Human Non-Small Cell Lung Cancer Cells Express a Type 2 Cytokine Pattern

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ABSTRACT

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. To characterize the expression profile of human non-small cell lung cancer (NSCLC)-derived cytokines, the mRNA expression of type 1 and type 2 cytokines in five human NSCLC lines was analyzed by reverse transcriptase-PCR. Expression of interleukin 6 (IL-6), transforming growth factor-β (TGF-β), interleukin 10 (IL-10), and tumor necrosis factor-α (TNF-α) was demonstrated in all tumor lines evaluated. Whereas type 1 cytokines promote cell-mediated responses, type 2 cytokines promote immunoglobulin production and inhibit the differentiation of type 1 cells and type 1 cytokine release (12–17). Recent studies indicate that a type 2 cytokine pattern is present at the tumor site and suggest that these cytokines may mediate immunosuppression (3, 18). The type 2 cytokine pattern at the tumor site has been ascribed to the lymphocytes infiltrating the tumor. The purpose of this study was to determine whether tumor cells also contribute Th2 cytokines to the lung tumor milieu. We report that NSCLC cell lines and fresh tumors produce a distinct type 2 cytokine pattern in vitro and in situ.

INTRODUCTION

Recent studies have evaluated the cytokine network involved in the local immune response to tumors (1–4). A variety of lung tumor-derived factors, including cytokines such as IL-6 (5), IL-8 (6–7), and TGF-β (8–9), may either regulate tumor growth or alter the antitumor immune response. Thus, human lung 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 (5–9).

In both murine models and human studies, T lymphocytes have been found to express two distinct cytokine patterns (10–11). Type 1 or Th1 cells produce IL-2 and IFN-γ, whereas type 2 or Th2 cells produce IL-4, IL-5, IL-10, and IL-13. Whereas type 1 cytokines promote cell-mediated responses, type 2 cytokines promote immunoglobulin production and inhibit the differentiation of type 1 cells and type 1 cytokine release (12–17). Recent studies indicate that a type 2 cytokine pattern is present at the tumor site and suggest that these cytokines may mediate immunosuppression (3, 18).

Human NSCLC Lines. Lung adenocarcinoma cell lines SKLU-1, 125, A1427, lung squamous cell carcinoma lines H520 and large cell lung carcinoma line H460 were obtained from Dr. J. A. Radosevich. Lung adenocarcinoma line A549 and lung squamous cell carcinoma lines SK-MES-1 and SW 900 were obtained from American Type Culture Collection (Rockville, MD). The human squamous cell carcinoma line RH2 was established in our laboratory (19). The cells were grown under an atmosphere of 5% CO2 in air as monolayers at 37°C in 25-cm2 tissue culture flasks containing 5.0 ml of RPMI 1640 (for SKLU-1, A549, 125, A1427, RH2, H460, H520) or EMEM medium (for SK-MES-1 and SW 900) supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin solution and 2 mM glutamine (JRH Biosciences, Lenexa, KS).

Human Cytokine PCR Primers. IL-10 and IL-13 PCR primers were synthesized by a DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). All other PCR primers were purchased from Clontech Laboratories (Palo Alto, CA). The sequences of the human cytokine PCR primers are: IL-2 sense primer 5′-ATG-TAC-AGG-AGG-CAA-CTC-CTG-TCT-T−3′ and antisense primer 5′-GTT-ACT-GAA-AGC-ATC-ATC-TCA-CTG-3′ (amplified DNA size, 458 bp); IFN-γ sense primer 5′-ATG-AAA-TAT-ATA-ATC-ATG-ATC-3′ and antisense primer 5′-GAT-GCT-CTG-CCA-GGT-GCA-3′ (amplified DNA size, 494 bp); TNF-α sense primer 5′-ATG-AGC-ACG-ATG-TGC-CTTG-3′ and antisense primer 5′-GCA-ATG-TGC-CCA-GAG-TAG-TGC-3′ (amplified DNA size, 695 bp); IL-4 sense primer 5′-ATG-GGT-GCT-ATC-AGC-ATG-CAG-CTG-3′ and antisense primer 5′-CCA-AGC-CTG-CTG-ATC-TCA-3′ (amplified DNA size, 456 bp); IL-5 sense primer 5′-GCT-TGT-GCA-TAT-TGC-TCT-GTC-3′ (amplified DNA size, 293 bp); IL-10 sense primer 5′-ATG-GGT-GGT-CTC-ACC-TCT-TCC-3′ and antisense primer 5′-GCA-GTG-ATC-AGA-GAC-ATC-3′ (amplified DNA size, 352 bp); IL-13 sense primer 5′-ATG-GGC-GAG-CTG-GCT-3′ and antisense primer 5′-GTC-CTG-CCA-GGG-GGG-GTT-TAC-GGC-CCC-3′ (amplified DNA size, 248 bp); TNF-α sense primer 5′-GCT-CTG-GGC-ACC-ACC-ACC-ACC-GAG-3′ and antisense primer 5′-GTC-CTG-CCA-GGG-GGG-GTG-GAG-GAG-3′ (amplified DNA size, 1126 bp), all used as controls.

Determination of Cytokine mRNA Expression by RT-PCR. We have shown previously that the initial copies of the cytokine templates within the range of 102 to 106 have a linear relationship to the amount of PCR amplification products (3). In preliminary studies, the PCR products amplified by each cytokine PCR primer pairs matched the expected length according to the published cytokine cDNA sequences. The authenticity of the amplified cytokine product was confirmed by agarose gel electrophoresis.
TUMOR-DERIVED TYPE 2 CYTOKINES

Kine PCR products was confirmed by hybridization to 32P-labeled cDNA or oligonucleotide probes in Southern blot analyses (data not shown). Total RNA isolation and RT-PCR were performed as described previously (20). Briefly, high quality total RNA from five human NSCLC cell lines was prepared by RNazol method according to the manufacturer's instructions (Tel-Test, Inc., Friendswood, TX). RT-PCR was performed by using a RNA-PCR kit supplied by Perkin Elmer Cetus (Norwalk, CT). Briefly, reverse transcription was performed at 42°C for 1 h in a solution containing 4 µl MgCl2 (25 mM), 2 µl 10× PCR buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.3), 2 µl dNTPs mix (10 mM of each), 1 µl RNase inhibitor (20 units/µl), 1 µl random hexamer (50 mm), 1 µl RT (50 units/µl), 9 µl sterile diethyl pyrocarbonate-treated ddH2O containing 1 µg of total RNA. For PCR amplification, the following components were added to the 20-µl RT reaction: 3 µl 10X PCR buffer, 2 µl of each PCR primer pair mix, 6 µl dNTPs mix (10 mM of each), 0.5 µl Taq polymerase (5 units/µl), 18.5 µl sterile diethyl pyrocarbonate-treated ddH2O. To each PCR reaction, 50-µl sterile mineral oil was added on top. A PTC-100-60 thermal cycler (MJ Research, Inc., Watertown, MA) was programmed as follows: 2 min at 94°C for 1 cycle; 1 min at 94°C for 35 cycles, 1 min at 58°C, 1 min at 72°C, and 7 min at 72°C for 1 cycle. Twenty-five µl of the PCR products from each reaction was analyzed by 1.5% agarose/ethidium bromide gel electrophoresis.

Determination of Cytokine Protein Synthesis by Immunoprecipitation.

The immunoprecipitation procedure was performed as described previously (21). Briefly, 3 × 106 cells of each line were plated in a 25-cm2 tissue culture flask and maintained in routine culture condition. After 24-h incubation, the medium from each culture was discarded, and the cell monolayers were washed three times with PBS. The cells were preincubated in methionine-free medium (JRH Biosciences, Lenexa, KS) for 2 h and labeled with 200 µCi/ml [35S] methionine (NEN Research Products, Wilmington, DE) for 24 h. The cultured medium from each flask was collected and anti-hIg-Lu, anti-hIg-S, anti-hIg-10, or anti-TGF-β mAbs (R&D Systems, Minneapolis, MN) were added to each sample, respectively, in a final concentration of 5 µg/ml. After incubation at 4°C overnight with gentle rotation, 50 µl of protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO) in 1:1 dilution with ddH2O was added and incubated at 4°C for 4 h. The samples were pelleted by centrifugation at 12,000 × g for 1 min and washed twice in buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, and 0.02% SDS, followed by three washes in the same buffer without Triton and SDS. The cytokine/anticytokine complexes adsorbed to protein A-Sepharose from each sample were released by boiling for 5 min in 50 µl buffer containing 60 mM Tris-Cl (pH 6.8), 2% SDS, 2% 2-mercaptoethanol, 0.01% bromophenol, and 10% glycerol before PAGE. The gel was then dried and exposed to Kodak XAR-5 film at 25°C.

Determination of Type 2 Cytokine Production from Fresh NSCLC Nodules and Normal Lung Specimens.

Fresh human NSCLC nodules and normal lung samples were obtained from fresh surgical specimens at the West Los Angeles Veterans Administration Medical Center (Los Angeles, CA). The specimens were rinsed in PBS with three changes. One g of tumor nodule or normal lung tissues in 1 ml PBS was homogenized, and the tissue homogenates were centrifuged at 12,000 × g for 1 min at 25°C. After centrifugation, the supernatant of each sample was collected for further analysis. Cytokine production from tumor nodules and normal lung specimens were quantified by cytokine-specific ELISA. Human IL-5 (R&D Systems, Minneapolis, MN), IL-2, IFN-γ, IL-4, and IL-10 (Biosource, Camarillo, CA) ELISA were performed as described by the manufacturers. Absorbance of each sample was determined with an ELISA plate reader (Dynatech, Chantilly, VA). The sensitivity of the assays is 15.6 pg/ml for IL-2 and IFN-γ, 7.8 pg/ml for IL-4 and IL-5, and 0.78 pg/ml for IL-10. The total protein content of tumor nodules and normal lung tissues was quantified with a protein micro determination kit (Sigma). The results are expressed as pg of cytokine/mg of total protein.

Immunostaining of Paraffin-embedded NSCLC Sections.

Specific biotinylated antibodies for immunostaining of IL-4, IL-5, and IL-10 proteins were obtained from R&D Systems (Minneapolis, MN). The tissue sections were deparaffinized and hydrated through xylenes and graded alcohol series. The sections were then incubated in 0.3% H2O2 in methanol for 30 min for quenching of endogenous peroxidase activity. After washing three times with PBS, the sections were blocked with 10% FBS/PBS for 20 min. After washing with the same buffer, biotinylated anti-hIg-Lu, anti-hIg-S, anti-hIg-10, or irrelevant control antibodies were added to the sections and incubated for 30 min. After washing, streptavidin-peroxidase was added to the sections and incubated for 1 h. After color development, the sections were washed in tap water, counterstained with hematoxylin, cleaned, and mounted for photography.

Induction of NSCLC-derived Type 2 Cytokines.

Recombinant IL-4, IL-5, IL-8, IL-10, TGF-β, and granulocyte-macrophage colony-stimulating factor were purchased from R&D systems (Minneapolis, MN). IL-6 and TNF-α were purchased from Genzyme Diagnostics, Cambridge, MA. IFN-γ was purchased from BioSource International (Camarillo, CA). IL-2 was supplied by Chiron (Emeryville, CA). IL-7 was supplied by Sterling, Inc. (Malvern, PA). Cells from NSCLC lines SKL1U1, A549, 125, A427, RH2, H460, SK-MES-1, SW900, and H520 were plated at a concentration of 200,000 cells/well in 12-well tissue culture plates (Corning, Corning, NY). The cells were maintained in RPMI 1640 supplemented with 5% human AB serum, glutamine, and antibiotics in the presence or absence of cytokines for 24 h. The cultured supernatants from each well were collected and analyzed by cytokine-specific ELISA. The results are expressed as pg/ml.

RESULTS

Human NSCLC Cell Lines Express Type 2 Cytokine mRNA.

Total RNA from five well established human NSCLC cell lines (SKLU-1, A549, 125, A427, and RH2) were prepared, and the cytokine mRNA expression profiles from these tumor cells were determined by RT-PCR using β-actin as a positive control (Fig. 1). IL-2 and IFN-γ were not detected after 35 cycle amplifications under the conditions described in “Materials and Methods.” In contrast, IL-5, IL-10, and TGF-β were present in all lines tested. IL-4 was present in three of five lines (SKLU-1, 125, RH2) and IL-13 was present in two of five lines (SKLU-1 and A549). To exclude the possibility of carry-over contamination, reactions containing all RT-PCR reagents without sample RNA were used as negative controls. No contamination was detected (Fig. 1, bottom). In addition, all of the NSCLC lines are devoid of lymphocytes and do not express CD3α mRNA by RT-PCR (data not shown).

Human NSCLC Cell Lines Produce Type 2 Cytokines.

To determine whether the protein synthesis of tumor-derived type 2 cytokines correlates with the mRNA expression, isotope incorporation into the cultured cells and immunoprecipitation with specific anticytokine mAbs were performed in these tumor lines. In addition to its high sensitivity and specificity, the advantage of immunoprecipitation is that it only determines the newly synthesized proteins, thus eliminating the possibility of cytokine contamination from serum or other sources. The cells were maintained in culture and labeled with [35S] methionine. New synthesis of tumor-derived type 2 cytokine proteins was confirmed by demonstrating the specific mAb-precipitated bands that corresponded to the appropriate molecular weight in PAGE (Fig. 2).

Fresh Human NSCLC Nodules Produce Type 2 Cytokines.

Tumor lines maintained in long-term in vitro culture may not accurately reflect the characteristics of tumors in situ. To evaluate whether fresh human NSCLC nodules also produce type 2 cytokines, we obtained fresh lung tumor nodules and normal lung specimens from surgical resections and assessed the content of type 1 and type 2 cytokines in tissue homogenates by cytokine-specific ELISA. As shown in Table 1, fresh human NSCLC tumor nodules contain significantly more type 2 cytokines than do normal lung tissues. IL-10 is the predominant type 2 cytokine contained in these tumor nodules.

Immunohistochemical Staining Reveals Localization of Type 2 Cytokines to Lung Tumor Cells.

Tumor lines contain a variety of different cell types that could contribute to cytokine production. To identify the cellular source of type 2 cytokine production, we performed immunohistology on sections from 10 lung tumors (5 squamous cell carcinomas and 5 adenocarcinomas). All 10 specimens revealed positive staining for type 2 cytokines within tumor cells.
TUMOR-DERIVED TYPE 2 CYTOKINES

Fig. 1. Cytokine mRNA expression pattern from five human NSCLC cell lines by RT-PCR. Top to bottom: human NSCLC cell lines SKLU-1, A549, 125, A427, RH2, and negative control (RT-PCR cocktails without RNA samples). Lane M, 100-bp molecular weight marker; Lane 1, β-actin (1.126 bp); Lane 2, IL-2 (458 bp); Lane 3, IFN-γ (494 bp); Lane 4, TNF-α (695 bp); Lane 5, IL-4 (456 bp); Lane 6, IL-5 (293 bp); Lane 7, IL-10 (352 bp); Lane 8, IL-13 (248 bp); Lane 9, TGF-β (161 bp); arrows, respective PCR products.

Representative immunohistochemical staining of tumors with primary localization of antigenic IL-4, IL-5, and IL-10 to individual tumor cells is shown in Fig. 3.

IL-4 and TNF-α Induce NSCLC IL-10 Production. To evaluate the regulation of tumor-derived type 2 cytokines, cell lines were exposed to recombinant cytokines in vitro. IL-4 and IL-5 were not induced by the following recombinant cytokines: IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, TGF-β, TNF-α, IFN-γ, and granulocyte-macrophage colony-stimulating factor (data not shown). In contrast, tumor-derived IL-10 was induced 2–14-fold in 5 of 9 NSCLC lines by recombinant IL-4 (250 units/ml) and 1.5–15-fold in 5 of 9 NSCLC lines by recombinant TNF-α (1000 units/ml) (Table 2). In addition, concanavalin A (10 µg/ml) induces tumor-derived IL-10 production 2–13-fold in all lines (Table 2).

DISCUSSION

An important emerging concept in tumor immunology is that two distinct cytokine patterns may be generated by T lymphocytes (10–11). Whereas type 1 lymphocytes promote cellular immunity by producing IL-2 and IFN-γ, type 2 lymphocytes produce IL-4, IL-5, IL-10, and IL-13 and suppress cellular immune responses (12–17). Th1 and Th2 cells oppose one another’s activity, cytokine production, and differentiation (12, 14, 22). For example, IFN-γ and IL-10 have opposing effects on macrophage antigen-presenting function (23). In addition, IL-10 prevents the dendritic cell costimulatory activity that leads to IFN-γ production by T lymphocytes and inhibits antigen-specific Th1 cell proliferation (24). Recent studies have also begun to define the molecular pathways that differentiate the Th1 and Th2 phenotypes (14, 22, 25). Differences in costimulatory molecule expression (25), extinction of IL-12 signaling (22), modification of promoter binding proteins (14), and soluble factors such as prostaglandins (26–27) have been reported to modulate the Th1/Th2 axis. The type 1 and type 2 cytokine patterns were originally thought to be limited to CD4+ lymphocytes (originally referred to as Th1 and Th2 lymphocytes), but it is now recognized that non-CD4+ lymphocytes can also express type 1 or type 2 cytokine patterns (28–29).

In the current study, we have determined that NSCLC cell lines as...
well as fresh tumors express a distinct type 2 cytokine pattern. Other investigations have shown cytokine expression at the tumor site by RT-PCR of tumor biopsy samples (3, 18). However, use of this method did not allow distinction of the cellular source of cytokine production, and the cytokine pattern has been attributed only to tumor infiltrating lymphocytes. For example, Yamamura et al. (3) reported that a type 2 cytokine pattern was observed in biopsy samples from basal cell carcinoma but not in benign cutaneous lesions.

NSCLC respond only minimally to immunotherapy, perhaps because NSCLC cells produce a variety of immunosuppressive factors and thus may escape immune recognition (8, 30–32). Some tumor-derived products, such as epithelial growth factors, may act in an autocrine manner to enhance tumor growth (8). Others such as prostaglandins (33–36) may regulate the cytokine release by infiltrating inflammatory cells and thus may augment antitumor immunity (19, 37–38). In addition to TGF-β, we found that NSCLC cells produce type 2 cytokines and speculate that this may also contribute to immunosuppression.

In particular, IL-10 possesses several properties that may be inhibitory to the generation of antitumor immunity (23, 39–44). IL-10 inhibits a broad array of immune parameters, including proinflammatory cytokine production by macrophages (40–41), antigen-presentation function (24, 42-43), Th1 cytokine production (16, 23), and lymphokine-activated killer cell function (15). Pretreatment with recombinant IL-10 protects tumor cells from lysis by tumor-specific cytotoxic T cells (45–46). In a murine model, Wang et al. (47) demonstrated that tumor cells transfected with the IL-10 gene produce local immunosuppression and prevent the induction of tumor-specific cytotoxic T lymphocytes.

Tumor-derived IL-10 has been documented in lymphoma (48–49), ovarian carcinoma (50), melanoma, neuroblastoma, renal cell and colon carcinoma (51), and NSCLC (32). Colon cancer–derived IL-10 significantly inhibited the proliferative response in a mixed lymphocyte reaction (51). NSCLC-derived IL-10 inhibited TNF-α and IL-6 production by peripheral blood monocytes (32). The secretion of tumor-derived IL-10 in vivo may inhibit antigen presentation to cytotoxic T lymphocytes (52). Recent clinical studies suggest that elevated IL-10 level in 153 patients with non-Hodgkin’s lymphoma (both EBV-positive and EBV-negative) was found to be an independent prognostic factor, correlating with poor prognosis in patients with intermediate or high-grade non-Hodgkin’s lymphoma (48).

Tumors produce IL-10 constitutively in vitro. In situ, within the complex tumor microenvironment, the tumor is also exposed to an array of cytokines produced by infiltrating inflammatory cells and the tumor stroma that may influence cytokine production by the tumor cells. Thus, these cytokines present within the tumor milieu may regulate tumor IL-10 production. Of the many cytokines that could be operative in these interactions we found IL-4 and TNF-α to be inducers of tumor-derived IL-10. This is in agreement with previous studies demonstrating an increase in IL-10 production from colon cancer cell lines in response to IL-4 and TNF-α (51). Thus, we have begun to define a possible cytokine network in the tumor microenvironment that may ultimately lead to diminished immune responses at the lung tumor site. We speculate that IL-4-producing tumor-infiltrating lymphocytes may promote tumor production of IL-10. IL-10 in turn could lead to down-regulation of proinflammatory cytokine expression, decreased antigen-presenting cell MHC expression, and maintenance of the type 2 lymphocytic infiltrate at the tumor site. This paracrine-inhibitory cycle could promote a greater type 2 lymphocytic infiltration and continue induction of tumor-derived IL-10. In addition, because IL-4 is also produced by NSCLC cells, autocrine induction of tumor-derived IL-10 may occur.

IL-4 is a pleiotropic type 2 cytokine that has been found to have both stimulatory and inhibitory effects on antitumor immune responses (53–61). Recombinant IL-4 decreases the proliferation of some solid tumor cell lines including lung cancers (54–55). In contrast, IL-4 is also an autocrine growth factor produced by lymphoma cells (56). Although IL-4 has been found to enhance the proliferation of tumor-infiltrating lymphocytes in vitro (57), IL-4 is also known to inhibit macrophage production of inflammatory cytokines such as TNF-α, IL-1, and IL-8 (58–60) and inhibit lymphokine-activated killer cell function (61). In addition, both IL-4 and IL-10 are key cytokines for the inhibition of the Th1 cytokine response and the development of the Th2 cytokine response (12–13, 62–63). Thus, tumor-derived IL-4 and IL-10 in the NSCLC microenvironment may favor the development of the type 2 cytokine response at the tumor site.
Fig. 3. Immunostaining of paraffin-embedded NSCLC sections. Sections from the same block were stained as follows: A, H&E staining; B, nonspecific antibody staining as negative control; C, specific anti-IL-10 mAb staining; D, specific anti-IL-4 mAb staining; E, specific anti-IL-5 mAb staining.

Little is known regarding the importance of IL-13 and IL-5 in the host anti-tumor response. IL-13, the most recently described type 2 cytokine, is similar to IL-4 in that it also decreases macrophage production of IL-1 and TNF-α (17, 64). Although the role of tumor-derived IL-5 remains unclear, one study demonstrated that IL-5-transduced murine tumors escaped rejection despite eosinophil infiltration (65).

Introduction of cytokine genes into tumors is now being investigated in animal models and in clinical trials. Before manipulation with cytokine genes, it is becoming more clear that tumors have an endogenous constitutive pattern of cytokine expression (5–6, 32). Thus, the alteration of endogenous tumor cytokine production induced by cytokine gene transfer may be one of the important parameters to be assessed in the design of cancer gene therapy (66). The development
of cytokine gene therapy for lung cancer may be aided by a more complete understanding of the functional significance and regulation of endogenous lung tumour-derived cytokines.

We conclude that NSCLC cells contribute to the type 2 cytokine pattern at the tumor site. Further in vivo studies will be necessary to define the immunoregulatory activities of tumor-derived type 2 cytokines in the lung cancer microenvironment.

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