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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※　　人類乳突病毒與血管新生:從臨床到基礎　　※

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計畫主持人：陳祈安

共同主持人：

本成果報告包括以下應繳交之附件：

☐赴國外出差或研習心得報告一份

☐赴大陸地區出差或研習心得報告一份

☐出席國際學術會議心得報告及發表之論文各一份

☐國際合作研究計畫國外研究報告書一份

執行單位：台大醫院婦產科

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中文摘要

為了要釐清人類乳突病毒及婦女生殖器官發炎反應之重要淋巴激素 IL-6,在子宮癌血管新生所扮演的角色,細胞 IL-6 濃度以酵素免疫分析(enzyme immunoassay)方式在 60 位早期子宮癌(Ib-IIa 期)病人測得, IL-6 及其受體以 immunohistochemical staining 方式分析, HPV DNA 則以 PCR 方式分析。IL-6 及血管內皮細胞生長因子的濃度在癌組織裏比臨近非癌組織高($p<0.01$),加入 recombinant IL-6 可以隨時間及濃度引發血管內皮細胞生長因子產生。IL-6 濃度在癌組織裏明顯的比較高。在 45 歲以上之病人($p<0.01$),腫瘤大於 2cm($p<0.01$),及有致癌 HPV 感染($p<0.01$)的病人。此外,有致癌 HPV 感染的病人之腫瘤內 IL-6 濃度也較無感染的病人為高($p=0.04$)。在 squamous cell carcinoma 的病人其 IL-6 明顯較高($p=0.02$)。因此局部 IL-6 及 HPV 有加成作用,在致癌過程中活化 angiogenic switch。此生物特性的抑制在未來可能做為治療的標的.

英文摘要

To elucidate the role of HPV and IL-6, a central proinflammatory cytokine involved in the female genital infection, in the angiogenic switch during uterine cervical carcinogenesis, cytosolic IL-6 levels were determined via enzyme immunoassay in 60 FIGO Stage IB-IIA patients. The levels of IL-6 and IL-6 receptor were analyzed by immunohistochemical staining and human papillomavirus (HPV) DNA was detected by PCR-based survey. There were consistently higher expressions of IL-6 and vascular endothelial growth factor (VEGF) in cancer tissues as compared with adjacent non-cancer tissues ($p<0.01$; respectively). Adding recombinant human IL-6, VEGF was induced at dose and time dependent manner in cervical cancer cell lines. Significant higher expression of IL-6 in cancer tissue was observed in patients older than 45 y/o ($p<0.01$), with tumor >2 cm ($p<0.01$), and oncogenic HPV infection ($p<0.01$). Patients with oncogenic HPV infection have higher intra-tumor IL-6 concentration than patients without ($p=0.04$). In patients with squamous cell carcinoma, significantly higher IL-6 production was observed in cancer tissue ($p=0.02$). Taken together, the focal IL-6 may have a synergistic effect with HPV and activate angiogenic switch during cervical carcinogenesis. Inhibition of its biologic activity may be of potential therapeutic benefit.

計畫緣由與目地

Cervical cancer is the second most common female cancer and is the leading cause of cancer-related death among women worldwide (Ponten *et al.*, 1995). Epidemiologic studies have suggested that the majority cases can be attributed to the infection with oncogenic HPV type 16 or 18. However, as compared with the extremely high lifetime cumulative incidence of cervical infection with HPV, only a minority of HPV infections progresses to malignancy. Therefore, the emergence of certain tumor-promoting factors, such as amplification of oncogenes (e.g., c-MYC, Ha-RAS, and ERB-2) (Pinion *et al.*, 1991) and/or up-regulation of proinflammatory cytokines (Tartour *et al.*, 1999; Tartour *et al.*, 1994), are necessary to enhance cervical intraepithelial neoplasm to progress to invasive cancer.

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is essential for the growth and metastasis of solid tumors (Folkman, 1990). In the uterine cervix, significant increases in microvessels density have been shown in severe cervical dysplasia as compared with low-grade lesions (Smith-McCune & Weidner, 1994), suggesting an active

angiogenesis was readily apparent in the dysplatic stage. Moreover, transgenic mouse models of tumorigenesis have revealed an angiogenic switch occurs during the early stages of tumor development, indicating that regulation of angiogenesis is a potentially rate-limiting step in the cervical carcinogenesis (Hanahan & Folkman, 1996).

Tumor angiogenesis is a complex process controlled by many growth factors and cytokines that act either directly or indirectly. In the uterine cervical cancers, angiogenic factors that derived from cancer cells or stromal cells included VEGF (Cheng *et al.*, 1999), platelet-derived growth factor (PDGF) (Fujimoto *et al.*, 1999), basic fibroblastic growth factor (bFGF) (Fujimoto *et al.*, 1997), and IL-8 (Fujimoto *et al.*, 2000). Among them, VEGF was found to be the main angiogenic factor associated with progression of intraepithelial neoplasm to early invasive uterine cervical cancer (Guidi *et al.*, 1995). VEGF is an endothelial cell-specific mitogen, and is the linking factor between tissue hypoxia and a compensatory angiogenic response in the context of underperfused tumors (Leung *et al.*, 1989; Shweiki *et al.*, 1992). However, the regulation of VEGF is still elusive during angiogenic switch of cervical carcinogenesis.

Uterine cervical cancer frequently develops in close association with chronic inflammation due to infection with a variety of sexually transmitted agents (Schmauz *et al.*, 1989). IL-6, a mediator of the inflammatory response, was found to be elevated in cervical secretions of women with pelvic inflammatory disease, and could also be used to predict the severity of genital tract infection as well as its long-term sequelae (Richter *et al.*, 1999). IL-6 has been shown elevated in tissues that undergo active angiogenesis. Therefore, we are prompted to study the potential role of IL-6 in tumor angiogenesis, and its association with HPV during cervical carcinogenesis.

結果

Sixty patients with early stage cervical carcinoma were enrolled in this study (FIGO stage IB-IIA). The age of the patients ranged from 23-70 years with a mean age of 48.7 years. Fifty-six patients were stage Ib, and 4 patients were stage IIa cervical cancer. The histologic types were squamous cell carcinoma in 49 patients (81.7%), adenocarcinoma in 8 patients (13.3%), adenosquamous carcinoma in 2 patients (3.3%), and small cell carcinoma in one patient (1.7%). HPV type 16 or 18 infections were detected in 43 patients (71.7%) by PCR-based survey. Intra-tumoral IL-6 levels ranged from 5.3-58.4 *pg*/mg (25-75% quantiles), with the median of 14.2 pg/mg. IL-6 levels in adjacent non-cancer tissues ranged from 2.9-58.6 *pg*/mg (25-75% quantiles), with the median of 6.0 *pg*/mg.

As shown in Table 1, significant higher concentrations of IL-6 in cancer than adjacent non-cancer tissues were observed in cervical cancer patients as determined by ELISA ($p<0.01$; Log transformed paired t-test). VEGF, but not PDGF, level is significantly elevated in cancer tissues as compared to non-cancer tissues (Table 1). Using log transformed paired t-test, significant higher expression of VEGF in cancer tissues was observed (11.6-70.6 vs 9.4-27.8 *pg*/mg, $p<0.01$), whereas there was no significant different expression of PDGF (23.4-41.9 vs 23.2-37.4 *pg*/mg, $p=0.43$), indicating that the major growth factor involved in the angiogenic switch during cervical carcinogenesis is VEGF. Since the level of IL-6 and VEGF is coincidentally increased in cancer tissues, we are prompt to investigate the effect of IL-6 on the expression of VEGF. We added recombinant human IL-6 to an HPV-negative cervical cancer cell line C33A, and showed that VEGF protein was induced by IL-6 at dose and time dependent pattern in C33A.

Table 2 showed the association between clinicopathological variables and the concentrations of IL-6. In the young age group (age<45 y/o), there was no significant different expression of IL-6 in cancer tissue (5.0-35.2 vs 3.3-54.7 *pg*/mg, $p=0.66$; Log transformed paired t-test). On the other hands, significant higher expression of IL-6 was

observed in patients older than 45 y/o (5.8-113.0 vs 2.8-79.9 *pg*/mg, p<0.01; Log transformed paired t-test). Though not statistically significant, the expression of IL-6 in cancer tissue was somewhat higher in old age group (median 20.4 vs 10.1 *pg*/mg, p=0.07; Log transformed t-test).

The association between the IL-6 expression and HPV-16 or-18 infection is strong (Table 2). Patients with HPV infection had higher intra-tumor IL-6 production (16.1vs 8.8 *pg*/mg, p=0.04; Log transformed t-test). Using log transformed paired t-test, we can observed that higher IL-6 expression in cancer tissue as compared with adjacent non-cancer tissue in patients with HPV infection (5.8-105.9 vs 2.8-23.7 *pg*/mg, p<0.01). In contrast, the distribution of IL-6 was not significant different between cancer and adjacent non-cancer tissue in HPV negative patients (4.6-20.5 vs 3.7-99.8 *pg*/mg, p=0.15; Log transformed paired t-test).

討論

In the present study, there are local over-expressions of IL-6 and their receptors in uterine cervical cancer tissues that undergo active angiogenesis, manifested by high tissue levels VEGF. We demonstrated that IL-6 could augment VEGF secretion of C33A cervical cancer cell line. In addition, significant suppression of VEGF secretion by SiHa cells could be achieved by using anti-IL-6 or anti-IL-6 receptor α chain antibodies. These findings are in line with previous reports on VEGF induction by IL-6 in Kaposi's sarcoma (Aoki *et al.*, 1999) and multiple myeloma (Padro *et al.*, 2000), suggesting the role of IL-6 in angiogenic switch of cervical carcinogenesis.

Uterine cervical cancer frequently develops with multiple, concurrent infection with HPV, herpes simplex virus, and chlamydia (Schmauz *et al.*, 1989). Inflammation and cell injury may cause release of proinflammatory cytokines such as IL-1 α and IL-6, which in turn may modulate tumor growth in the host (Castrilli *et al.*, 1997). IL-6 is a multifunctional growth-regulatory cytokine, which exerts diverse biological effects depending on the target cell types. In malignant cells, IL-6 may act as a potent growth activator for myeloma (Klein *et al.*, 1989), ovarian carcinoma (Watson *et al.*, 1993), or growth inhibitor for melanoma (Lu *et al.*, 1992), and lung cancer (Takizawa *et al.*, 1993). In uterine cervical cancer, IL-6 may promote tumor cell proliferation by either autocrine (Iglesias *et al.*, 1995) or paracrine (Tartour *et al.*, 1994) mechanism. With the over-expression of IL-6 in cervical cancer tissue and marked IL-6 receptor expression in less differentiated cells at peripheral of tumor cell nests (Fig. 1D), it is believed that the IL-6 signal cascade is initiated and provides growth advantages at tumor site.

Recent in vitro study reported that HPV-16 E7 oncoprotein enhances the release of angiogenic cytokines, including TNF-α, IL-1β and IL-6 by macrophage and dendritic cells (Le Buanec *et al.*, 1999). In the current study, we find higher IL-6 production in patients with oncogenic HPV infection (Table 2), and higher levels of IL-6 were observed in cancer than adjacent non-cancer tissues in oncogenic HPV infected patients. Thus, it seems that IL-6 may have synergistic effect to oncogenic HPV infection; as tumors grow in size, different expressions of tissue levels of IL-6 become more significant. In our study, there were no significant differences in the expression of IL-6 with respect to depth of stromal invasion, endocervical invasion, vaginal invasion, lymphovascular emboli and lymph node metastasis. This implies that the relative contribution of IL-6 in cervical carcinogenesis may be an early event, that is, from dysplastic stage to early invasive cancer.

No risk factors have been found in case-control studies to distinguish invasive cancer from CIN2-3, with the exception of age (Schiffman & Brinton, 1995). Our results, demonstrating the expression of IL-6 is highly associated with patients' age, shed some light on the underline molecular events for this phenomenon. Elevated IL-6 levels in the genital tract are reliable markers of pelvic inflammatory disease (Richter *et al.*, 1999). During host defense

responses to infections, IL-6 is induced in the infected cells and in cells of the immune system by microbial products such as bacterial lipopolysaccharides, viral RNAs, and viral proteins. Chronic cervicovaginal inflammation may result in high microenvironmental IL-6, which may increases the oncogenicity of HPV infection through activates the angiogenic switch.

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TABLE 1.

Tissues Levels of IL-6, VEGF, and PDGF by Enzyme Immunoassay

	Median (25-75% quantiles)	p*
IL6 (cancer tissues)	14.2 (5.3-58.4)	<0.01
IL6 (adjacent non-cancer tissues)	6.0 (2.9-58.6)	
VEGF (cancer tissues)	26.7 (11.6-70.6)	<0.01
VEGF (adjacent non-cancer tissues)	16.6 (9.4-27.8)	
PDGF (cancer tissues)	29.8 (23.4-41.9)	0.43
PDGF (adjacent non-cancer tissues)	25.8 (23.2-37.4)	

* Log transformed paired t-test

TABLE 2.

The Association between Clinicopathological Characteristics and IL-6 Expression

	N	Cancer tissue Median (25-75% quantiles)	Adjacent non-cancer tissue Median (25-75% quantiles)	p[a]
Age				
≤45	26	10.1 (5.0-35.2)	6.6 (3.3-54.7)	p=0.66
>45	34	20.4 (5.8-113.0)	6.0 (2.8-79.7)	**p<0.01**
p[b]		p=0.07	p=0.83	
HPV type 16 or 18 status				
Negative	17	8.8 (4.6-20.5)	9.5 (3.7-99.8)	p=0.15
Positive	43	16.1 (5.8-105.9)	6.0 (2.8-23.7)	**p<0.01**
p[b]		**p=0.04**	p=0.14	
Histologic type				
Squamous	49	15.4 (5.8-94.4)	7.1 (3.4-62.6)	**p=0.02**
Non squamous	11	8.7 (3.2-33.4)	3.7 (1.5-16.3)	p=0.20
p[b]		p=0.09	p=0.18	
Patho. Tumor size (cm)				
≤2	35	10.0 (5.0-47.7)	7.2 (2.8-70.8)	p=0.32
>2	25	20.3 (8.5-61.5)	4.7 (2.9-27.9)	**p<0.01**
p[b]		p=0.23	p=0.47	

[a] Log transformed paired t test

[b] Log transformed t test