A7r5 在高糖狀態加上 SDF-1α的刺激能促使 migration 明顯增加。其 pathway 並非經過 CXCR4 的表現量增加，而是透過 CTGF。CTGF 在之前的研究 (Huang et al)發現在高糖狀態下能促成 VSMC migration 的現象。本研究仍無法釐清巨噬細胞和血管內膜平滑肌細胞之遷移之間 SDF-1α的關係，因我們沒有在 macrophage 的 supernatant 發現 SDF-1α的表現。未來可能須用 Boyden Chamber 方式進一步探討之間的關係。另外，STZ rat model 發現其血清的 SDF-1 表現量明顯增加，但人體血清則無明顯差異，血清的表現量和動脈粥狀硬化過程的意義仍仍缺乏解釋，或許須進一步探討動脈粥狀硬化血管的病理組織變化及 SDF-1α/CXCR4 的表現。相信這些發現及研究結果也將提供探討動脈粥狀硬化機轉更多的研究方向。

關鍵詞: Stromal-derived-factor 1α、動脈粥狀硬化、高血糖、血管內膜平滑肌細胞、遷移、趨化細胞激素。

英文摘要及關鍵詞

The prevalence rate of type 2 diabetes mellitus (T2DM) in Taiwan is about 5-10%. It has become the 4th leading cause of death in Taiwan. Besides, cerebrovascular and cardiovascular diseases, the 2nd and the 3rd, are strongly associated with T2DM. The occurrence of coronary heart disease (CHD) increased 2 to 4 folds in diabetic patients, while the mortality is 2 folds higher in diabetic patients with CHD, indicating that T2DM is CHD equivalent at risk. The risk for atherosclerosis begins to increase in pre-diabetic stage. From the DECODE study, hyperglycemia, including fasting and postprandial hyperglycemia, was also documented to be an independent risk factor for CHD.

Early atherosclerotic lesions are characterized by accumulation of inflammatory cells and intimal smooth muscle cell proliferation and migration, as well as extracellular matrix deposition. All these phenomenon might be exacerbated in the setting of diabetes because of long term of hyperglycemia. Realization of the link between inflammation, atherosclerosis, and diabetes highlights the potential therapeutic targets to inhibit atherosclerosis, especially in diabetic patients. Chemokines are small chemotactic peptides instrumental in attracting leukocytes, monocytes, progenitor cells to specific tissues. Stromal-derived-factor 1α (SDF-1α) is one of many chemokines noted in the human. It has been demonstrated that SDF-1α has enhanced migratory effects in tumor cells. Early atherosclerotic lesions are characterized by accumulation of inflammatory cells and vascular smooth muscle cell (VSMC) proliferation and migration. Many mechanisms remain unknown. Therefore, we studied the effect of SDF-1α in VSMC proliferation and migration. Our results demonstrated that expression of SDF-1α was present in high glucose treated macrophage, however, not in supernatant. The receptor CXCR4 was present in VSMC, however, glucose did not influence it expression. High glucose and presence of SDF-1α may enhance VSMC migratory effect. The possible pathway was through collagen-tissue growth factor (CTGF), CXCR4 was not the major pathway. From our result, we suggest another VSMC proliferation pathway, however, future is needed to confirm the relationship of SDF-1α/CXCR4 in macropahge and VMSC.

Key words: hyperglycemia, atherosclerosis, stromal-derived factor-1α (SDF-1α), vascular smooth muscle cells, high-glucose, migration

報告內容：

前言

台灣地區糖尿病的盛行率約在 4.9％~9.2％ 之間，死亡率則高居十大死因第四位，位居第二、三位的腦血管疾病與冠心症，與糖尿病也密切相關。糖尿病患者之冠心症罹病率及死亡率與非糖尿病者相比約增加 2~4 倍，且一旦合併心血管疾病，糖尿病之死亡率將增為單獨罹患心血管疾病者的兩倍以上，因此糖尿病目前已被視為等同於冠心症之同等級危險因子。此外，許多研究也證實，在糖尿病的前期如血糖耐受不良時，其動脈粥狀硬化之危險性已開始增加，由 DECODE 研究報告，更知高血糖包括飯前或飯後高血糖皆是糖尿病引起冠心症之獨立危險因子。早期動脈粥狀硬化的特徵包括發炎細胞聚集，單核細胞藉助特殊接受體與血管壁之內皮細胞相結合轉形為巨噬細胞，血管內膜平滑肌細胞之增生和遷移，以及細胞間質的沉積等，而這些現象在糖尿病患者加上高血糖影響之後可能更加嚴重。其中為何高血糖影響會更加嚴重，有些仍待驗證的機制。因此本研究主要是希望探討 SDF-1α/CXCR4 在動脈粥樣硬化發生過程中，在巨噬細胞，血管內膜平滑肌細胞之增生和遷移所扮演的角色。

研究目的

主要是希望探討 SDF-1α/CXCR4 在動脈粥樣硬化發生過程中，巨噬細胞，血管內膜平滑肌細胞之增生和遷移所扮演的角色。假說: 巨噬細胞受高糖刺激會分泌 SDF-1 藉由血管平滑肌細胞中的 CXCR4 刺激血管平滑肌細胞，導致血管平滑肌細胞遷移。其中的機轉則進一步探討。

文獻探討

(一) 糖尿病現況

由於全球面臨糖尿病盛行率不斷增加，死亡率提高，第二型糖尿病患年輕化，血糖控制不佳，合併多重的併發症，世界衛生組織將糖尿病視為全球人類健康的重要威脅。根據實證數據顯示，全球現有一億七千多萬糖尿病病患，並推估二十五年後，糖尿病患將會超過現今病患數的兩倍，達三億六千六百萬。而國內糖尿病目前已高居十大死因第四位[1]。且位居十大死因第二、三位的腦血管疾病與冠心症，都和糖尿病密切相關。許多文獻資料顯示，糖尿病與冠心症的罹病率及死亡率與非糖尿病者相比，約增加2~4倍[2]，由Haffner及Whiteley等學者的研究，目前已認為糖尿病約相等於冠心症之同等級危險因子 (CHD risk equivalent) [3,4]，這些證據一再顯示糖尿病及其所引發的併發症危害健康甚鉅。

(二) 動脈粥狀硬化

糖尿病約相等於冠心症之同等級危險因子。而冠心病中動脈粥狀硬化更是主要的致死原因[5,6]。動脈粥狀硬化 (atherosclerosis) 的發生為一種複雜且為漸進式的致病過程，包括脂質進入血管壁、脂蛋白氧化、單核細胞活化、泡沫細胞形成與堆積，血管平滑肌細胞遷移 (migration) 和增生 (proliferation) 等[7]。在動脈粥狀硬化的病理致病機轉研究中，動脈粥狀硬化的形成機制是一種脂質與發炎細胞間的聚集，伴隨著血管平滑肌細胞增生與細胞外間質液分泌所引起的細胞內膜纖維變性[8]，早期發展的過程"低密度脂蛋白" (LDL) 受到自由基的作用產生脂質過氧化作用修飾成"氧化型低密度脂蛋白" (ox-LDL) [9-11]，並且堆積在血管壁的細胞內膜，這些氧化型低密度脂蛋白會影響血管內皮細胞 (endothelial cell) 的正常功能，使內皮細胞變得容易被血液內的細胞吸附[12]。其

次，這些氧化態的低密度脂蛋白也會吸引血液中的單核細胞 (monocyte)，由血管內轉移至血管內皮細胞外的內膜 (intima)。單核細胞會向血管內皮表面黏著，這種黏著現象是藉由存在於白血球與內皮細胞的黏著分子互相作用而形成的，單核細胞與內皮表面黏著之後，會移動到內皮下間隙，並分化成巨噬細胞 (macrophage) [13-15]；巨噬細胞對氧化態低密度脂蛋白，進行無限制的吞噬而形成泡沫細胞 (foam cell) [16,17]，這些泡沫細胞的集中所形成的脂肪條紋 (fatty streak)，是粥狀硬化過程中最早可以辨識的病灶，這些脂肪條紋會緩慢的形成纖維斑[18]。而纖維斑轉變的發生，是由於血管平滑肌細胞 (vascular smooth muscle cell, VSMC) 受到氧化態低密度脂蛋白的刺激，分泌酵素，逐步分解彈性纖維層後，由血管中層移動到血管內膜層，且在內膜當中大量增生[19,20]。其中，血管平滑肌細胞也會透過吞噬氧化態低密度脂蛋白而形成泡沫細胞[21]。在這些病灶中，由巨噬細胞以及血管平滑肌所形成的泡沫細胞持續增加造成細胞的死亡，進而形成一個明顯以脂質為核心，外環包圍著死亡巨噬細胞的粥樣瘤狀病變。正常的血管內壁並無腫塊，而動脈粥狀硬化是指動脈血管壁中，因粥樣瘤狀的現象惡化變大，導致血管管徑變小、血流阻力增大、血流量變少，使組織間氧氣與養分供應減少，血管壁所承受壓力過大，再加上血小板的作用，誘導血栓的形成 (thrombosis) [22,23]，促使阻塞情形更加惡化，而血栓更是引起中風的潛在因子，這些現象都是導致晚期動脈血管壁粥狀塊呈現複雜、具不可逆性，造成治療上困難的原因。所以低密度脂蛋白所受到的氧化修飾及血管平滑肌細胞的增生與遷移，在動脈粥狀硬化的早期都扮演著很重要的角色。因此，如何藉由阻止低密度脂蛋白的氧化修飾及抑制血管平滑肌細胞的增生與遷移作用，被視為一種有效預防動脈粥狀硬化發生的方法。此外，加上近年來由於飲食習慣及環境改變的種種因素，導致了許多慢性病的高發生率，而其中的致病機轉不外乎與自由基及過氧化物的生成有關[24,25]。

（三）單核細胞與動脈粥狀硬化的關連性

單核細胞與血管壁內皮細胞之接觸與結合:單核細胞藉助特殊接受體與血管壁之內皮細胞相結合。結合過程之導火線,膽固醇(Cholesterol)及氧化變性之 LDL(Ox-LDL)則占重要角色。單核細胞之增兵;單核球轉形為巨噬細胞。單核細胞及脂蛋白趁細胞分裂,青黃不接之時,由細胞間隙穿越內皮進入內皮下層。單核細胞之大舉招兵買馬及入侵內皮與單核球趨化蛋白-1 有關,單核細胞趨化蛋白-1 來自動脈平滑肌細胞(ASMC),單核細胞及巨噬細胞。此期間氧化變性之 LDL(Ox-LDL)之居間興風作浪實難辭其咎。可能由 T 淋巴球釋放細胞激動素(Cytokines)引導單核球轉化成巨噬細胞 [26]。

(四) 平滑肌細胞與動脈粥狀硬化的關連性

1. 平滑肌細胞的遷移在動脈粥狀硬化扮演之角色

平滑肌細胞的遷移主要是受到化學趨引 (chemoattractant) 所移動[27]。目前牽涉細胞的遷移最被常用的有兩個說法：Chemotaxis 和 Haptotaxis。Chemotaxis 指的是細胞遷移是透過可溶性 (soluble) 的化學趨引因子濃度的增加所造成，例如：血漿中的因子。Haptotaxis 則是指透過不可溶性 (insoluble) 趨引方式，例如：鍵結到基質(matrix)上。在文獻上也指出，誘導細胞遷移的因子很多，例如：血小板生長因子、fibronectin、Matrix Metalloproteinases (MMP)等。這其中當然也涉及了許多的訊息傳遞，其中主要是透過兩個 Receptor-coupled 系統： Guanosine 5’-triphosphate-binding proteins-coupled 和 Tyrosine kinase - coupled proteins。而其中可能參與遷移的訊息傳遞蛋白相當多[27]。因此，平滑肌細胞的遷移在動脈粥狀硬化是一個重要的過程，而抑制平滑肌細胞的遷移可作為治療動脈粥狀硬化的一個方向。

2. 平滑肌細胞的增生在動脈粥狀硬化扮演之角色

平滑肌細胞的增生在動脈粥狀硬化中是一個重要的現象[28]。在正常的動脈中，平滑肌細胞會停留在不增生的靜止期而且呈現良好的分化收縮的型態 (differentiated contractile phenotype)；當血管受到損傷後，平滑肌細胞會由分化的形態轉成合成的型態 (synthetic phenotype)，並伴隨著進入細胞週期 (cell cycle) 使得平滑肌細胞增生。細胞的增生主要是受到細胞週期的調控。細胞週期可分為四期 (G_1、S、G_2 及 M 期)：（1）在 G_1 (Gap1) 時期細胞維持正常代謝並且繼續生長，再進入 S (synthesis) 時期之前會檢查染色體 (chromosome) DNA 是否受到破壞以進行修補 (repair) 的工作，此時期需要花十到十二小時。細胞也可能由此脫離細胞週期進入不生長的休止狀態(G_0 時期)，需要有適當的訊息才會重新投入細胞週期；（2）當細胞進入 S 時期會花六到八小時進行 DNA 的合成，將原本的二十三對染色體複製另一份；（3）當細胞進入 G_2 (Gap2) 時期需要花三到四小時，除了繼續生長並且合成蛋白質之外，細胞也會負責檢查染色體 DNA 的複製是否完整以準備進行有絲分裂 (mitosis)；（4）進入 M (Mitosis) 時期之後細胞會由一個母細胞變成兩個子細胞，已複製完整的染色體會各自分配到子細胞內，使得子細胞內的染色體與母細胞完全一樣，此時期只有一小時。之後子細胞再開始進入下一個細胞週期。而細胞週期是經過 cyclin 與 Cdk 嚴密的調控，因為大量合成與累積的 cyclin 與 Cdk 結合後會導引 Cdk 到目標蛋白質上，Cdk 會把磷酸基加在調節 DNA 複製或引發細胞分裂的關鍵蛋白質上，藉由改變蛋白質的功能而進一步控制細胞週期。細胞週期除了受到 cyclin 與 Cdk 的調控外，亦受到其他因子的調控，如：Cdk 的抑制劑 (inhibitor)、p53 等。因此，有效的抑制平滑肌細胞的增生將是阻斷動脈粥狀硬化的重要元素之一。

(五) 高血糖與冠心症關係密切

糖尿病患者易發生動脈粥狀硬化，咸認肇因於脂質異常。例如高三酸甘油脂血症及低血濃度之高密度脂蛋白膽固醇[29]，尤其是 small dense LDL [30]。在最近以糖尿病之脂質異常為治療目標的研究中，statin 藥物能顯著降低死亡及非死亡冠心症及中風的發生率[31,32]，然而卻仍有部分 statin 治療試驗無法達到治療目標[33,34]。糖尿病患者之血管常呈現多段處狹窄，形成動脈硬化的原因複雜[35]，除了血脂異常造成的結果之外，其他因子如高血糖，自由基等原因之探討，亦有助於糖尿病患者血管病變之治療。

在以非糖尿病患者的研究中，Coutinho 學者指出，當血糖值由 75 mg/dL 上升至 110 mg/dL，或上升至 140 mg/dL 時，其冠心症事件發生率分別增加至 1.33 及 1.58 倍[36]。文獻也指出糖尿病的高血糖嚴重程度與冠心症的死亡率密切相關[37-39]。

當糖尿病病人發生急性心肌梗塞時，以胰島素積極降低控制高血糖，更可降低整體死亡率[40]。Kosiborod 等學者也提出報告，當老年人因急性心肌梗塞住院時，常被發現高血糖值，且其值將增加此類病人之死亡率[41]。

此外，許多研究也證實，在糖尿病的前期：血糖耐受不良 (impaired glucose tolerance：IGT) 時，動脈粥狀硬化之危險性已開始增加[42-45]，可見高血糖包括飯前高血糖[46]及飯後高血糖是糖尿病引起冠心症之獨立危險因子[47]，著名的 DECODE 研究，更清楚指出飯後高血糖與冠心症之關聯性[48]。

在飯後高血糖的治療中，一項以 α-glucosidase inhibitor 的治療試驗 STOP-NIDDM，已清楚看出，降低飯後血糖，不但減少糖尿病的發生率，更減少了心血管事件達 49 % [49]，同時減緩了頸動脈內膜中層厚度 (intima-media thickness；IMT) 的惡化[50]。此外，除了第

二型糖尿病外，對第一型糖尿病患者而言，積極控制血糖，也證實能降低冠心症的發生率[51]。

(六)高血糖導致血管病變

至少有四條路徑與高血糖造成血管傷害有關[52]:

(1) 促進 polyol 活性，使得 sorbitol 及 fructose 堆積；

(2) 增加 advanced glycation end products (AGE)；

(3) 活化 protein kinase C (PKC) 及 nuclear factor κβ (NF-κβ)；

(4) 增加 hexosamine pathway flux。

而飯後高血糖將減少第二型糖尿病的心臟血液灌流，且其受損程度與糖尿病患者之小血管或大血管併發症有關[53]。高血糖也抑制了內皮細胞功能，同時降低內皮細胞之血管擴張功能[54-56]。同時高血糖增加內皮細胞 superoxide anion 的形成，而導致內皮細胞功能異常[57,58]；也增加血循環中 ICAM-1 的濃度[59]，一般認為這可能與 oxidase stress 增加有關[60,61]，而更值得一提的是急性高血糖或大的血糖高低變動幅度比慢性長期的高血糖(如高的糖化血色素)更易引動 oxidative stress [62]。

AGE 是由還原糖與蛋白質胺基酸 (通常是 α-NH_3) 所形成的老化蛋白。反應過程稱為 Millard reaction，不需酵素調控。此種蛋白會因高血糖而慢慢堆積，最後導致結構與生理性的異常[63]。AGE 對心血管的影響包括: 內皮功能不良、血管傷害反應、動脈硬化斑塊與血管硬度增加[64]。以 AGE 抑制劑作用的 STZ 糖尿病鼠，可降低動脈斑塊，其中 α-sm actin、growth factor、total collagen 亦將隨之降低[65]。高血糖可使內皮細胞 AGE 增加，而 Bcl2 與 antioxidant 則可抑制脂質過氧化與 AGE 產生[66]。

除了慢性高血糖及 AGE_S 的刺激會促進單核細胞球之 NF-κβ 活化之外[67,68]，短暫的高血糖亦會使血管平滑肌細胞之 NF-κβ 活化[67]，同時使週邊血液單核球細胞 ($PBMC_S$) 的 ras, p42/44 MAPK 及 NF-κβ 活化[69]。

（七）糖與單核細胞

高濃度糖可藉ICAM-1、VCAM-1 與CD18，而促使單核細胞黏附至內皮細胞[70]。亦有學者指出，高糖引發單核細胞黏附至內皮細胞主要是藉由lipooxygenase 與其產物所致，ICAM-1、VCAM 本身在此過程中的變化不大；然而阻絕ICAM-1 的counterreceptor leukocyte function-related antigen-1 與VLA-4，卻抑制了lipooxygenase與細胞黏附[71]。 Glycated albumin 可藉ICAM-1、VCAM、ELAM-1 刺激單核細胞黏附內皮細胞，此類黏附分子則因NF-κB 而增加轉錄[72]。此外，有關活化單核細胞的傳訊機轉，尚包括 MEK、ERK1/2、PI3K 等[73,74]。

(八) 糖與血管平滑肌細胞

高濃度糖會促使 VSMC 生長與表現 ECM 基因[75]。高糖藉活化 PKCβ 來消除 estrogen 抑制 VSMC 生長的現象[76]。於 STZ 糖尿病鼠與相關體外實驗中亦發現，高糖藉活化 PKCβ 與 α2 而抑制 VSMC 凋亡[77]。而升高糖類代謝，可使 GSK3β 失活，進而抑制 VSMC 凋亡與 neo intima 生成[78]。Campbell 等人的研究指出，高糖可藉由 PI3K、MAPK 活化而增加 VSMC 的移動[79]。許多文獻也顯示 PI3K 或 ERK1/2 對 VSMC 增生與移動的重要性，藉由 MAPK 抑制 p53，也造成對 VSMC 的抑制[79]。此外，PKCα 與 PTEN 亦與 VSMC 的活動有關[79]。然而糖的作用是直接誘導或只是協同加強，尚有爭議。高糖可透過 GSH 降低與 PKC 活化而加強 TNF-α 誘導 NF-κB 的表現，亦可加強 IL-1 活化 cox，引致 PG 大量產生[67,79]。然而亦有其他研究報告指出，高糖本身其實並無作用，但可透過 tyr receptor 來增

強 PDGF、TGF 刺激 VSMC 增生[80]。

(九) Stromal-derived Factor-1α (SDF-1α)

趨化細胞激素就像是小型趨化性胜肽合成器，可以將白血球、單核白血球、原生細胞等，吸收到某個特定組織內。血管疾病所引起的動脈粥樣硬化，以及單核細胞反射增進作用，讓我們開始體會到趨化細胞激素在血管生物學裏，扮演了重要的角色 [81]。 除了初期動脈硬化時的各項功能之外，對於細胞重組及新生內膜形成，各種血管傷害、動脈栓塞併發症、心肌梗塞和組織損傷成因、血管生成與再生...等反應，趨化細胞激素都發揮強大的作用。 因此，趨化細胞激素在血管生物學，各種不同階段的發展過程中，都扮演著主要角色，也可能影響各個不同階段狀況 [82,83,84]。因 SDF-1α 所具備的多項特性，使其有別於其他趨化細胞激素。 首先，雖然 SDF-1α擁有標準 CXC 基本構造，但它顯示出 CC 和 CXC-趨化因子完全等距序列的同源性。 SDF-1α具備獨特系統發生關聯功能，可以延續連接 CXCR4 受容器，這也使得 SDF-1α 與 CC 和 CXC-趨化因子受容器，同時擁有類似的等距同源性。 SDF-1α 趨化細胞激素是 CXCR4 受容器唯一的配合基 [85]。 SDF-1α的主要生物功效和趨化細胞激素產生趨化性反應能力、能動性、SDF-1α同源細胞附著力，都有著密切關係 [86,87]。SDF-1α在單核白血球與淋巴球趨化活體，展現高度活躍力[88]；它也是人類內皮細胞強大趨化力量，具備這樣的功能，協助血管受傷後的內皮細胞，能夠增生成長[89]。 SDF-1α屬於陽性單核白血球，超高效能的趨化細胞激素[90]。SDF-1α，具有淋巴球和單核白血球的趨化因子，檢查其組織結構，可以看到容易破裂、動脈硬化血小板，並且對於動脈粥樣硬化，具有舉足輕重的影響。許多研究也顯示， SDF-1α 可能是導致許多發炎性疾病的媒介，其中包括動脈粥樣硬化的發病與惡化[88]。 然而，在嗜中性白血球的研究反而發現 SDF-1α透過調節嗜中性白血球體內平衡，SDF-1α表現會有動脈 remoldeling 的作用 [90]。所以至今仍無法定論 SDF-1α 在動脈粥樣硬化的發病與惡化中所扮演的角色。 SDF-1α/CXCR4 軸已經成為重要多種細胞類型之間的聯繫調節器，針對 SDF-1α/CXCR4 軸進行各種不同的研究方法，視為未來治療潛在目標。在血糖過高、動脈粥樣硬化惡化期，SDF-1α/CXCR4 在整個高糖狀況、動脈粥樣硬化過程，是如何發揮作用，尚不可知。無論 SDF-1α/CXCR4 是否具備促進發炎或保護作用的特性，也不管是否 SDF-1α/CXCR4 具有診斷價值，或者可做為治療目標，對血糖過高、動脈粥樣硬化惡化期的研究，勢必更加重要。

研究方法

Cell culture

A7r5, a rat thoracic aorta smooth muscle cell line, was obtained from ATCC (ATCC number: CRL-1444). A7r5 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin–streptomycin, and 1.5 g/l sodium bicarbonate (all from Gibco/BRL (Gaithersburg, MD)). All cell cultures were maintained at 37 ℃ in a humidified atmosphere of 5% CO2. The medium was changed to DMEM supplemented with 5% FBS containing normal (N, 5.5mM) or high glucose (C, 25 mM). The high glucose DMEM is normal glucose medium added with 19.5mM glucose. Mannitol (19.5mM) was added in normal concentration (N, 5.5mM) DMEM to keep the same osmolarity.

Cell viability analysis

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine cell viability. Cells were seeded in 6-well culture plates at a density of 1×105 cells/well

and incubated in normal or high glucose medium for 48 h. Thereafter, SDF-1α at various concentrations up to 60.0 μM as indicated was added in high glucose medium for 48 h to evaluate the dose dependent effect of SDF-1α on VSMC growth and viability. In addition, SDF-1αat 40.0 μM was added for indicated times to evaluate the time-dependent effect of SDF-1αon VSMC. Thereafter, 0.1mg/ml MTT was added at 37 ℃ for another 4 h. Following solubilization with isopropanol, the viable cell number is directly proportional to the production of formazan measured spectrophotometrically at 563 nm.

Western blot analysis

For Western blot analysis, we used specific antibodies to evaluate the expression of p27 (F-8), p21 (F-5), p16 (F-12) (all from Santa Cruz Biotechnology, Santa Cruz, Calif, USA) and α-actin (Sigma, St. Louis, MO, USA). After the indicated SDF-1α treatment, equal amounts of cell lysate (50_g protein) were separated by electrophoresis on 8–12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were incubated with Tris-buffered saline (TBS) containing 1% (W/V) nonfat-milk and 0.1% (V/V) Tween-20 (TBST) for 1 h to block non-specific binding then washed with TBST for 30 min. Each membrane was incubated with the appropriate primary antibody for 2 h followed by horseradish peroxidase-conjugated second antibody (Sigma, St. Louis, MO, USA) for 1 h and developed by ECL chemiluminescence (Millipore, Bedford, MA, USA), using AlphaImager Series 2200 software. Results are representative of at least three independent experiments.

Migration assay

The *in Vitro* migratory activity of VSMCs was measured using a wound migration assay. Cells were seeded at 5 × 105 cells/well in a 6-well plate and obtained 50-70% confluence. An injury line was created with a single scratch at the center of a VSMC monolayer (50-70% confluence) using a sterile 1.15 mm diameter pipet tip. Thereafter, VSMCs were continuously cultured and photographed with phase-contrast microscopy (model CK40, Olympus) at 24 and 48 hours, respectively, with five images of randomly selected field for each preparation. The distance between cells at both sides of the wound was measured for five pairs of cells per image. Cell migration was expressed in the percentage of initial wound width.

Electrophoresis and Immunoblotting

Cells were harvested into lysis buffer containing 50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 5% mercaptoethanol and then lysed by sonication. Equal amounts of protein (50 mg/lane) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transfected to nitrocellulose (NC) membranes. Subsequently, NC membranes were blocked with 5% bovine serum albumin with 0.05% Tween 20 in phosphate-buffered saline and then were incubated with the first antibody for 1 h. NC membranes were washed 3 times with 0.05% Tween 20 in phosphatebuffered saline and incubated with the secondary antibody conjugated to anti-mouse horseradish peroxidase (Amersham Life Science, Buckinghamshire, U.K.). Bands were visualized using the enhanced chemiluminescent (ECL) Western blotting detection system (Perkin-Elmer Life Sciences, Boston, MA). Protein quantities were determined by densitometry through AlphaImager Series 2200 software (Alpha Innotech, San Leandro, CA).

結果與討論（含結論與建議）

一、Cytotoxicity of SDF-1α on A7r5 cells was measured by MTT assay. Proliferation of cells cultured in high glucose (control group) (C, 25mM) was increased as compared to cells cultured in normal glucose (N, 5.0 mM). Following 48 h incubation with 100 ug and 300 ug SDF-1α in high glucose medium, cell survival ranged from approximately similarly and was dose-dependent. Furthermore, a time-dependent increase in SDF-1α induced cytotoxicity was also observed at a dose of 500 ug. The results indicated that SDF-1α had a significant inhibitory effect on VSMC growth in a dose- and time-dependent manner in high glucose status as compared to control group. According to these results, we used the concentrations of 100 ug and 300 ug of SDF-1α, which had no cytotoxic effects in the following investigations. (Figure 1)

二、Expression of SDF-1α was increased macrophage J774 cell. The SDF-1α expression was increased in 24 hours with high glucose comparing to the control and decreased 48 and 72 hours of incubation. However, the supernatant did not increased expression. The possible explanation is that high glucose in 24 hours could induce SDF-1α, but not enough to secrete it. (Figure 2)

三、Expression of CXCR4 in normal glucose and high glucose with SDF-1α in VSMC. There was expression of CXCR4 in VSMC, however, neither glucose nor SDF-1α influence its concentration. It may indicate that VMSC migratory pathway was not through increase expression of receptor, therefore, other signal pathways were determined. (Figure 3)

四、Effect of SDF-1α on the migration of VSMC in normal glucose and high glucose. Figure showed that high glucose induced the cell migration above two fold, further stimulation with SDF-1α 100 ug, the cell migration was increased by 2.5 fold. Indicating that SDF-1α could enhance VSMC migration effect in normal glucose and high glucose. (Figure 4A,B)

五、Effect of SDF-1α on the expression of MMP-2、CDC42、RAC1, RHO, CTGF and RAGE in normal glucose and high glucose. Collagen-tissue growth factor (CTGF) mediated downstream of in normal and high glucose SDF-1α stimulated migration. To further investigate if SDF-1α in high glucose status could enhance cell migratory effect in vascular smooth muscle cell, what could be the target affected. Proteins reported to be involved with cell migration were tested. Our data demonstrated that CTGF expression was increased with SDF-1α, and high glucose, more prominent in both high-glucose and SDF-1α. The expression of MMP-2、CDC42、RAC1, RHO and RAGE were all negative. Indicating that the vascular smooth muscle cell migration effect induced by high glucose and SDF-1α was mediated by CTGF. (Figure 5A,B)

六、The serum SDF-1α was increased in animal model, but did not show significant change in human, neither diabetic nor atherosclerotic patient. To correlate cell study to animal and human study, we also examined the serum level of SDF-1α in animal model (STZ rat) and diabetic and atheroslcerotic patient. The SDF-1α was only increased in STZ rat model, did not show significant change in the two groups of patients. A possible explanation in human may be the expression of SDF-1α is in the local area, further investigation is needed in animal model and human tissue. (Figure 6A,B)

七、Proposed scheme of our model: in cell condition with high glucose, SDF-1α may enhance migratory effect of VSMC, a possible pathway was through CTGF, with CXCR4 or not could not be determined)

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附表及附圖

Figure 1. Effect of different concentrations of SDF-1α in VSMC A7r5 cell viability

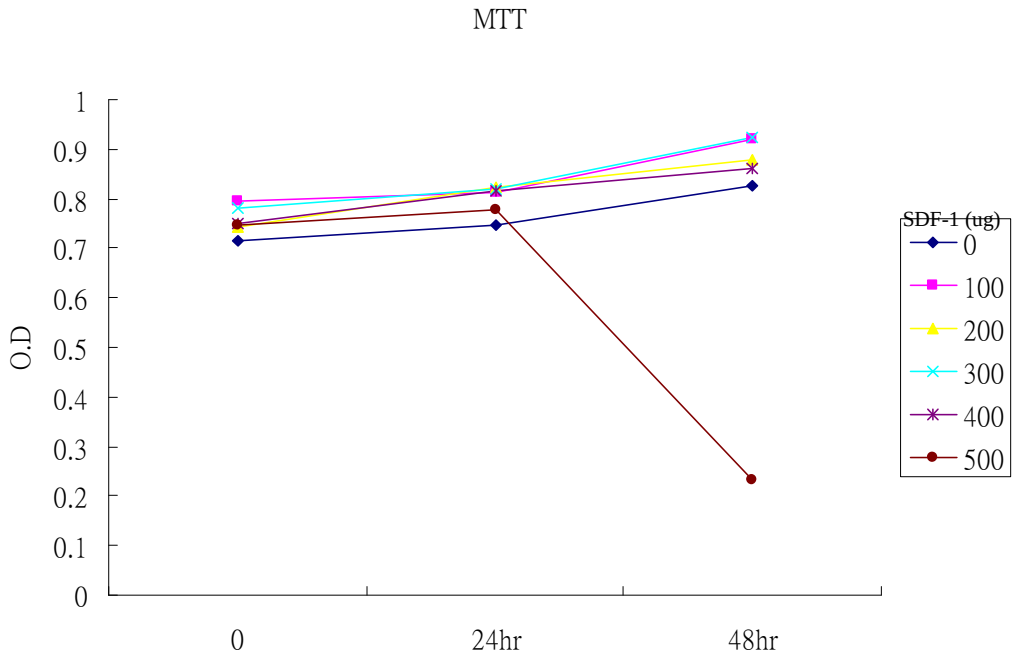


Figure 2. SDF-1α and CXCR4 expression in macrophage J774 cell.

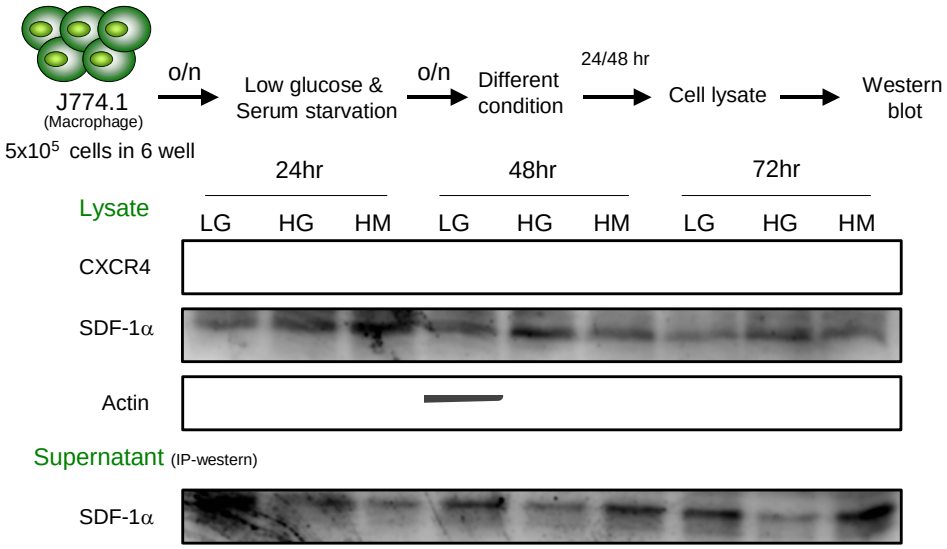


Figure 3. Expression of CXCR4 expression in 6,24, and 48 hours in VSMC A7r5 cell

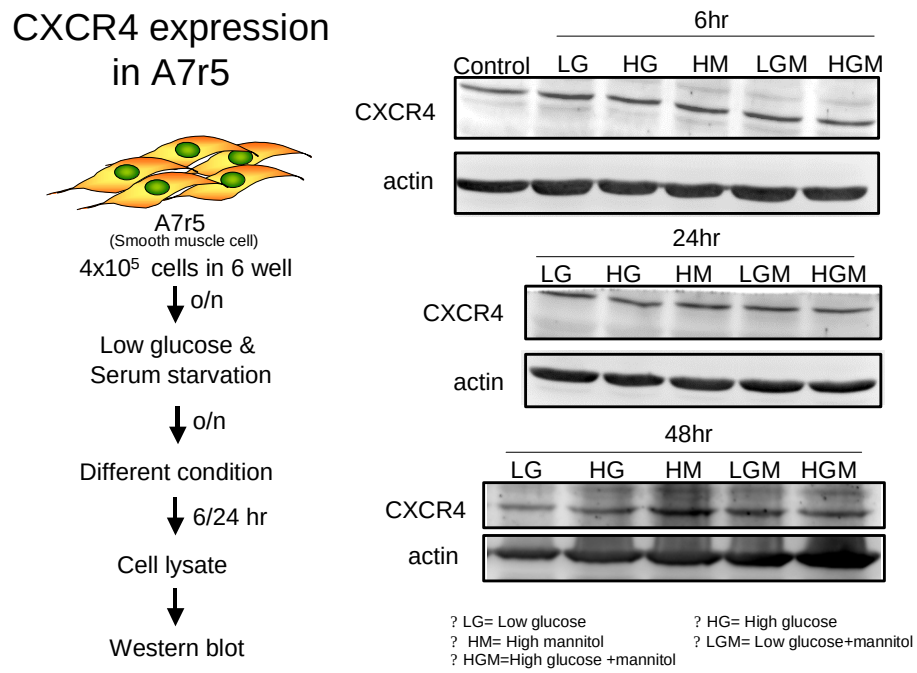


Figure 4A. VSMV A7r5cells treated with glucose 5 and 25 mM and SDF-1α in different concentrations at 48 hours, which indicated migratory effect increased with higher concentration of SDF-1α, in low and high glucose, more prominent in high glucose.

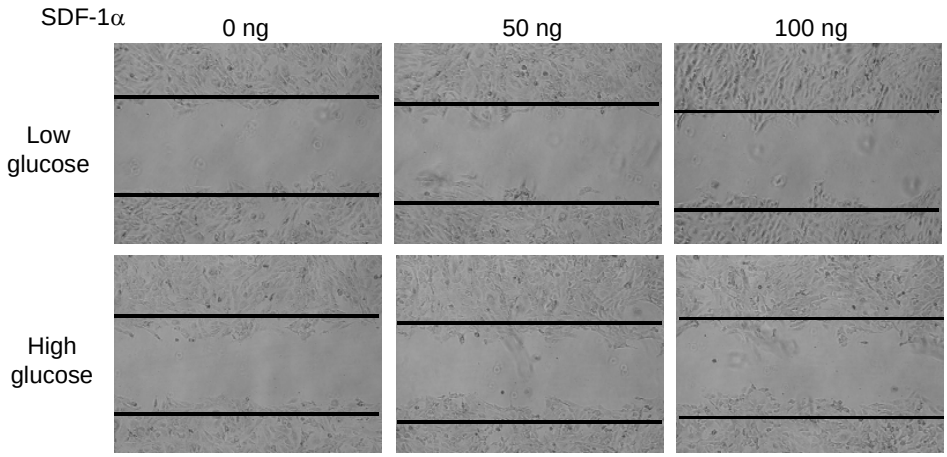


Figure 4B. Bar graph showed the results of 4A, data were presented as means ± SD

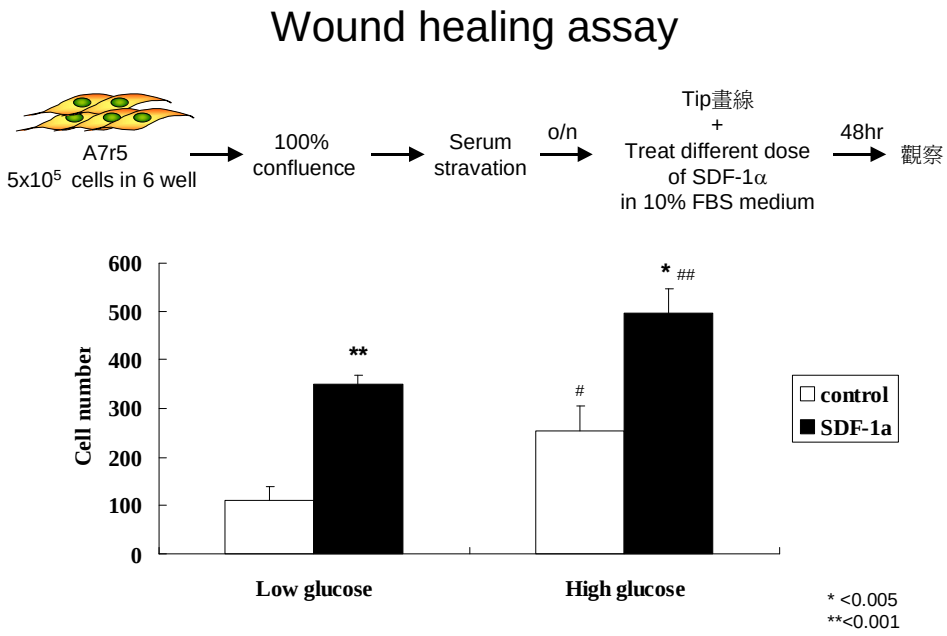


Figure 5A Expression of different pathways expression in control, SDF-1α, glucose, and glucose plus SDF-1α 100 ug

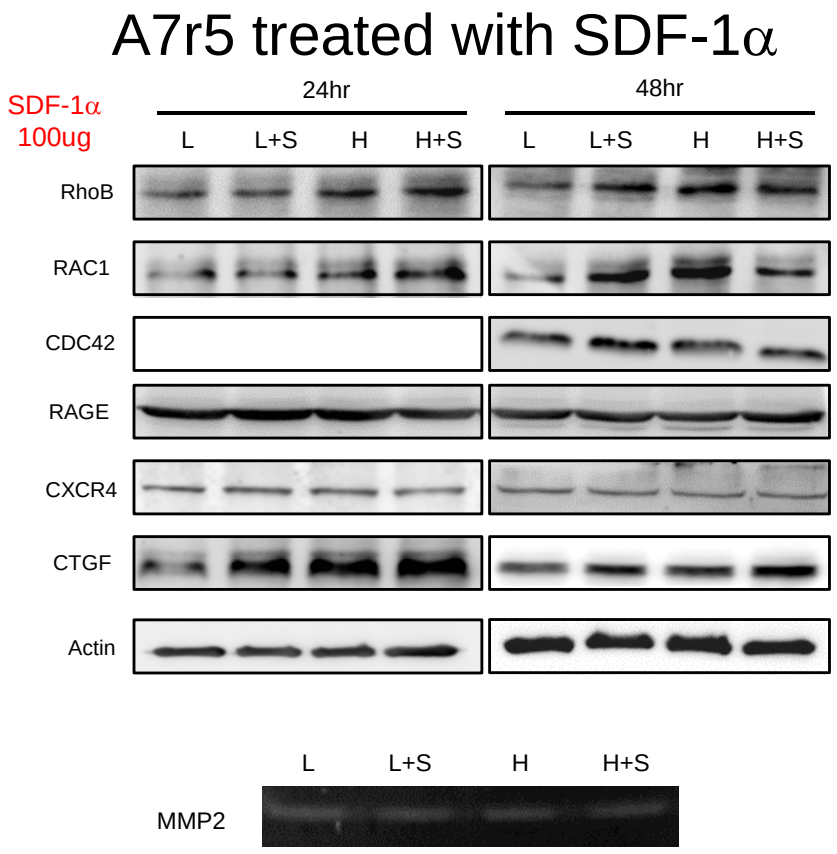


Figure 5B Expression of different pathways expression in control, SDF-1, glucose, and glucose plus SDF-1α 300 ug

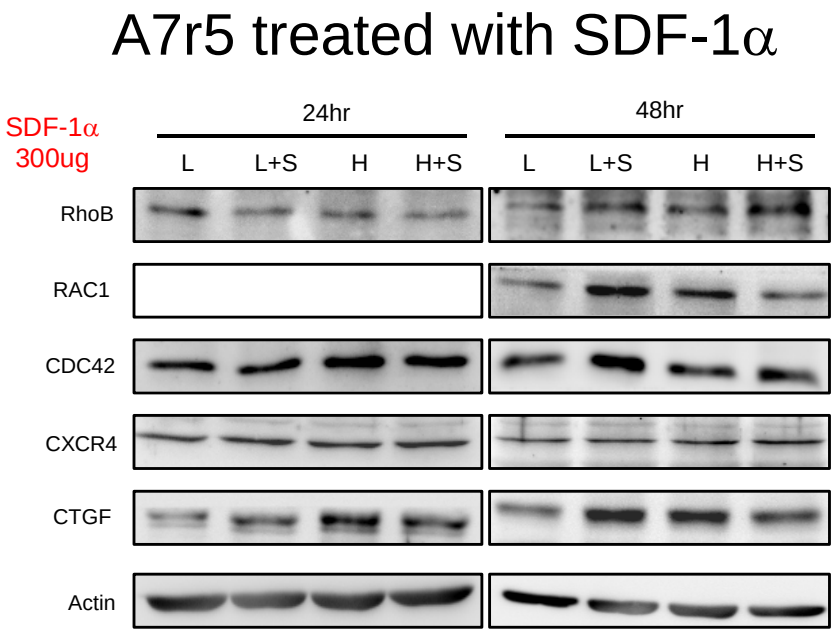


Figure 6A. SDF-1α in control and STZ rat model, serum.

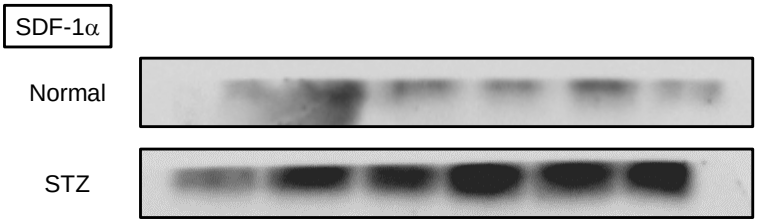


Figure 6B. SDF-1α in Human serum expression of SDF-1 in control, diabetic patient, and atherosclerotic patient.

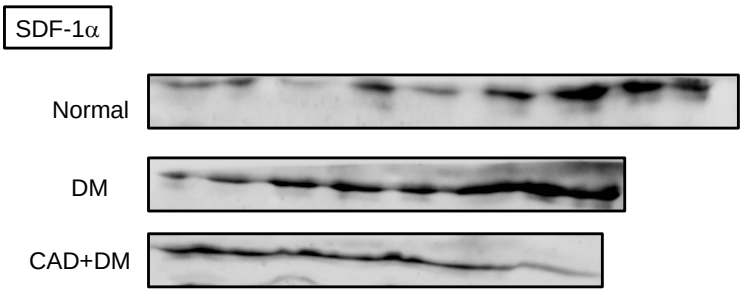
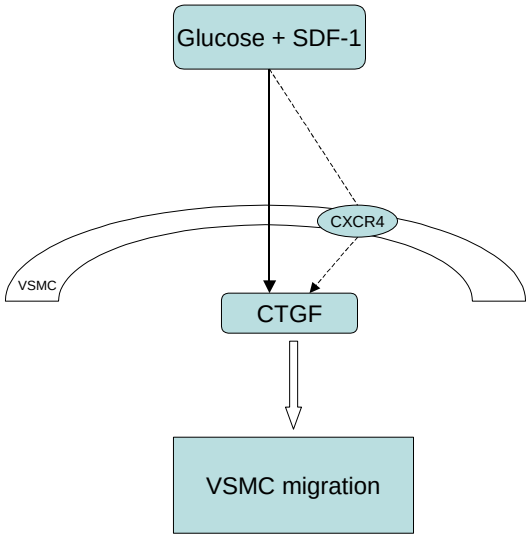


Figure 7 Scheme of our model



計畫成果自評

本子計劃設定在細胞、動物、人體模式的研究，釐清SDF-1α/CXCR4在動脈粥樣硬化發生過程中，巨噬細胞，血管內膜平滑肌細胞之遷移所扮演的角色。首先實驗設計包含觀察在高糖之下，血管平滑肌細胞A7r5和下巨噬細胞J774內CXCR4 和SDF-1α的表現量、細胞增生跟migration的情形。在高糖之下處理加入SDF-1α，巨噬細胞J774有SDF-1α的表現，但單高糖刺激似乎仍不足以使SDF-1α分泌至細胞外。血管平滑肌細胞A7r5在高糖狀態能促進細胞增生跟migration的情形。血管平滑肌細胞A7r5在高糖狀態加上SDF-1α的刺激能促使migration 明顯增加。其pathway並非經過CXCR4的表現量增加，而是透過CTGF。CTGF在之前的研究 (Huang et al)發現在高糖狀態下能促成VSMC migration的現象。本研究仍無法釐清巨噬細胞和血管內膜平滑肌細胞之遷移之間SDF-1的關係，因我們沒有在macrophage的supernatant發現SDF-1的表現。未來可能須用Boyden Chamber方式進一步探討之間的關係。另外，STZ rat model發現其血清的SDF-1表現量明顯增加，但人體血清則無明顯差異，血清的表現量和動脈粥狀硬化過程的意義仍仍缺乏解釋，或許須進一步探討動脈粥狀硬化血管的病理組織變化及SDF-1α/CXCR4的表現。相信這些發現及研究結果也將提供探討動脈粥狀硬化機轉更多的研究方向。