

Significance of Expression of TGF- β in Pulmonary Metastasis in Non-small Cell Lung Cancer Tissues

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Recent studies have evaluated the cytokine network involved in the local immune response to tumors. In addition to infiltrating inflammatory cells, tumors also produce cytokines and growth factors that may alter tumor growth and tumor immunogenicity. Ninety-one samples of NSCLC were used in this study. We measured the expression of VEGF, TNF- α , TGF- β , IL-6, IL-8, IL-12, INF- γ , and MCP-1 in NSCLC tissues, by ELISA. The expression of IL-6 and IL-8 were significantly higher in squamous cell carcinoma than in adenocarcinoma ($p=0.016$ and $p<0.001$, respectively). The expression of TGF- β , MCP-1 and IL-8 were significantly higher in pulmonary metastasis positive than negative cases ($p=0.002$, $p=0.001$, and $p=0.008$, respectively). In multivariate logistic regression analysis, the expression of TGF- β was an independent risk factor for the occurrence of pulmonary metastasis ($p=0.008$, 95% CI=1.002-1.011). We confirmed that tumor infiltrating stromal cells were major sources of TGF- β by immunohistochemical analysis. The expression of VEGF and IL-8 were significantly higher in cases with central necrosis ($p=0.006$ and $p=0.011$, respectively). We speculated that TGF- β expression in tumor infiltrating stromal cells may regulate the occurrence of spontaneous pulmonary metastasis in NSCLC. (*Ann Thorac Cardiovasc Surg* 2003; 9: 295–300)

Key words: cytokine, TGF- β , pulmonary metastasis, non-small cell lung cancer

Introduction

Recent studies have evaluated the cytokine network involved in the local immune response to tumors,¹⁻⁴⁾ In addition to infiltrating inflammatory cells, tumors also produce cytokines and growth factors that may alter tumor growth, tumor immunogenicity, and the host immune response. A variety of tumor-derived factors, including cytokines such as interleukin (IL)-4,⁵⁾ IL-6,⁶⁾ IL-8,⁷⁾ IL-10,⁵⁾ transforming growth factor (TGF)- β ,⁸⁾ vascular endothelial growth factor (VEGF),⁹⁻¹¹⁾ and monocyte chemoattractant protein (MCP)-1¹⁰⁻¹²⁾ may either regulate tumor growth or alter the anti-tumor immune response. Thus, human cancer growth may be regulated by a vari-

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ety of cytokines via both autocrine and paracrine pathways. Regulation of tumor growth by cytokines may occur directly by regulation of cell proliferation or indirectly through effects on angiogenesis or host immunity.

Cytokine responses have been broadly categorized into two main types. T helper 1 (Th1) cytokines, such as IL-2, IL-12, interferon (INF)- γ , and tumor necrosis factor (TNF)- α are responsible for cell-mediated immunity. Th2 type cytokines, such as IL-4, IL-5, IL-6, IL-10, and IL-13, are capable of stimulating humoral immunity. Recent studies have indicated a Th2 cytokine pattern at the tumor site and suggested that these cytokines may mediate immunosuppression.^{3,5,13)} In this study, we assessed the expression of several cytokines, such as IL-6, IL-12, and INF- γ , chemokines, such as IL-8 and MCP-1, and angiogenic factors, such as VEGF, TNF- α , and TGF- β in tumor tissues and evaluated the correlation with clinicopathological factors in human non-small cell lung cancer (NSCLC).

Materials and Methods

Lung cancer tissues

Tissues from 91 randomly selected primary NSCLC cases, resected surgically at Tokyo Medical University from May 2000 to July 2001, were used in this investigation. Representative parts of the specimens were frozen in liquid nitrogen immediately after surgical resection and stored at -80°C until preparation of tissue extracts. The main characteristics of the patients are described in Table 1. Informed consent was obtained from all patients.

Sample preparation for ELISA

Tumor tissues were homogenized in 10 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl_2 , and 50 μM potassium phosphate and then centrifuged at $10,000 \times g$ for 15 min. The supernatant was stored at -80°C until use. A portion of the supernatant was dialyzed overnight at 4°C in a buffer containing 20 mM potassium phosphate (pH 7.4) and 1 mM 2-mercaptoethanol and then used for enzyme-linked immunosorbent assay (ELISA).

Measurement of cytokines, chemokines, and angiogenic factors

The concentrations of Th1 cytokines (IL-12 and $\text{INF-}\gamma$), Th2 cytokines (IL-6), chemokines (IL-8 and MCP-1), and angiogenic factors (VEGF, $\text{TNF-}\alpha$, and $\text{TGF-}\beta$) in the tumor extracts were measured by ELISA (BioSource International, Camarillo, CA). The measurements were performed according to the manufacturer's instructions. Absorbance of each sample was determined with an ELISA plate reader (Model 450 Microplate Reader, Bio-Rad Laboratories). The minimal detection limits for the various factors were as follows: IL-12+p40, 1.0 pg/ml; $\text{INF-}\gamma$, 4.0 pg/ml; IL-6, 2.0 pg/ml; IL-8, 5.0 pg/ml; MCP-1, 20.0 pg/ml; VEGF, 5.0 pg/ml; $\text{TNF-}\alpha$, 1.7 pg/ml; and $\text{TGF-}\beta$, 2.0 pg/ml. The total protein concentration of the supernatants extracted from tumor tissue was determined with a DC protein assay kit (Bio-Rad Laboratories). The results are expressed as pg of cytokine/mg of total protein.

Immunohistochemical analysis of $\text{TGF-}\beta$

The 3-5 μm sections of paraffin-embedded tumor tissues were applied to indirect anti-peroxidase immunohistochemical assay, Strep ABC technique (Dako, Carpinteria, CA) for assessing the expression of $\text{TGF-}\beta$. Analysis of $\text{TGF-}\beta$ was carried out using anti-human

Table 1. Patient characteristics

Enrolled patients	91
Age (mean and range)	65.8 (33-84)
Sex M/F	57/34
Pathological type	
Adenocarcinoma	64 (70%)
Squamous cell carcinoma	19 (21%)
Large cell carcinoma	8 (9%)
p-stage I/II/III/IV	54/13/20/2
Tumor size (mean and range, cm)	3.3 (0.8-13.0)
p-T factor T1, T2/T3, T4	73/18
p-N factor N0/N1/N2/N3	68/14/8/1
p factor p0/p1/p2/p3	63/12/7/9
pm factor pm0/pm1	82/9
Central necrosis grade 0/1/2	47/23/19

p, pathological; pm1, intrapulmonary metastasis in only same lobe; pm0, intrapulmonary metastasis negative.

$\text{TGF-}\beta$ antibody (Genzyme/Techne, Cambridge, MA) at 1:100 dilutions. On each section five microscopic fields, which had the most accumulated positive signals (hot spots) were selected. The percentage of stained cells in these areas was counted, and mean value of these percentages was used as an expression value of $\text{TGF-}\beta$. For further analysis, values of each sample were categorized into two groups (negative, 0-30%; positive, >30%). Immunohistochemical and pathological assessments were conducted by two investigators, completely blinded to any clinical information.

Pathological status

Central necrosis grade was classified by H&E staining as follows: negative, no central necrosis; positive, moderate to marked central necrosis. Intrapulmonary metastasis (pm) was classified according to Classification of Lung Cancer of the Japan Lung Cancer Society: pm0, no intrapulmonary metastasis; pm1, metastasis to the same lobe as that of the primary tumor.¹⁴⁾

Statistical analysis

Unpaired groups were compared by Student's t-test. For qualitative variables, Pearson chi-square coefficient was calculated. A p value of less than 0.05 was taken to indicate a statistically significant difference. Multivariate logistic regression analysis was performed to evaluate possible associations between different variables and the occurrence of pm1. Only variables significant by univariate analysis were included in the logistic regression model. All statistical analyses were carried out using StatView 5.0 software (SAS Institute Inc.).

Table 2. Expression of cytokines, chemokines, and angiogenic factors in 91 NSCLC tissues

Biological markers	Median (range)
VEGF	171.834 pg/mg (0.000-894.734)
TNF- α	1.744 pg/mg (0.000-21.578)
TGF- β	97.294 pg/mg (0.000-749.804)
IL-6	261.533 pg/mg (0.000-1600.066)
IL-8	236.870 pg/mg (0.000-1970.393)
IL-12	194.717 pg/mg (0.000-1557.222)
IFN- γ	0.000 pg/mg (0.000-0.000)
MCP-1	212.556 pg/mg (24.757-529.946)

VEGF, vascular endothelial growth factor; TNF- α , tumor necrosis factor- α ; TGF- β , transforming growth factor- β ; IL, interleukin; IFN- γ , interferon- γ ; MCP-1, monocyte chemoattractant protein-1.

Results

Expression of cytokines, chemokines, and angiogenic factors

VEGF, TNF- α , TGF- β , IL-6, IL-8, IL-12, and MCP-1 were variously secreted in lung cancer tissues, which were measured by ELISA. There were only three detectable cases of TNF- α in 91 cases. No case had detectable levels of IFN- γ in this series. These factors were therefore excluded from the analysis. The median values and the range of each biological marker are shown in Table 2.

Correlation between biological markers and pathological type

The expression of IL-6 and IL-8 were significantly higher in squamous cell carcinoma than in adenocarcinoma ($p=0.0164$ and $p<0.001$, respectively) (Table 3).

Correlation between biological markers, clinicopathological factor, and pm factor

Pulmonary metastasis in only one lobe: pm1 was detected in 9 of 91 cases (9.9%). The size of pulmonary metastasis nodules ranged from 0.3 to 1.0 cm. On the other hand, the size of primary lesions ranged from 2.0 to 13.0 cm. Among clinicopathological factors, tumor size and pathological nodule involvement significantly correlated with pm1 ($p=0.0117$ and $p=0.0296$, respectively). Among biological markers, the expression of TGF- β , IL-8, and MCP-1 were significantly higher in primary lung cancer tissues of cases with pm1 ($p=0.0017$, $p=0.0075$, and 0.0013 , respectively) (Table 4). Moreover, in multivariate logistic regression analysis, the expression of TGF- β was an independent risk factor of the occurrence of pm1 (Table 5).

Correlation between biological markers and central necrosis

Expression of VEGF and IL-8 were significantly higher in central necrosis cases than in cases without central necrosis ($p=0.006$ and $p=0.011$, respectively) (Table 6).

Expression of TGF- β NSCLC tissues by immunohistochemical analysis

We evaluated TGF- β expression in randomly selected 36 NSCLC tissues by immunohistochemical analysis. TGF- β expression in tumor cells was observed in 21 out of 36 cases (58%) (Fig. 1a). Twenty-seven out of 36 cases (75%) showed TGF- β expression in stromal cells, especially tumor-infiltrating inflammatory cells in tumor stroma (Fig. 1b). High expression of TGF- β in tumor cells and stromal cells was found in 9 (25%) and 13 (36%) of cases, respectively.

Discussion

Human cancer growth may be regulated by a variety of cytokines via both autocrine and paracrine pathways. Regulation of tumor growth by cytokines may occur directly by regulation of cell proliferation or indirectly through effects on angiogenesis or host immunity. In this study, we assessed the expression of several mediators, including immune cytokines, such as IL-6, IL-12, and IFN- γ , chemokines, such as IL-8 and MCP-1, and angiogenic factors, such as VEGF, TNF- α , and TGF- β by the ELISA technique in 91 samples of NSCLC tissues to clarify the role of the cytokine network in the tumor tissues for the development of human NSCLC.

Several studies reported expression of various cytokines in NSCLC tissues by using immunohistochemical stains or RT-PCR or ELISA method.^{3,13} In this study, the expression of MCP-1, IL-6, and IL-8 was high, while the expression of VEGF, TGF- β , and IL-12 was moderate in NSCLC tissues. However, the expression of TNF- α , which is one of the major anti-tumor cytokines, was measurable in only three cases, and that of IFN- γ , which is also one of the major anti-tumor cytokines, was not detectable in this series (Table 2). Anti-tumor cytokines, such as TNF- α and IFN- γ were a few secreted in tumor tissues, compared with in serum in NSCLC, which a previous study reported.¹⁵

With regard to the correlation with clinicopathological variables, we found three significant correlations in the present study. First, the expression of IL-6 and IL-8 were significantly higher in squamous cell carcinoma than

Table 3. Correlation between biological markers and pathological type

	Adenocarcinoma (n=64) mean±SD (pg/mg)	Squamous cell carcinoma (n=19) mean±SD (pg/mg)	p value
VEGF	185.213±223.355	141.981±165.381	0.438
TGF-β	102.990±154.510	90.987±118.452	0.763
IL-6	209.303±209.034	359.708±286.989	0.016 ^a
IL-8	145.392±242.168	479.741±418.348	<0.001 ^a
IL-12	184.421±281.625	122.477±120.508	0.355
MCP-1	239.880±273.944	197.594±150.741	0.522

Statistical analysis calculated according to Student's t-test.

^a: Statistically significant

VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; IL, interleukin; MCP-1, monocyte chemoattractant protein-1.

Table 4. Correlation between biological mediators, clinicopathological factor and pm factor

	pm1 ^a (n=9) mean±SD (pg/mg)	pm0 (n=82) mean±SD (pg/mg)	p value
Tumor size (cm)	4.889±4.622	3.154±1.391	0.012 ^b
p-N status (+/-)	5/4	18/63	0.030 ^b
VEGF	209.301±277.879	167.671±195.918	0.821
TGF-β	236.186±258.531	79.688±113.742	0.002 ^b
IL-6	388.957±507.945	248.463±241.459	0.171
IL-8	538.473±633.028	202.939±305.256	0.008 ^b
IL-12	30.905±17.024	155.172±185.838	0.107
MCP-1	539.344±630.332	223.084±201.540	0.001 ^b

^a: pm1 nodule size = 0.3-1.0 cm

^b: Statistically significant

VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-β; IL, interleukin; MCP-1, monocyte chemoattractant protein-1.

Table 5. Relationship between occurrence of pm1 and clinical factors or cytokine expression by multivariate logistic regression analysis

Clinical factors and cytokines	p value	Odds ratio	95% CI
Tumor size (cm)	0.086	1.719	0.955-2.027
p-N status (+/-)	0.055	1.922	0.962-53.478
TGF-β	0.008 ^a	2.665	1.002-1.011
IL-8	0.249	1.152	0.999-1.004
MCP-1	0.275	1.092	0.999-1.003

^a: Statistically significant

TGF-β, transforming growth factor-β; IL, interleukin; MCP-1, monocyte chemoattractant protein-1.

in adenocarcinoma in NSCLC tissues, which has not previously been reported. IL-6 is one of the inflammatory cytokines, which may contribute to an increased expression of MHC molecules, while IL-8 is one of the major CXC chemokines capable of inducing chemotactic migration of neutrophils. It is important to note that these specific findings may be reflected in the different clinical

behaviors and immune response of squamous cell and adenocarcinomas.

The second result was that the expression of TGF-β, IL-8, and MCP-1 positively correlated with the occurrence of pulmonary metastasis, pm1, in addition to clinicopathological factors, such as tumor size and p-N status, by univariate analysis (Table 4). Moreover, multivari-

Table 6. Correlation between biological mediators and central necrosis

	Central necrosis [mean \pm SD (pg/mg)]		p value
	+	-	
VEGF	228.963 \pm 224.828	113.174 \pm 162.611	0.006 ^a
TGF- β	77.276 \pm 110.156	111.359 \pm 166.156	0.290
IL-6	301.710 \pm 270.896	237.027 \pm 298.484	0.296
IL-8	318.161 \pm 350.443	138.073 \pm 303.993	0.011 ^a
IL-12	132.934 \pm 168.101	159.141 \pm 196.881	0.514
MCP-1	233.790 \pm 319.926	178.386 \pm 154.618	0.319

Statistical analysis were calculated according to Student's t-test.

^a: Statistically significant

VEGF, vascular endothelial growth factor; TNF- α , tumor necrosis factor- α ; TGF- β , transforming growth factor- β ; IL, interleukin; MCP-1, monocyte chemoattractant protein-1.

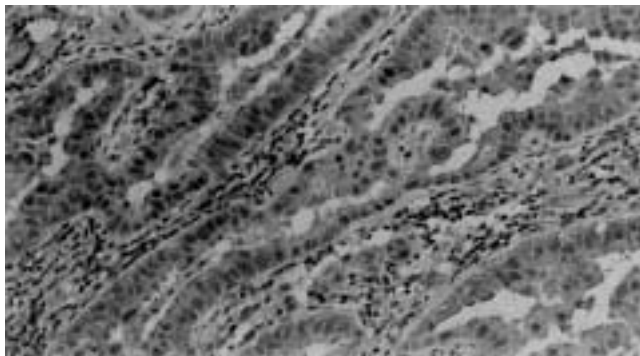
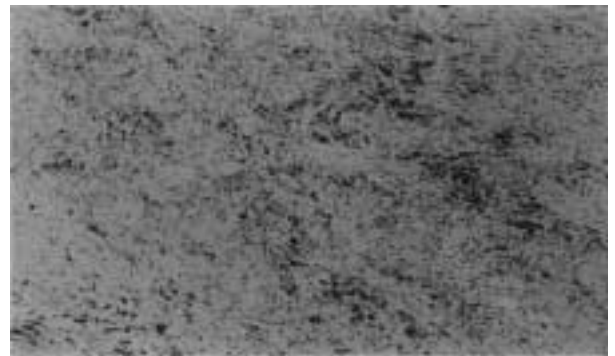
**a****b**

Fig. 1. Immunohistochemical analysis of TGF- β in NSCLC tissues.

Positive staining of TGF- β was seen in tumor cells (a) and in tumor infiltrating stromal cells (b).

ate logistic regression analysis revealed that the expression of TGF- β remained as an independent risk factor of the occurrence of pm1 (Table 5). We confirmed that both tumor cells and tumor infiltrating stromal cells were major sources of TGF- β by immunohistochemical analysis (Fig. 1). Furthermore, the expression status of TGF- β in stromal cells by immunohistochemical stain significantly positively correlated with the value of TGF- β in tumor tissues by ELISA ($p=0.0279$), as compared with immunohistochemical expression of TGF- β in tumor cells ($p=0.8282$), using Student's t-test analysis. TGF- β has been reported to be a multifunctional growth factor, synthesized by a wide variety of both normal and malignant cells. The effect of TGF- β on tumor growth is controversial, and it is able to directly inhibit proliferation of epithelial cancer cells, but its immunosuppressive properties can also promote tumor growth. In this study, we suggested that expression of TGF- β in the tumor microenvi-

ronment promotes the development of spontaneous pulmonary metastasis by its immunosuppressive properties in NSCLC.

Last, we showed that VEGF and IL-8 expression have a significant relationship with the status of central necrosis (Table 6). Some studies have reported that hypoxia in tumor tissues, such as central necrosis, induced tumor angiogenesis in breast cancer and NSCLC.^{16,17)} Hypoxia increased the expression and secretion of VEGF and IL-8 in vitro.¹⁸⁾ The hypoxia-induced neovascularization was found to be mediated by IL-8, a multifunctional cytokine that shows potent angiogenic activities in vitro and in vivo, and possibly by VEGF, a strong specific mitogen for endothelial cells.¹⁹⁻²¹⁾ We assumed that our result supports this hypothesis.

In addition, recent studies reported that serum levels of proinflammatory cytokines, such as IL-1 β , IL-6, and TNF- α ,¹⁵⁾ anti-tumor cytokines, such as IL-2 and IL-10,²²⁾

and chemokines, such as IL-8²³) have prognostic significance for survival of NSCLC patients. We could not detect any factors that correlated with the status of nodal involvement, which is known to be the worst prognostic factor in NSCLC in this series (data is not shown). We did not assess the prognostic significance of these cytokines, because of short median follow-up terms such as 2.1 years to less than 3 years.

Cytokine expression in tumor microenvironments is thought to be important in the mediation of both host immune response and tumor survival. The source of these cytokines includes tumor cells, infiltrating leukocytes, fibroblasts, and other stromal elements. Therefore, understanding the basis of molecular and cellular cross-talk between tumor cells and the immune system of the host in local area remains a crucial issue in the effective use of cytokines in the biotherapy of cancer.

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