行政院國家科學委員會專題研究計畫　成果報告

胃癌血管新生之機制:介白質-6 與血管內皮生長因子之角色及相互作用(2/2)

計畫類別： 個別型計畫
計畫編號： NSC93-2314-B-002-041-
執行期間： 93 年 08 月 01 日至 94 年 07 月 31 日
執行單位： 國立臺灣大學醫學院一般醫學科

計畫主持人： 吳明賢
共同主持人： 郭明良

報告類型： 完整報告

處理方式： 本計畫可公開查詢

中　華　民　國 94 年 12 月 19 日

行政院國家科學委員會專題研究計畫成果報告

胃癌血管新生之機制：介白質-6與血管內皮生長因子之角色及相互作用 (2/2)

The mechanism of angiogenesis in gastric carcinoma: the role and interaction between interleukin-6 and vascular endothelial growth factor (2/2)

計畫類別：■個別型計畫　□整合型計畫

計畫編號：NSC92-2314-B-002-133

執行期間：　93年 8月 1日至 94年 7月 31日

計畫主持人：吳明賢醫師

處理方式：　□可立即對外提供參考
■一年後可對外提供參考
□兩年後可對外提供參考
(必要時，本會得展延發表時限)

執行單位：台大醫學院一般醫學科

中華民國 94 年 8 月 26 日

中 文 摘 要

血管新生(angiogenesis)目前熟知為惡性腫瘤的生長、侵犯、及轉移所必須。過去研究發現胃癌腫瘤血管新生與其病程、淋巴及遠處轉移、手術後再發等有相關性。許多血管新生因子及抗血管新生因子已被發現在此血管新生過程很重要。在這許多的血管新生因子中，血管內皮生長因子(vascular endothelial growth factor, VEGF)在胃癌生成之血管新生居樞紐地位。然而，目前尚無細胞外分子證實在胃癌可以調控增加血管內皮生長因子。介白質-6(Interleukin-6, IL-6)在幽門螺旋桿菌胃炎中表現增加，也曾被報告與胃癌疾病狀態有關，但其機制仍不清楚。我們較早之研究發現胃癌病人血清之介白質-6 與血管內皮生長因子有正相關，此結果暗示介白質-6 可能透過與血管內皮生長因子產生之互動進而在胃癌之血管新生扮演某個角色。因此吾人假設：介白質-6 經由誘導產生血管內皮生長因子而刺激胃癌之腫瘤血管新生。於此計劃中，我們將先藉由臨床研究驗證介白質-6、生血管內皮生長因子、腫瘤血管之相關性，再藉由胃癌細胞株實驗性研究探討此三者之關係。

本計劃的第一年，我們著重在驗證介白質-6、生血管內皮生長因子、腫瘤血管在胃癌之臨床相關性。胃癌樣本收集自臺大醫院之外科手術檢體，該樣本以免疫組織化學染色(immunohistochemical staining)染介白質-6、生血管內皮生長因子、及腫瘤血管。免疫組織化學染色活性分級後根據不同臨床病理特性加以分析，我們發現介白質-6、生血管內皮生長因子、腫瘤血管在不同次分組胃癌有正相關性（Table 1, 2）。而這實驗性研究中，我們以 ELISA 評估不同劑量及時間之介白質-6 刺激在不同胃癌細胞株中所產生之血管內皮生長因子（Figure 2a,b）。三種訊息傳遞路徑：JAK/STAT、Raf/MAPK、及 PI3K/Akt 中介白質-6 刺激之細胞訊息。因此我們利用 firefly luciferase reporter gene assay 研究哪一條訊息傳遞路徑負責傳導介白質-6 刺激活化生血管內皮生長因子之啟動子（Figure 3）。在研究介白質-6 對 *in vitro* 血管新生的影響，我們利用介白質-6 刺激胃癌細胞後收集之培養液來培養人類臍靜脈內皮細胞，評估其生長及形成管狀結構之效應（Figure 4, 5a-d）。在研究介白質-6 對 *in vivo* 血管新生的影響，我們利用 Matrigel plug assay 來評估。Matrigel 與介白質-6 刺激胃癌細胞後收集之培養液混合後注射於小鼠皮下，收取此 Matrigel plug 後觀察其巨觀差異，並測定其血紅素密度以代表腫瘤血管密度（Figure 6a-e）。我

們的結果顯示介白質-6 可以刺激胃癌細胞株產生血管內皮生長因子，並進一步證實其訊息傳遞係經由 JAK/STAT 路徑。同時亦證明介白質-6 可以增加胃癌的 in vitro 及 in vivo 血管新生。

在本計劃的第二年，我們已收集胃癌病人 250 例及健康對照 250 例，抽取白血球 DNA，分析兩組介白質-6 基因單核甘酸多型性之差異性，結果發現國人在 IL-6 基因-174G/C 位置上基因型全為同一型(-174G/G)，目前正在分析另一位置-634C/G 上之基因型。此外，我們也嘗試於手術檢體進行 IL-6 及 VEGF 的免疫組織化學染色並將其表現狀況與胃癌臨床病理特徵和血管密度做比較，以進一步証實 IL-6 在胃癌血管新生之角色及了解其與 VEGF 之交互作用機轉

關鍵詞：血管新生，血管內皮生長因子，介白質-6，胃癌，基因單核甘酸多型性

Abstract

Angiogenesis is currently well known to be essential for the growth, invasion, and metastasis in malignancies. Earlier studies showed that tumor angiogenesis of gastric carcinoma (GC) correlates with disease stage, lymphatic and distant metastases, recurrence after operation. A wide variety of angiogenic and antiangiogenic factors have been found to be important in this angiogenic process. Among the vast of angiogenic factors, vascular endothelial growth factor (VEGF) is reported to be pivotal for angiogenesis in gastric carcinogenesis. However, no extracellular molecule has yet been documented as being capable of up-regulating VEGF in GC. Interleukin-6 (IL-6) increases in Helicobacter pylori gastritis and is reported to be associated with the disease status of GC, but the mechanism underlying this association remains unclear. Our earlier research has found a positive association between the serum levels of IL-6 and VEGF in GC patients, which suggested that IL-6 might play a role for angiogenesis in GC via an interaction with VEGF production. Accordingly, we propose a hypothesis that IL-6 simulates tumor angiogenesis in GC via inducing VEGF. In this project, we will verify the association of IL-6, VEGF, and tumor vasculature by the clinical studies on tumor tissues and investigate their relationship by the experimental studies using GC cell lines.

During the first year of this project, we focus on the verification of the clinical association among IL-6, VEGF, and tumor vasculature in GC. GC samples obtained from surgical specimens at National Taiwan University are collected for immunohistochemical examination of IL-6, VEGF, and tumor microvessels. The immunoreactivities will be graded and then analyzed according to the different clinicopathologic characteristics. We found that there was a positive association among IL-6, VEGF, and tumor vasculature in different subsets of GC (Table 1, 2). In the experimental studies, we first assessed the effects of dose and duration of IL-6 simulation on VEGF production in different GC cell lines by ELISA (Figure 2a,b). Three signaling pathways, JAK/STAT, Raf/MAPK, and Akt/PI3K, mediate the cellular signals of IL-6 stimulation. Therefore, we next employed firefly luciferase reporter gene assay to determine the signaling pathway involved in the activation of the promoter of VEGF gene by IL-6 (Figure3). To study the effect of IL-6 on

angiogenesis *in vitro*, the growth and tube formation of human umbilical vein endothelial cells cultured with conditioned media obtained from the GC cells after IL-6 stimulation are to be assessed (Figure 4, 5a-d). In assessment of the effect of IL-6 on angiogenesis *in vivo*, Matrigel plug assay will be used. Matrigel plugs were harvested from mice injected subcutaneously with the mixture of Matrigel and conditioned media for gross examination and determination of hemoglobin densities, which stand for tumor vasculature (Figure 6a-e). We found that IL-6 indeed induced VEGF production in GC cells, which was mediated through JAK/STAT pathway. Meanwhile, we also demonstrated that IL-6 can increase in vitro and in vivo angiogenesis in GC.

During the second year of this project, we have enrolled 250 GC patients and 250 health controls and obtain their WBC DNA to compare the single nucleotide polymorphisms (SNPs) of IL-6 between these two groups. Our results demonstrated all Taiwanese showed IL-6 -174G/G genotypes. The SNP of IL-6 at another locus (-634C$\rightarrow$G) was also analyzed and in progress. In addition, immunohistoclemical stainings of IL-6 and VEGF have been performed in surgical specimens of GC. The expression of IL-6 and VEGF will be correlated with each other and related to the clinicopathologic features and microvessel density of GC. These results will further elucidate the role of IL-6 in angiogenesis of GC and shed light on its interaction with VEGF.

Keywords: angiogenesis, vascular endothelial growth factor, interleukin-6, gastric carcinoma, single nucleotide polymorphism

結果：Tables & Figures

Table 1	Table 2
Figure 2a	Figure 2b
Figure 3	Figure 4
Figure 5a	Figure 5b
Figure 5c	Figure 5d
Figure 6a-d	Figure 6e

Table 1. VEGF immunoreactivity and MVD in gastric carcinoma with different IL-6 immunoreactivities

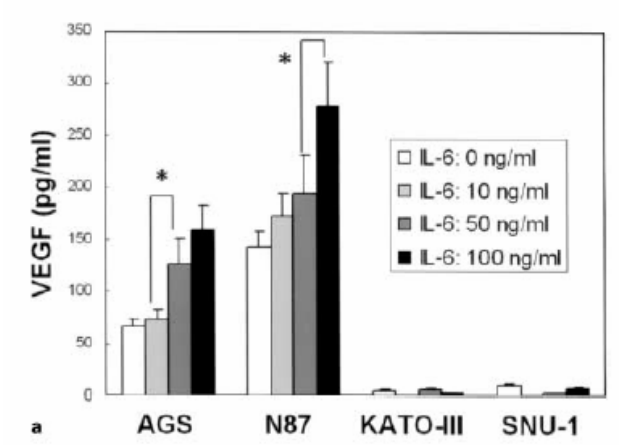
	Total	IL-6 immunoreactivity		
		high	low	p value
Cases	54	42	12	
Age, years	59.9 ± 13.5	62.1 ± 12.5	59.3 ± 13.9	0.539
Gender (M:F)	28:26	20:22	8:4	0.332
VEGF (H:L)	44:10	38:4	6:6	0.005
MVD[c]	37.8 ± 31.4	42.5 ± 32.0	21.5 ± 23.3	0.040

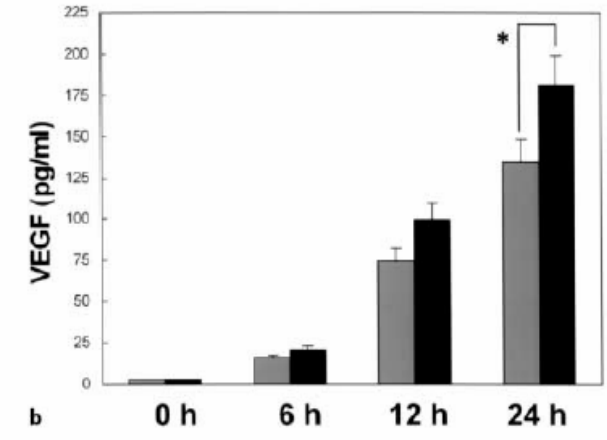
High = High immunoreactivity of IL-6 (– or +); low = low immunoreactivity of IL-6 (++ or +++); M = male; F = female; H = high immunoreactivity of VEGF; L = low immunoreactivity of VEGF.

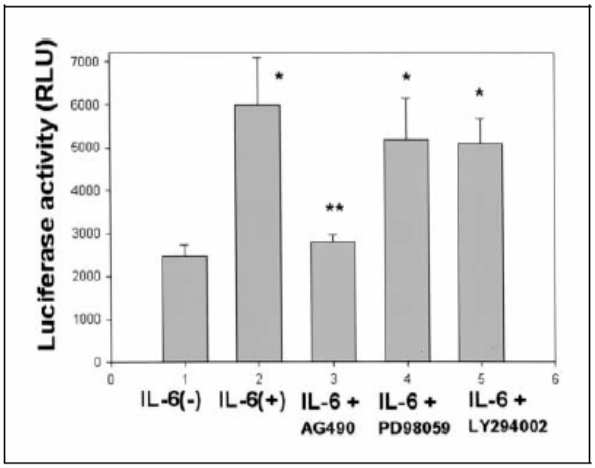
Table 2. Relationship between IL-6 and VEGF immunoreactivities in different subsets of gastric carcinoma

	High IL-6 high:low VEGF	Low IL-6 high:low VEGF	p value
Tumor stage			
Advanced	35:3	6:4	0.027*
Early	3:1	0:2	0.400
Laurén's type			
Intestinal type	23:1	4:4	0.009*
Diffuse type	15:3	2:2	0.210
Location			
Non-cardiac	31:4	5:6	0.006*
Cardiac	7:0	1:0	
H. pylori			
Present	23:4	1:4	0.009*
Absent	15:0	5:2	0.091

High:low VEGF = High/low immunoreactivity of VEGF.
* $p < 0.05$ by Fisher's exact test.







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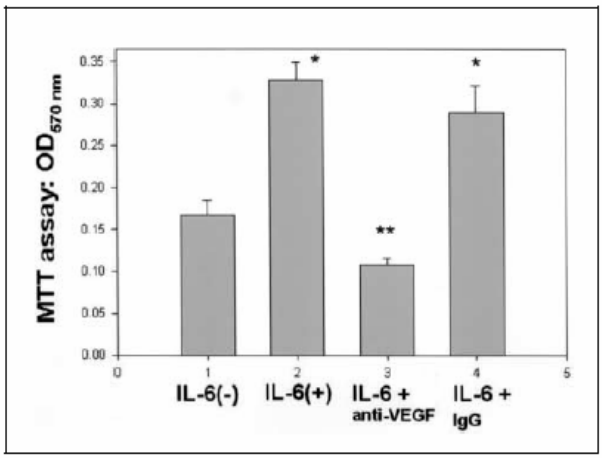

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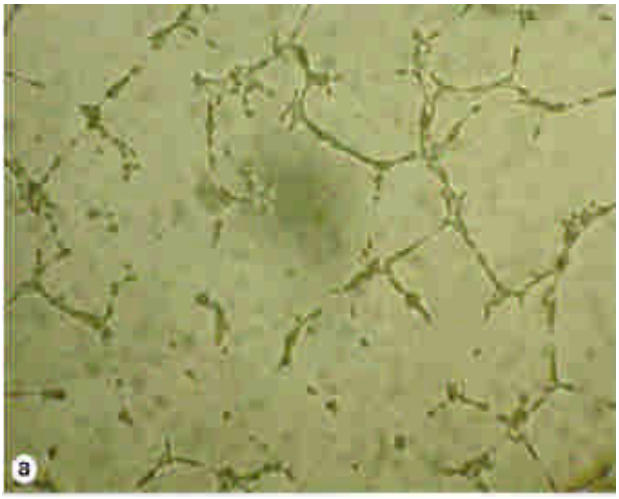
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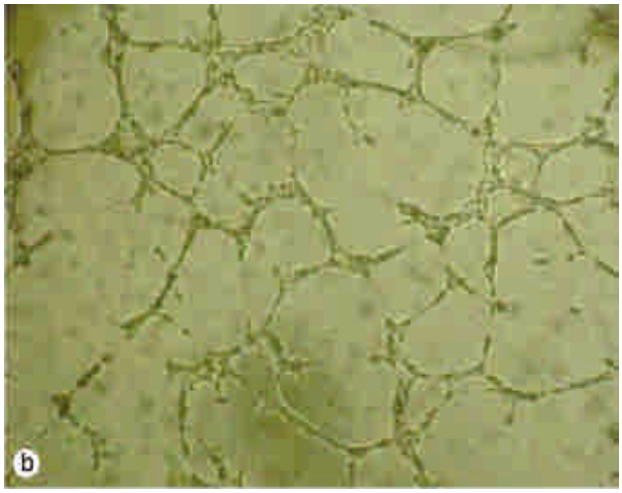
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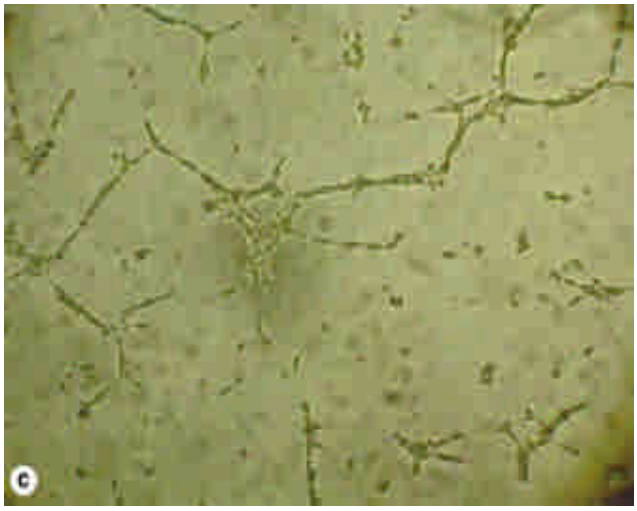
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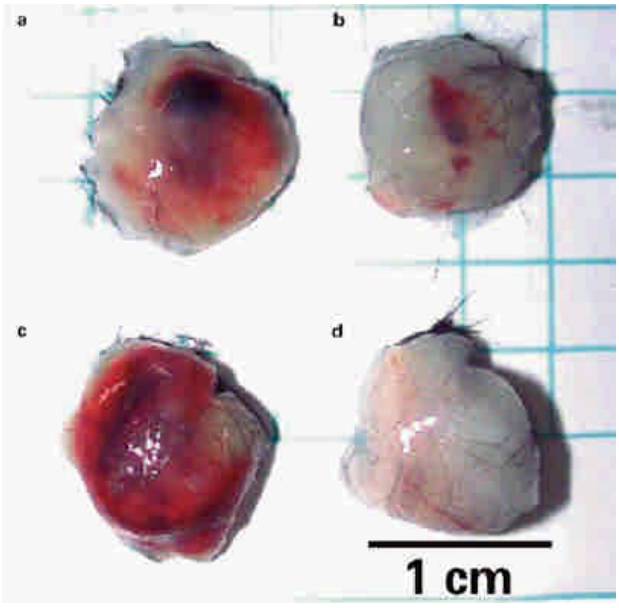
a
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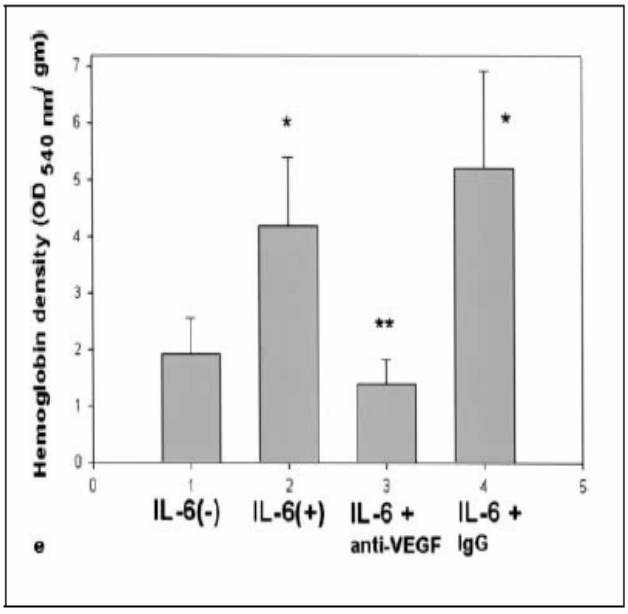
Hemoglobin density (OD 540 nm/ gm)
0
1
2
3
4
5
6
7
*
*
**
IL-6(-)
IL-6(+)
IL-6 + anti-VEGF
IL-6 + IgG
e

6

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