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Lack of Interleukin-10 Expression Could Predict Poor Outcome in Patients with Stage I Non-Small Cell Lung Cancer[1]

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ABSTRACT

Purpose: **Interleukin-10 (IL-10) may play an important role in controlling tumor growth and metastasis. Some reports have shown that IL-10 can be a potent inhibitor of tumor growth, but others suggest that IL-10 expression by the tumor is an adverse prognostic factor. Because normal bronchial epithelial cells constitutively produce IL-10, we decided to test the prognostic value of IL-10 in a well-defined population of patients with stage I non-small cell lung cancer (NSCLC) treated in a single institution.**

Patients and Methods: **Using immunohistochemical analysis, we retrospectively analyzed IL-10 expression in specimens from 138 patients with completely resected clinical/radiographic stage I NSCLC for whom clinical follow-up data were available.**

Results: **IL-10 expression was retained (IL-10 labeling index ≥ 10%) in 94 patients (68.1%) and lost in 44 patients (31.9%). The duration of overall, disease-specific, and disease-free survival in the 44 patients lacking IL-10 expression was worse than in the 94 patients with IL-10 expression ($P = 0.08$, 0.02, and 0.05, respectively; Log-rank test). Interestingly, IL-10 expression was observed more frequently in tumors with squamous cell histology than in tumors of other histological subtypes ($P = 0.04$; χ^2 test). Multivariate analysis confirmed the independent prognostic value of IL-10 expression for disease-specific survival ($P = 0.04$).**

Conclusion: **Lack of IL-10 expression by the tumor was associated with a significantly worse outcome of early stage NSCLC. The mechanisms underlying this clinically and biologically important finding need to be further explored.**

INTRODUCTION

Lung cancer is a major cause of mortality worldwide. Last year, in the United States alone, an estimated 169,400 new cases of lung cancer were diagnosed, and an estimated 154,900 deaths from lung cancer occurred (1). Improving the survival rate of patients with this disease requires a better understanding of tumor biology and the subsequent development of novel therapeutic strategies. One area of intense lung cancer research has been in assessing the prognostic factors of NSCLC,[3] focusing on stage I disease and molecular factors (2–5). This avenue of investigation may lead to the identification of patients with the highest risk stage I NSCLC or of those who are most likely to benefit from adjuvant or chemopreventive approaches.

IL-10 is thought to play a potential pathogenic or therapeutic role in a number of human conditions, such as inflammation, autoimmunity, and cancer (6). The immunomodulatory effects of IL-10 have yielded mixed results in various tumor systems. On one hand, because many tumor types express IL-10, its role in helping tumors evade immunosurveillance has been suggested (7, 8). IL-10 inhibits the tumoricidal capacity of macrophages and the cytotoxicity and cytokine production of tumor-specific T cells and blocks the presentation of tumor antigens by antigen-presenting cells (9, 10). On the other hand, *in vivo* studies in different animal models have demonstrated that IL-10 is a potent inhibitor of tumor growth and metastasis (11–14). Additionally, systemic administration of IL-10 has inhibited tumor metastasis and stimulated antitumor immune responses in murine models (15). Nevertheless, recent data generated by analyzing human lung tissue samples suggest that IL-10 produced by NSCLC is a predictor of poor outcome (16).

Because IL-10 is constitutively expressed in normal bronchial epithelial cells, we hypothesized that loss of IL-10 expression by lung tumors might be a prognostic factor for survival. Therefore, we decided to analyze the prognostic value of IL-10 expression in a homogeneous population of 138 patients with stage I NSCLC.

PATIENTS AND METHODS

Study Population. A total of 595 consecutive patients with stage I NSCLC underwent definitive surgical resection, defined as a lobectomy or a pneumonectomy, from 1975 to 1990

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma; TNM, Tumor-Node-Metastasis; CI, confidence interval; IL, interleukin; TIMP, tissue inhibitor of metalloprotease; NK, natural killer; MMP, matrix metalloprotease.

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Table 1 IL-10 status in stage I NSCLC tumors according to clinicopathological features of patients

	No. of patients (n = 138)	IL-10 expression Positive (n = 94)	IL-10 expression Negative (n = 44)	P
Age: median (range)	64 (37–82)	64 (37–82)	65 (45–76)	0.28
Sex				
Male	106	68	38	0.07
Female	32	26	6	
Race				
Caucasian	120	84	36	0.22
Other	18	10	8	
Smoker				
Yes	119	79	40	0.27[a]
No	9	8	1	
Unknown	10	7	3	
Histology of tumors				
SCC	58	45	13	0.04
Adenocarcinoma and others	80	49	31	
TNM stage				
$T_1N_0M_0$	65	44	21	0.92
$T_2N_0M_0$	73	50	23	
5-year overall survival rate	51.5%	56.6%	40.9%	
(95% CI)	(43.8%, 60.6%)	(47.3%, 67.7%)	(28.7%, 58.4%)	

[a] P calculated comparing smoking *vs.* nonsmoking patients.

at The University of Texas M. D. Anderson Cancer Center. We retrospectively examined 138 cases for which both tissue samples and a median follow-up period of >5 years were available at the time of this study. The patient population was identified through a search of the Tumor Registry database maintained by the Department of Medical Informatics at The University of Texas M. D. Anderson Cancer Center. Survival status was verified and updated from Tumor Registry records as of December 1, 2000.

The study population consisted of 106 men and 32 women; 120 patients were Caucasian, and 18 patients were of other ethnicities (Table 1). The mean age of patients at surgery was 63 years. Histological subtypes included 58 cases of SCC, 60 cases of adenocarcinoma, 10 cases of bronchioalveolar carcinoma, 5 cases of large cell carcinoma, 3 cases of adenosquamous carcinoma, and 2 cases unclassified.

Immunohistochemical Staining for IL-10 Protein. All available tissue blocks from each patient were reviewed for the presence of tumor by a thoracic pathologist (B. L. K.). Paraffin-embedded, 4-μm-thick tissue sections from all 154 primary tumors were stained for IL-10 protein using a primary goat polyclonal antihuman IL-10 antibody (AF-217-NA; R&D Systems, Minneapolis, MN). Slides were deparaffinized through a series of xylene baths. Rehydration was performed using graded alcohol. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 20 min to block the endogenous peroxidase activity and incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated overnight at 4°C with primary anti-IL-10 antibody at a dilution of 1:75 (1.33 μg/ml). The sections were then processed using a standard avidin-biotin immunohistochemical assay according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Routinely processed tissue sections of normal lymph nodes and tonsils were used as positive staining controls and also stained with the primary antibody omitted to confirm staining specificity. Normal bronchial epithelial cells that constitutively produce IL-10 were also used as internal positive controls (17).

The IL-10 labeling index was defined as the percentage of tumor cells displaying cytoplasmic immunoreactivity and calculated by counting IL-10-stained tumor cells among ≥1000 tumor cells for each section. Representative areas of each tissue section were selected, and cells were counted in at least four fields (magnified 400 times) in these areas. On the basis of previous reports, if ≥10% of the tumor cells were positive for IL-10, the case was considered to be IL-10 positive (18, 19). Tumors with <10% of the cells stained were counted as negative. All slides were scored concomitantly by a pathologist (X. T.) and another investigator (J-C. S.). Immunohistochemical analysis was performed in a blinded manner with respect to clinical information about the subjects.

Statistical Analysis. Overall, disease-specific, and disease-free survival were analyzed in this study. Survival curves were estimated by the Kaplan-Meier method. The Log-rank test was used to compare patient's survival time between groups. The Fisher exact and χ^2 tests were used to analyze the association between two categorical variables. The Wilcoxon rank-sum test was used for differences in median of age. The Cox proportional hazards model was used for univariate analysis to evaluate the association between survival time and risk factors and for multivariate analysis to model the risks of IL-10 expression on survival time, with adjustment for clinical and histopathological parameters (age, sex, race, tumor histology,

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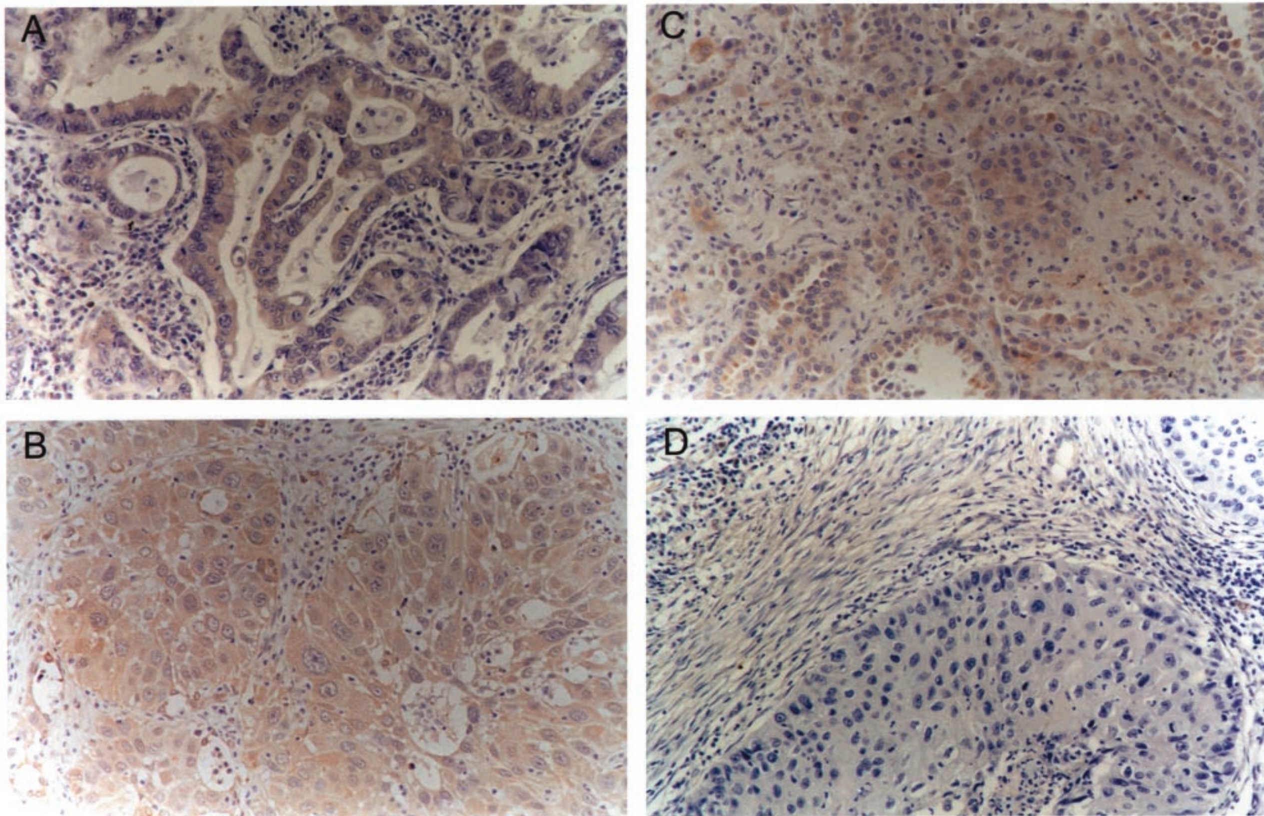

Fig. 1 Immunohistochemical staining patterns of IL-10 in stage I NSCLC. *A,* a well-differentiated adenocarcinoma with most cancer cells expressing IL-10 in the cytoplasm. *B,* a SCC with most carcinoma cells positive for IL-10. *C,* a bronchioalveolar carcinoma tumor with IL-10 expression. *D,* a SCC tumor negative for IL-10 expression (original magnification, ×400).

and tumor size). All statistical tests are two sided, and a $P <$ 0.05 was considered to be statistically significant.

All survival curves were calculated from the date of surgery. Overall survival took all deaths (cancer related or not) into account. Disease-specific survival time was calculated from the date of surgery to death from cancer-related causes. Disease-free survival time was calculated from the date of surgery to relapse or death from cancer-related causes.

RESULTS

A total of 138 formalin-fixed, paraffin-embedded NSCLC tumor specimens was stained using a standard immunohistochemical technique reported previously for the identification of IL-10 expression (18, 19). The usual pattern of positive staining for IL-10 in NSCLC was cytoplasmic and not nuclear (Fig. 1, *A–C*). Even if tumors cells were negative for IL-10 staining, normal bronchial epithelial cells in the section were positive and used as an internal positive control of the staining for IL-10. In peribronchial gland cells or alveolar pneumocytes, IL-10 expression was not detectable. Lymphoid cells of tumor areas were occasionally immunostained. Only 20 of 138 samples displayed tumor-infiltrating lymphocytes, therefore hindering any relevant analysis of IL-10 production by infiltrating immune cells. In the positive control tissues (tonsil), the normal stratified squamous epithelium displayed IL-10-positive cells. In the adjacent lymph nodes, IL-10-positive cells were localized predominantly in the germinal centers (data not shown). IL-10 immunohistochemical staining showed a wide heterogeneity from rare scattered cells to a homogeneous pattern for the vast majority of cells examined, suggesting that phenotypic heterogeneity is a major feature in NSCLC (Fig. 1).

IL-10 expression was observed in 94 (68.1%) of the 138 stage I NSCLC specimens. Lack of staining was observed in 44 tumors (31.9%). Table 1 shows the relationships between the expression of IL-10 and clinicopathological factors. There were no statistically significant differences in TNM stage, sex, smoking status, age, and race between the groups with IL-10-positive and -negative staining. Interestingly, IL-10 expression was more prevalent in the SCC subtype than it was in other histological subtypes. Forty-five (77.6%) of the 58 cases of SCC exhibited IL-10 expression, whereas 49 (61.3%) of 80 patients with non-SCC tumors (mainly adenocarcinoma) showed IL-10 expression ($P = 0.04$; χ^2 test).

We subsequently analyzed the relationship between IL-10 expression and length of survival. The median follow-up time for the patient population was 10.6 years. Fig. 2*A* shows the Kaplan-Meier overall survival curves for patients whose tumors were IL-10 positive and negative. Patients with tumors that were

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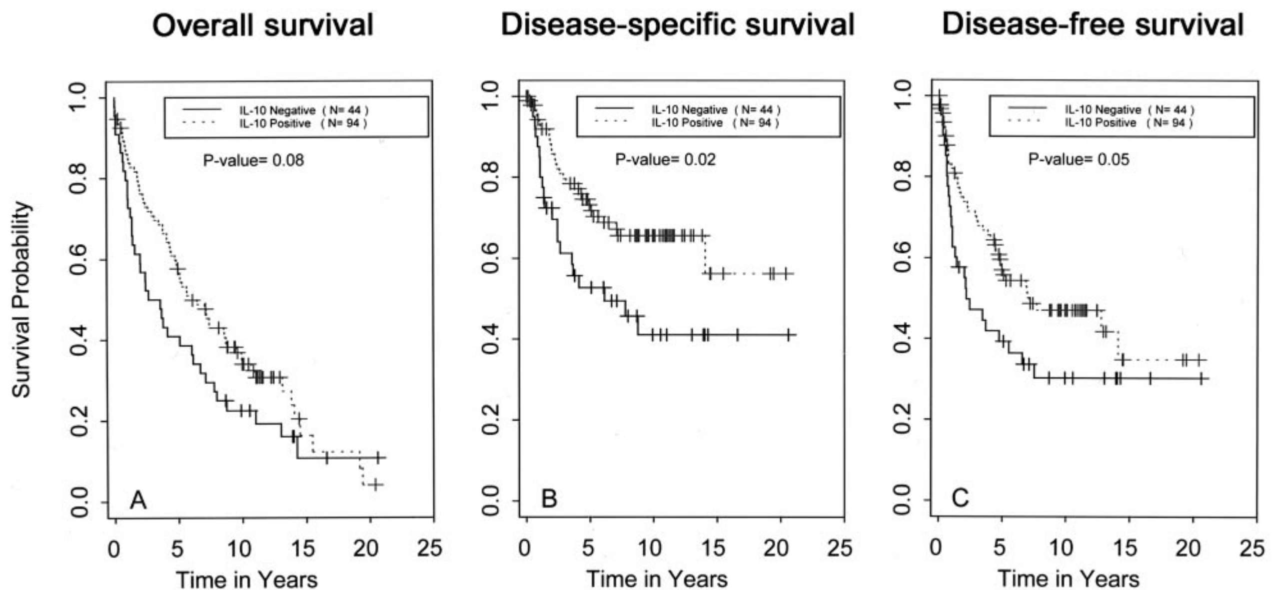

Fig. 2 Survival curves of patients with IL-10-positive and -negative NSCLC. The patients lacking IL-10 expression (*solid line, n* = 44) had worst outcomes than the patients with IL-10 expression (*broken line, n* = 94) for overall (*A*), disease-specific (*B*), and disease-free survival (*C*).

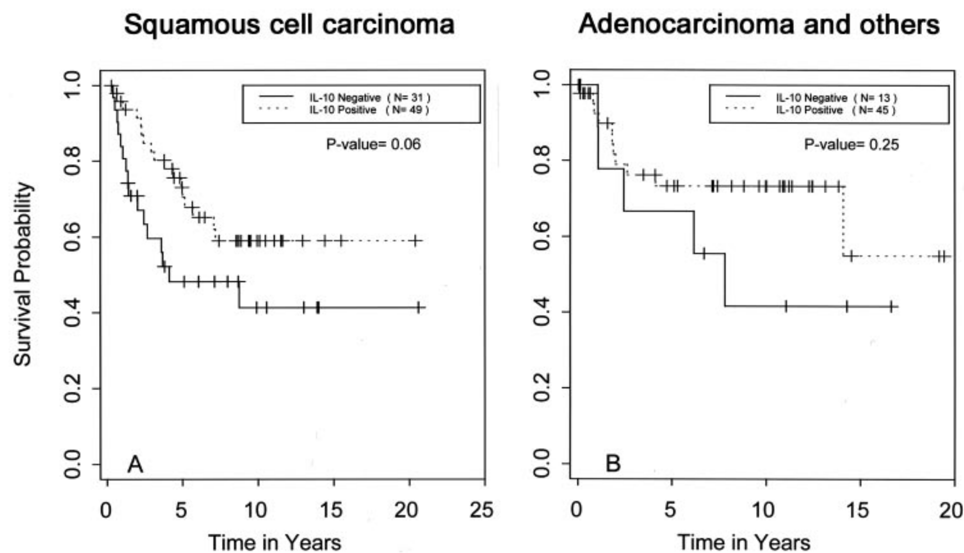

Fig. 3 *A,* disease-specific survival curve of patients with stage I SCC according to IL-10 expression; *B,* disease-specific survival curve of patients with adenocarcinoma or other histological subtypes according to IL-10 expression.

IL-10 negative had a shorter survival time than did patients with tumors that were IL-10 positive (P = 0.08; Log-rank test). Five-year overall survival rate for patients whose tumors were IL-10 positive was 56.6% (95% CI = 47.3–67.7%) and 40.9% (95% CI = 28.7–58.4%) for patients whose tumors were IL-10 negative (Table 1). Fig. 2*B* shows that patients with negative IL-10 expression had significantly shorter disease-specific survival times than did patients with positive IL-10 expression (P = 0.02; Log-rank test). A comparison of disease-free survival curves in IL-10-negative and -positive patients yielded similar results (P = 0.05; Log-rank test; Fig. 2*C*). The prognostic significance of IL-10 expression on disease-specific survival was further explored in each major histological subtype. IL-10 negativity was a borderline significant adverse prognostic factor among patients with non-SCC tumors (P = 0.06; Log-rank test; Fig. 3*A*). A similar trend was observed for patients with SCC of the lung, but this trend was not significant (P = 0.25; Log-rank test; Fig. 3*B*). Univariate Cox proportional hazards model was used to evaluate the association between IL-10, clinicopathological variables (age, sex, race, histological subtype, and TNM), and survival time. Table 2 shows the results on disease-specific survival. In multivariate Cox proportional hazards model, among all clinicopathological variables, IL-10 expression was the only significant independent prognostic indicator

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Table 2 Univariate and multivariate Cox proportional hazards model on disease-specific survival

	Univariate			Multivariate		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Age	1.0	0.97–1.03	0.92			
Sex (Male or female)	0.88	0.48–1.64	0.69			
Race (Caucasian or other)	0.49	0.25–0.99	0.047	0.58	0.28–1.2	0.13
Histological subtype (SCC or other)	0.72	0.39–1.30	0.27			
Tumor size (T1 or T2)	0.73	0.42–1.28	0.28			
IL-10 (+ or −)[a]	0.51	0.29–0.89	0.02	0.55	0.31–0.98	0.04

[a] +, IL-10 labeling index ≥ 10%; −, IL-10 labeling index < 10%.

for disease-specific survival. The hazard of cancer death for patients whose tumor was IL-10 positive was only 55% of the hazard for patients whose tumor was IL-10 negative ($P = 0.04$, Cox model).

DISCUSSION

Human lung cancer displays an extremely aggressive clinical course and represents the leading cause of malignancy-related mortality in the United States (1). This behavior may reflect an increased capacity to evade detection and containment by host immune response. Because IL-10 demonstrates *in vitro* immunosuppressive activities (9, 10), some groups have hypothesized that IL-10 production by cancer cells may help the tumor evade immunosurveillance (7, 8). Nevertheless, IL-10 is also able to inhibit tumor growth and metastasis in various tumor models (11–14). These conflicting results imply that it is all about "fine tuning" in case of IL-10-mediated immunosuppression or immunostimulation. Because normal bronchial epithelial cells constitutively express IL-10 (17), loss of IL-10 expression by lung cancer cells would represent a specific change in the tumor as compared with its normal epithelial counterpart. In the present study, we explored the prognostic value of IL-10 expression by lung cancer cells in a large and homogeneous population of 138 completely resected clinical/radiographic stage I NSCLC for whom a median follow-up of 10.6 years was available. We have demonstrated that IL-10 is retained in a significant percentage of stage I NSCLCs. Overall, 94 (68.1%) of 138 tumors expressed IL-10 in ≥10% tumor cells, whereas loss of IL-10 expression was observed in 44 patients (31.9%). Our data show that lack of IL-10 expression is a poor prognostic factor in patients with stage I NSCLC. The poor prognostic value of lack of IL-10 expression was observed for disease-specific and -free survival, with a trend for overall survival. Furthermore, multivariate analysis confirmed the independent prognostic value of lack of IL-10 expression. The prognostic value of IL-10 was retained even when we changed the cutoff level of positivity from 10% (18) to 5 or 15%. Our results are in contrast to a previous report by Hatanaka *et al.* (16), who suggested that IL-10 expression by the tumor was an indicator of poor prognosis. We have analyzed IL-10's prognostic value in a large and homogeneous population of patients with early stage lung cancer ($n = 138$), whereas Hatanaka *et al.* performed their analysis using a smaller and more heterogeneous population that included 82 patients with stage I–IIIb disease. Furthermore, the fact that all of the patients in our study were treated at a single institution and received lengthy follow-up care after surgery helps to increase the credibility of our survival analysis. Finally, Hatanake *et al.* (16) evaluated IL-10 expression by RT-PCR as opposed to immunohistochemistry in this study. Thus, our different results may be related in part to differences in patient population and the technique used to evaluate IL-10 expression. Indeed, we evaluated IL-10 expression at the protein level as compared with the mRNA level for Hatanake *et al.* Furthermore, both studies were retrospectively conducted and therefore potentially subject to some degree of selection bias.

We have analyzed IL-10 expression by performing immunohistochemical analysis with a polyclonal antihuman IL-10 antibody reported previously (18, 19). Other anti-IL-10 antibodies have also been used to evaluate IL-10 expression in paraffin-embedded tissue sections (17, 20). A good concordance between reverse transcription-PCR analysis and immunohistochemical analysis for IL-10 has been suggested in different reports (7, 17). We have used internal and external positive controls to assess the specificity of the staining. We found that even if tumor cells were negative for IL-10 staining, normal bronchial epithelial cells in the section (when present) were positive (17), thus ruling out a false negative result.

Although the mechanisms underlying the current data are not clear, there are several potential explanations for the poor outcome of patients with IL-10-negative tumors. Several laboratories have demonstrated that IL-10 is a potent inhibitor of tumor growth and metastasis in multiple animal models and tumor types, including melanoma, breast and prostate cancers, and Burkitt's lymphoma (11, 13, 20, 21). *In vivo,* the effects of IL-10 may be multifold. They can be related to direct inhibition of IL-10 on the angiogenic process *per se* or indirectly by affecting the angiogenic capacity or signals from tumor and/or tumor-infiltrating cells. Compelling evidence indicates that the antiangiogenic effect of IL-10 results from the inhibition of angiogenic factor release and production by the tumor and/or stromal cells. IL-10 induces production of TIMP-1, an inhibitor of angiogenesis, and inhibits MMP-2 and MMP-9 secretion by cancer cell lines, blocking the induction of microvessel formation *in vitro* (20, 22). It has also been suggested that IL-10 can directly inhibit endothelial cell response to angiogenic factors (21). Moreover, in murine mammary tumors, the antimetastatic and antitumor activity resulting from IL-10 gene transfer is related to enhanced production of nitric oxide (23).

One of the major roles of IL-10 in the regulation of immune responses involves its deactivating effect on macrophages (6). From the many cells and cell products within a tumor that serve as inducers or modulators of angiogenesis, macrophages

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have emerged as a major component. IL-10 secreted by the tumor cells may prevent the migration of macrophages from the periphery into the tumor tissue, thus preventing macrophage infiltration (24). IL-10 also inhibits the expression of angiogenic factors (vascular endothelial growth factor, IL-1β, tumor necrosis factor-α, IL-6, and MMP-9) in tumor-associated macrophages (25). These changes correlate with decreased neovascularization of the tumors. Alternatively, the inhibitory effect of IL-10 on tumor metastasis has been suggested to be mediated through a NK cell-dependent mechanism (6, 12). IL-10 is able to affect the activities of NK cells, and NK cells were recently shown to contribute to the antiangiogenic effects of IL-12 through the killing of endothelial cells (26).

The observation that IL-10 expression differs among histological subtypes highlights the biological differences among different subtypes of NSCLC. Different abnormalities in oncogenes and tumor suppressor genes among histological subtypes of NSCLC are well known. Indeed, K-*ras* mutations are much more common in adenocarcinomas than in SCCs, whereas cyclin B1 overexpression or the *p53* mutant immunophenotype is more frequent in SCCs than in adenocarcinoma (2, 27, 28).

In conclusion, we found that a lack of IL-10 expression is a prognostic factor of poor outcome in stage I NSCLC. This result may be explained by the antitumor effects of IL-10, which contrast with the immunomodulatory effects that this cytokine displays *in vitro*. The mechanisms behind IL-10 antitumor effects might include inhibition of angiogenesis, stimulation of TIMPs, inhibition of MMP secretion, and inhibition of macrophage activity (20–25). Nevertheless, this result needs to be interpreted with caution because of potential limitations in the present study: (*a*) IL-10 production by tumor-infiltrating lymphocytes was not addressed because only a small fraction of our tissue samples displayed immune infiltrating cells; and (*b*) the role of IL-10 in cancer progression or regression might be very different according to the level of cytokine produced by the tumor and infiltrating immune cells. Additional studies are clearly required to confirm the present data and resolve the role of IL-10 in tumor growth and metastasis. We plan to conduct additional studies that will help in assessing the clinical importance of the present IL-10 findings and in understanding their possible mechanisms. These studies will be conducted using resected tissue from patients with stage I NSCLC, analyze the expression of MMPs and TIMPs, microvessel density, and the presence of tumor-infiltrating lymphocytes and their phenotype, and relate these factors to IL-10 expression by the tumor and to overall prognosis.

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