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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INTERLEUKIN-6 在人類子宮頸癌癌化過程所扮演的角色(2/2)

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計畫類別：個別型計畫
計畫編號：NSC　NSC 90-2314-B-002-208
執行期間：90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：謝長堯 教授
共同主持人：魏凌鴻
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一、中文摘要

介白質-6 是一個很重要的原發炎細胞激素，已被指出和子宮頸癌有關。然而，其在子宮頸癌發展的機制仍不清楚。在這個研究裡，我們藉由介白質-6 在子宮頸癌細胞株 C33A 過度表現來探討介白質-6 在子宮頸癌癌化機轉中扮演的角色。我們發現介白質-6 可以促進腫瘤在裸鼠體內的生長，但是不會改變在體外細胞生長的速率；在細胞株 C33A 體內或體外測試，介白質-6 都可以促進血管新生。由六天的 Matrigel Plug assay 可以看出，介白質-6 過度表現的 C33A 細胞株比轉殖對照載體的細胞株更具有促進血管新生的作用。我們也證實在多種血管新生因子中，介白質-6 特別和血管內皮細胞生長因子的表現有關。從 Luciferase assay 中得知介白質-6 可以增加血管內皮細胞生長因子 promoter 的活動力，而且這種誘導作用與與缺氧機制是無關的。此外，加入 anti-VEGF 抗體可以在體內體外測試中，阻斷介白質-6 刺激引起的血管新生現象。這些結果符合在子宮頸癌病人身上介白質-6 和血管內皮細胞生長因子表現強度的相伴增加。總結來說，介白質-6 可以獨立於缺氧機制，活化血管內皮細胞生長因子調控的血管新生進而促進子宮頸癌的癌化。

關鍵詞：介白質-6、子宮頸癌、腫瘤血管新生

Abstract

Interleukin-6 (IL-6) has received particular attention in the pathogenesis of cervical cancer, though the underlying mechanism remains elusive. This study revealed that IL-6 promotes *in vivo* tumor growth of human cervical cancer C33A cells but does not substantially alter their *in vitro* growth kinetics. The *in vivo* angiogenic assays showed that IL-6 increases angiogenic activity in human cervical cancer cells, an effect which is specifically associated with up-regulation of vascular endothelial growth factor (VEGF). Also, using anti-VEGF antibody to blockade VEGF function significantly inhibited IL-6-mediated angiogenesis and tumor growth in nude mice,

strongly supporting the critical role of VEGF in the IL-6-mediated cervical tumorigenesis. Accordingly, the signaling pathway downstream of IL-6/IL-6R responsible for the regulation of VEGF was investigated. Notably, pharmacological inhibition of PI3-K or MAPK failed to inhibit IL-6-mediated transcriptional up-regulation of VEGF. Meanwhile, blocking STAT3 pathway with dominant-negative mutant STAT3D effectively abolished IL-6-induced VEGF mRNA. In transient transfections, a luciferase reporter construct containing the full-length 1.5-kb VEGF promoter or a 1.2-kb fragment lacking the known hypoxic-response element also exhibited the same degree of response to IL-6. Additionally, transient transfection of STAT3D down-regulated the 1.2-kb VEGF promoter luciferase reporter stimulated by IL-6. Based on the above phenomenon combined with the concomitant increased tumor expression of IL-6 and VEGF in cervical cancer tissues, we conclude that IL-6 may promote cervical tumorigenesis by activating VEGF-mediated angiogenesis via a STAT3 pathway.

Keywords: angiogenesis, cervical cancer, cytokine, interleukin-6, STAT3, vascular endothelial growth factor

二、緣由與目的

Cervical cancer is a leading neoplastic disease that inflicts women worldwide. An etiologic relationship between high-risk human papillomavirus (HPV) and cervical cancer has been firmly established (Zur Hausen, 2002). The local immune system of the genital tract is assumed to be important in the monitoring of HPV-related cervical cancer. Consequently, various cytokines have been implicated in the pathogenesis of cervical cancer, among which interleukin-6 (IL-6) has received particular attention. Epidemiologic studies have shown that the development of cervical cancer is associated with chronic inflammation resulting from multiple sexually transmitted agents (Schiffman and Brinton, 1995). Viral infection and exposure to proinflammatory cytokines such as IL-1α and tumor necrosis factor α can stimulate IL-6 gene expression by the keratinocytes (Iglesias *et al*, 1995). IL-6 expression in tumor tissues is high and correlates with the severity of

cervical cancer (Tartour *et al,* 1994; Tjiong *et al,* 1999; Wei *et al,* 2001a). Also, IL-6 may contribute to a local immunosuppressive effect that protects the tumor cells from the host immune system (Tilg *et al,* 1997). These works support the hypothesis that IL-6 promotes the development of cervical cancer, though the underlying mechanism remains elusive.

IL-6 is a secreted, multi-functional glycoprotein. Through binding to α-chain (IL-6-Rp80) and subsequently recruiting the β-chain (gp130) of the receptor, IL-6 performs various biological functions (Kishimoto *et al,* 1995). Specifically, the IL-6/IL-6Rp80 complex initiates homodimerization of gp130, activates a cytoplasmic tyrosine kinase bound to gp130 (Murakami *et al,* 1993), and then triggers signaling cascades through the Jak/STAT, Ras/MAPK, and PI 3-K/Akt pathways (Hirano *et al,* 1997). The diversity of IL-6 signaling mediated via gp130 explains its functional pleiotrophy. IL-6 regulates inflammatory reactions, immune responses, hepatic acute-phase protein synthesis, and several other important physiological processes (Taga and Kishimoto, 1997). Interestingly, the influence of IL-6 in human cancers is varied depending on the cell types. For example, IL-6 has been demonstrated to promote growth of multiple myeloma, Kaposi's sarcoma and prostatic cancer cells, while inhibiting the proliferation of lung and breast cancer cells (Danforth and Sgagias, 1993; Klein *et al,* 1995; Nicholas *et al,* 1997; Okamoto *et al,* 1997; Takizawa *et al,* 1993).

Angiogenesis is essential for the development, growth, and progression of cervical cancer (Hanahan and Folkman, 1996; Sillman *et al,* 1981; Smith-McCune *et al,* 1997; Smith-McCune and Weidner, 1994). The angiogenic process is balanced by various positive (such as bFGF, VEGF, and PD-ECGF) and negative (such as thrombospondin-1, platelet factor-4, and interferon α/β) regulatory molecules of endothelial proliferation and migration (Carmeliet, 2000). Previous investigations have confirmed that IL-6 is important in both physiological and pathological angiogenesis (Mahnke *et al*, 2000; Mateo *et al*, 1994; Motro *et al*, 1990), raising the possibility that elevated IL-6 in the uterine cervix promotes the development of cervical neoplasm

via angiogenesis stimulation.

This work focused on the angiogenic and tumorigenic potential of IL-6 overexpressed C33A cervical cancer cells. The experimental results revealed that IL-6 overexpressed C33A cells displayed accelerated tumor growth and neovascularization in nude mice. Furthermore, blockage of VEGF function was found to inhibit IL-6-mediated angiogenesis and reduce IL-6-induced tumor growth in nude mice. IL-6 specifically up-regulates VEGF in C33A cells via a STAT3 pathway. Earlier studies have demonstrated consistently higher expression of IL-6 and VEGF in cervical cancer tissues (Wei *et al*, 2001a). Therefore, we believe strong evidence exists to support the hypothesis that IL-6 facilitates tumorigenesis of cervical cancer via VEGF-mediated angiogenesis.

三、研究成果

Growth properties of IL-6 overexpressed cervical cancer cells

Five C33A clones with stable IL-6 gene transfection were established and designated as C33A/IL-6 cells (C33A/IL-6-c1, -c2, -c3, -c4, and -c5), while that with control vector were designated as C33A/*neo* cell. ELISA confirmed that C33A/IL-6 cells produced significantly higher levels of IL-6 (> 2,000 *pg*/ml) than did C33A/*neo* cells (< 50 *pg*/ml). Neither the IL-6 overexpressed cells nor the *neo* control cells caused changes in IL-6 receptor levels, which were measured by immunoblotting with an antibody that recognized the ligand binding subunit of the IL-6 receptor (Wei *et al,* 2001b). Also, the *in vitro* growth properties of C33A/IL-6 and C33A/*neo* cells were examined. Figure 1a shows that IL-6 overexpression did not affect cell proliferation rate. Additionally, treatment with exogenous IL-6 did not influence the proliferation rate of parental C33A cells (data not shown). Collectively, the above results suggest that overexpression of IL-6 does not affect receptor levels and anchorage-dependent growth in C33A cells.

IL-6 accelerated tumor growth in nude mice

Next the influence of IL-6 on *in vivo* tumorigenesis was examined. Two IL-6 overexpressed cells (C33A/IL-6-c1 and –c5) and *neo* control cells were subcutaneously injected into 4 to 5 week-old nude mice. Then tumorigenicity was assessed weekly according to tumor volume. The tumors

derived from C33A/IL-6-c1 cell grew faster than those from C33A/*neo* cell after 2 to 3 weeks of initial development. The mean tumor size in C33A/IL-6-c1 transplanted nude mice was around 3500 mm^3 at the 6th week post-transplantation, but in nude mice injected with C33A/*neo* cells mean tumor size reached merely 1100 mm^3 (Figure 1b). In a separate experiment, the mean volume of C33A/IL-6-c5 tumors grew to a similar size to C33A/IL-6-c1 tumors at the 6th week and was approximately 7 times the size of C33A/*neo* tumors at the 8th week after transplantation (Figure 1c). These experimental results clearly verify that IL-6 can enhance *in vivo* tumor growth of cervical cancers without significantly altering their *in vitro* growth rate.

Angiogenic activity of C33A cells is augmented by IL-6

Angiogenesis is a prerequisite to the rapid clonal expansion associated with the formation of macroscopic tumors. Gross examination of the peripheral segments of C33A xenografts revealed that the tumors produced by the IL-6 overexpressed C33A cells were highly vascularized, whereas those produced by the C33A/*neo* cells displayed slight new blood vessel growth (data not shown). The *in vivo* model of chick embryo CAM assay can be used to clarify the influence of IL-6 on cervical tumor angiogenesis. Upon dissection of the CAM of a 12-day-old chick embryo, low angiogenic activity in CAM was observed using the conditioned-medium (CM) from parent C33A or *neo* control cell cultures (Figure 2a). Importantly, high angiogenic activity was found on CAM treated using CM from IL-6 transfectant. To quantify the different types of tumor angiogenic activity before the appearance of a grossly visible tumor, *in vivo* Matrigel plug assays were performed. The experimental results revealed that CM from IL-6 overexpressed cells displayed more vascularization in the matrigel plug on the 7th day after inoculation (Figure 2b, left). The degree of vascularization can be measured based on the hemoglobin contents (Figure 2b, right). These biological assays confirm that IL-6 may eincrease angiogenic activity in human cervical cancer cells.

Up-regulation of VEGF by IL-6 in cervical cancer cells and tumors

RT-PCR was used to assess expression of various angiogenic factors, including VEGF, bFGF, PDGF, and IL-8, that previously have been reported to contribute to tumor angiogenesis in cervical cancer (Cheng *et al,* 1999; Fujimoto *et al,* 1997; Fujimoto *et al,* 2000; Fujimoto *et al,* 1999a; Kodama *et al,* 2001). Of the genes tested, VEGF-A mRNA but not other factors, was elevated in IL-6 overexpressed C33A cervical cancer cells (Figure 3a). Consistent with the mRNA level, CM contained a significant amount of VEGF-A protein from IL-6 overexpressed C33A cells (Figure 3b). Moreover, the gene expression of VEGF was measured in tissue from C33A/*neo* and C33A/IL-6 tumors transplanted to nude mice. RT-PCR revealed that tumors from the IL-6 overexpressed C33A cells had significantly higher VEGF mRNA levels than those from *neo* control cells (Figure 3c). These experimental results indicate that VEGF is the major mediator of IL-6-induced tumor angiogenesis.

Blockage of VEGF function inhibits IL-6-mediated angiogenesis and inhibits IL-6-induced tumor growth in nude mice

To clarify whether VEGF influenced IL-6-mediated tumorigenesis, 10 mg/kg anti-VEGF antibody was intraperitoneally injected on alternative days specifically to block VEGF function in nude mice transplanted with IL-6 overexpressed C33A cells. From the second week after tumor inoculation, tumor growth was significantly delayed in the anti-VEGF antibody treated group (Figure 4a). The tumors in the C33A/IL-6-c5 and control mouse IgG antibody groups (10 mg/kg) grew steadily during the experiment, but those in the anti-VEGF antibody treated group did not. On day 29 after tumor implantation, the tumor weight was 43±5 mg in the anti-VEGF antibody group compared to 625±155 mg in the untreated group ($p<0.003$) and 201±50 mg in the control antibody group ($p<0.006$). Meanwhile, the tumor weight in the C33A/*neo* group reached just 123±58 mg. Furthermore, the relative mRNA expression of mouse PECAM-1 in C33A/IL-6-c5 and C33A/*neo* tumors was examined using RT-PCR. The experimental results revealed that expression of PECAM-1 mRNA was considerably up-regulated in C33A/IL-6-c5 tumors (Figure 4b), which was

consistent with the more vascularized appearance of IL-6 overexpressed tumors. Treatment with anti-VEGF antibody led to a marked down-regulation of PECAM-1 mRNA expression in the C33A/IL-6-c5 tumors as compared to the control antibody (Figure 4b). These works indicate that VEGF is responsible for IL-6-mediated tumorigenicity in cervical cancer.

STAT3 pathway is essential for VEGF up-regulation by IL-6

To define the signaling pathway involved in transcriptional up-regulation of VEGF by IL-6, C33A cells were treated with different pharmacological inhibitors. Treatment with PD98059 and LY294002, which specifically inhibit MEK1 and PI3-K respectively, did not influence IL-6-mediated up-regulation of VEGF (Figure 5a). Consequently, the involvement STAT3 pathway in the IL-6-mediated VEGF up-regulation was examined. To address this issue, control vector and STAT3 dominant-negative mutant (STAT3D), in which E434 and E435 of STAT3 were replaced with alanines (Horvath *et al*, 1995), were introduced into C33A cells. As shown in figure 5b, transfection of STAT3D in C33A cells significantly inhibited the VEGF expression as compared to the parent C33A and *neo* control cells, suggesting that STAT3 is crucial to the IL-6-mediated VEGF up-regulation. Since tissue hypoxia is a potent inducer of VEGF induction, reporter gene assay was used to examine whether the IL-6 up-regulated VEGF through a hypoxia-dependent mechanism. Figure 5c indicated that only the 1.5kb promoter responded to cobalt chlorides, but both the1.5kb and 1.2kb (lacking HIF-1 biding site) promoters of VEGF were markedly activated by IL-6 treatment, suggesting IL-6 is capable of stimulating VEGF promoter through a HIF-1-independent mechanism. However, when STAT3D transiently transfected into C33A cells, the activation of VEGF promoter by IL-6 reduced significantly (Figure 5d).

Co-expression of IL-6 and VEGF in human cervical cancer

The close association between IL-6 and VEGF revealed above prompted investigation of the expression of IL-6 and VEGF in cervical cancer tissues. To address this issue, biopsies of cervical cancer tissues and adjacent noncancer tissues were taken from four patients with invasive cervical cancer to analyze the levels of IL-6 and VEGF proteins by Western blotting analysis. Figure 6

displayed that both IL-6 and VEGF expressions were significantly more abundant in cancer tissues than in noncancer tissues. This finding provides clinical evidence to support a close relationship between IL-6 and VEGF in the development of cervical cancer.

五、參考文獻

Aoki Y, Jaffe ES, Chang Y, Jones K, Teruya-Feldstein J, Moore PS and Tosato G. (1999). *Blood*, **93,** 4034-43.

Belec L, Gherardi R, Payan C, Prazuck T, Malkin JE, Tevi-Benissan C and Pillot J. (1995). *Cytokine*, **7,** 568-74.

Carmeliet P. (2000). *Nat. Med.*, **6,** 389-95.

Castrilli G, Tatone D, Diodoro MG, Rosini S, Piantelli M and Musiani P. (1997). *Br. J. Cancer*, **75,** 855-9.

Chen Z, Malhotra PS, Thomas GR, Ondrey FG, Duffey DC, Smith CW, Enamorado I, Yeh NT, Kroog GS, Rudy S, McCullagh L, Mousa S, Quezado M, Herscher LL and Van Waes C. (1999). *Clin. Cancer Res.*, **5,** 1369-79.

Cheng WF, Chen CA, Lee CN, Wei LH, Hsieh FJ and Hsieh CY. (2000). *Obstet. Gynecol.*, **96,** 721-6.

Cheng WF, Chen CA, Lee CN, Chen TM, Hsieh FJ & Hsieh CY. (1999). *Obstet. Gynecol.*, **93,** 761-5.

Danforth DN Jr and Sgagias MK. (1993). *Cancer Res.*, **53,** 1538-45.

Dankbar B, Padro T, Leo R, Feldmann B, Kropff M, Mesters RM, Serve H, Berdel WE and Kienast J. (2000). *Blood*, **95,** 2630-6.

Dobbs SP, Hewett PW, Johnson IR, Carmichael J and Murray JC. (1997). *Br. J. Cancer*, **76,** 1410-5.

Eustace D, Han X, Gooding R, Rowbottom A, Riches P and Heyderman E. (1993). *Gynecol. Oncol.*, **50,** 15-9.

Ferrara N and Davis-Smyth T. (1997). *Endocr. Rev.*, **18,** 4-25.

Fujimoto J, Ichigo S, Hori M, Hirose R, Sakaguchi H and Tamaya T. (1997). *Cancer Lett.*, **111,** 21-6.

Fujimoto J, Sakaguchi H, Aoki I and Tamaya T. (2000). *Cancer Res.*, **60,** 2632-5.

Fujimoto J, Sakaguchi H, Hirose R, Ichigo S and Tamaya, T. (1999a). *Br. J. Cancer*, **79,** 1249-54.

Fujimoto J, Sakaguchi H, Hirose R, Ichigo S and Tamaya T. (1999b). *Br. J. Cancer*, **80,** 827-33.

Guidi AJ, Abu-Jawdeh G, Berse B, Jackman RW, Tognazzi K, Dvorak HF and Brown LF. (1995). *J. Natl. Cancer Inst.*, **87,** 1237-45.

Hanahan D and Folkman J. (1996). *Cell*, **86,** 353-64.

Hirano T, Ishihara K and Hibi M. (2000). *Oncogene*, **19,** 2548-56.

Hirano T, Nakajima K and Hibi M. (1997). *Cytokine Growth Factor Rev.*, **8,** 241-52.

Horvath CM, Wen Z and Darnell JE Jr. (1995). *Genes Dev.*, **9,** 984-94.

Iglesias M, Plowman GD and Woodworth CD. (1995). *Am. J. Pathol.*, **146,** 944-52.

Jee SH, Shen SC, Chiu HC, Tsai WL and Kuo ML. (2001). *Oncogene*, **20,** 198-208.

Jiang BH, Jiang G, Zheng JZ, Lu Z, Hunter T and Vogt PK. (2001). *Cell Growth Differ.*, **12,** 363-9.

Kishimoto T, Akira S, Narazaki M and Taga T. (1995). *Blood*, **86,** 1243-54.

Klein B, Zhang XG, Lu ZY and Bataille R. (1995). *Blood*, **85,** 863-72.

Kodama J, Hashimoto I, Seki N, Hongo A, Yoshinouchi M, Okuda H and Kudo T. (2001). *Clin. Cancer Res.*, **7,** 2826-31.

Kodama J, Seki N, Tokumo K, Hongo A, Miyagi Y, Yoshinouchi M, Okuda H and Kudo T. (1999). *Eur. J. Cancer*, **35,** 485-9.

Lee JS, Kim HS, Jung JJ, Lee MC and Park CS. (2002). *Gynecol. Oncol.*, **85,** 469-75.

Lopez-Ocejo O, Viloria-Petit A, Bequet-Romero M, Mukhopadhyay D, Rak J and Kerbel RS. (2000). *Oncogene*, **19,** 4611-20.

Mahnke JL, Dawood MY and Huang JC. (2000). *Fertil. Steril.*, **73,** 166-70.

Masood R, McGarvey ME, Zheng T, Cai J, Arora N, Smith DL, Sloane N and Gill PS. (1999).