GENERAL THORACIC SURGERY

FUNCTIONAL INTERLEUKIN 4 RECEPTOR AND INTERLEUKIN 2 RECEPTOR COMMON γ -CHAIN ON HUMAN NON-SMALL CELL LUNG CANCERS: NOVEL TARGETS FOR IMMUNE THERAPY

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Objective: The interleukin 4 receptor has been demonstrated on the surface of human non-small cell lung carcinoma cell lines and tumor specimens. Interleukin 4 causes G1-phase cell-cycle arrest of non-small cell lung cancer cell lines expressing the interleukin 4 receptor; the effect directly correlates with the expression of the interleukin 4 receptor and is seen within 48 hours after treatment. We examined signal transduction pathways used by the interleukin 4 receptor that may account for growth arrest of the cell line LUsT but had no effect on another non-small cell lung cancer cell line, SK-MES-1. **Methods:** Western blot analysis was performed on both LUsT and SK-MES-1 cell lines cultured in the presence of interleukin 4 (500 U/mL). Cells were lysed, protein extracted, and electroblotted; blots were then probed with murine monoclonal antibodies to specific intracellular proteins. **Results:** Western blotting of the cell lines with antiphosphotyrosine antibody (4G10) demonstrated multiple (140 kd, 100-130 kd, and 65 kd) phosphoproteins seen only in the interleukin 4-treated LUsT cell line and not observed in the SK-MES-1 cell lines. Immunoprecipitation and blotting of the LUsT cell line with specific secondary antibodies demonstrated that the 140-kd phosphoprotein was the interleukin 4 receptor, the 130-kd phosphoprotein was Janus kinase 1, the 116-kd phosphoprotein was Janus kinase 3, and the 65-kd phosphoprotein was the interleukin 2 receptor γ -chain. Specific binding was not observed in the non-small cell lung cancer cell line SK-MES-1, suggesting that a functional interleukin receptor γ -chain was not present. Southern blotting with complementary DNA probes to interleukin 2 receptor γ -chain confirmed the absence of this receptor on cell line SK-MES-1. **Conclusions:** These results suggest that non-small cell lung cancer cells may express functional cytokine receptors, including the interleukin 2 receptor γ -chain commonly found in association with the lymphocyte interleukin 2 receptor. These receptors may be novel targets for directing cytokine-based immune therapy. (J Thorac Cardiovasc Surg 2000;119:10-20)

Interleukin 4 (IL-4) is primarily a T-lymphocyte and mast cell-derived cytokine with a variety of biologic functions. Originally described for its ability to stimu-

late B cells, IL-4 has been found to activate cytotoxic T cells, modulate macrophage function, and cause B-cell immunoglobulin class switching.^{1,2} Our results and the

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work of other investigators demonstrate that IL-4 can inhibit growth of a variety of nonhematopoietic malignancies, including melanoma, renal,⁴ gastric,⁵ and colorectal carcinomas.⁶ Topp and colleagues⁷ reported that IL-4 inhibited the growth of lung carcinoma cell lines expressing IL-4 receptor (IL-4R) α . Tungekar and colleagues⁸ demonstrated the presence of IL-4R on 35% of fresh surgical specimens of human squamous and adenocarcinoma lung cancers and found that the effect of IL-4 was mediated through its receptor. We have previously demonstrated that IL-4 produces G0/G1 cell-cycle arrest of IL-4R⁺ cell lines.⁹ Yet the molecular mechanisms that regulate the downstream processing of the IL-4 signal in nonhematopoietic cells are not well understood.

The complementary (c)DNA for the human IL-4R α has been cloned, is well characterized, and is part of the hematopoietic superfamily of receptors.¹⁰ Crosslinking studies demonstrate that IL-4 binds to the high-affinity 140-kd IL-4R α and 65-kd IL-2R γ -chain in hematopoietic cells,¹¹ although this process has not been demonstrated in nonhematopoietic malignancies. The intracellular domains of the IL-4R α have no consensus sequences for tyrosine or serine-threonine kinases, although tyrosine phosphorylation (170-kd, 140-kd, and 110-kd proteins) has been observed after IL-4 activation of murine lymphoid cell lines.¹² These studies suggest the 140-kd protein is the IL-4R α , and this protein activates both the 170-kd insulin receptor substrate 1 (IRS-1), the Janus kinase (JAK) family of transcription factors, and the signal transducers and activators of transcription (STAT) 6.^{12,13} In the current investigation we examined the growth-inhibitory effects of IL-4 on 2 human non-small cell lung carcinoma (NSCLC) cell lines. Our data indicate that the IL-4-responsive NSCLC cell line LUST demonstrated growth inhibition in the presence of IL-4 with tyrosine phosphorylation of multiple proteins. Although IRS-1 is constitutively expressed in both the LUST and SK-MES-1 cell lines, immunoprecipitation of the IL-2R γ -chain along with IL-4R α in the LUST cell line suggests that this receptor complex is necessary for activation of JAK1 and JAK3, along with STAT6. The absence of IL-2R γ -chain in the nonresponsive cell line SK-MES-1 may explain the inability of this cell line to respond to IL-4 and be useful for the design of therapies directed at IL-4R⁺ lung cancers.

Methods

Cell lines. Human NSCLC cell lines LUST (established at the University of California, Los Angeles, gift of G. Juillard) and SK-MES-1 (American Type Cell Culture, Rockville, Md)

were cultured in RPMI-1640 culture medium (JRH Biosciences, Lenexa, Kan) or Dulbecco's modified Eagle's medium (Life Technologies Inc, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Gemini Bioproducts, Calabasas, Calif) and antibiotics (100 U/mL each of penicillin and streptomycin) in 75-cm² flasks. The human gastric cancer cell line CRL 1739 (ATCC) and colon carcinoma line (Spiro or CoSp) established at our laboratory served as positive controls in some studies because they have shown growth inhibition in the presence of IL-4. All cell cultures were maintained in continuous exponential growth by weekly passage, as previously described.⁵

Cytokines and reagents. Recombinant human IL-4 (1.8×10^7 U/mg) was the generous gift of Schering Plough (Kenilworth, NJ). Mouse monoclonal IgG2b antiphosphotyrosine (clone 4G10), agarose-conjugated antiphosphotyrosine 4G10 antibody, IgG anti-JAK1, and anti-IRS-1 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal IgG anti-IL-4R α , anti-IL-4 Stat (STAT6), anti-IL-2R γ -chain, and anti-JAK3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Anti-IL-2R γ -chain monoclonal antibody was a kind gift of Dr K. Sugamura (Tohoku University School of Medicine, Sendai, Japan).

RNA extraction. Total cellular RNA was extracted by using the UltraSpec isolation system (Biotecx, Houston, Tex), as described by the manufacturer. Briefly, cells were trypsinized, washed in 1 $\times$ phosphate-buffered saline (PBS), lysed in 2 mL of UltraSpec reagent by repetitive pipetting, and placed on ice for 5 minutes in an RNase-free Eppendorf tube. Four hundred microliters of chloroform was added, and after vigorous mixing for 15 minutes, the solution was placed on ice for 5 minutes and then centrifuged at 12,000g at 4°C for 15 minutes. The aqueous phase was transferred into another RNase-free Eppendorf tube; one volume of isopropanol was added, and the solution was precipitated at 4°C for 30 minutes. The tube was centrifuged at 12,000g at 4°C for 20 minutes to obtain an RNA pellet. The sample was washed with 70% ethanol, dried, and resuspended in 50 μ L of diethyl pyrocarbonate-treated TRIS-hydroxymethylamino methane (TRIS)-ethylenediamine tetraacetic acid (EDTA) buffer. All RNA extraction procedures were performed in a designated laminar flow hood under sterile conditions. Polymerase chain reaction (PCR) reagent set-up and gel electrophoresis were performed in separate rooms to avoid potential RNA contamination.

Reverse transcription PCR. Total sample RNA was reverse transcribed by using the reverse transcription (RT) mixture consisting of Moloney murine leukemia virus reverse transcriptase with oligo (dT) primer, as previously described.⁵ Three micrograms of sample RNA was used in the reactions. All reagents were obtained from Promega (Madison, Wis). The reaction was incubated at 37°C for 2 hours, at 99°C for 5 minutes, and on ice for 5 minutes.

PCR amplification. Oligonucleotide primers were synthesized and purified by Gibco BRL (Gaithersburg, Md). Oligonucleotide 5' and 3' primers for individual genes were

designed as follows: IL-2R γ -chain, 5'-GGCCACACAGAT-GCTAAACT-3' and 3'-GAACAATGACTTATGGTGGCC-5'; IL-4R α , 5'-ATGGGGTGGCTTTGCTCTGGG-3' and 3'-ACCTTCCCGAGGAAGTTCGGG-5'; and β -actin-5'-CCTTCCTGGGCATGGAGTCCTG-3' and 3'-CTTC-TAGTTCTAGTAACGAGG-5'. The PCR cDNA products of IL-2R γ -chain, IL-4R α , and β -actin were 492 bp, 345 bp, and 202 bp, respectively. The PCR was performed as previously described.⁵ The annealing temperature for primers was designed by using OLIGO Primer Analysis Software 5.0 (Plymouth, Minn), and the PCR conditions were set up as follows: 95°C for 5 minutes followed by the cycle of 95°C for 1 minute, 67°C for IL-2R γ -chain (64°C for IL-4R α and 55°C for β -actin) for 1 minute, and 72°C for 1 minute, with a repeat of the three 1-minute phases for 35 cycles and a final 72°C for a 10-minute extension time and final soaking at 4°C. The PCR reaction was performed in an OmniGene temperature cyclor (Hybaid, Middlesex, England). The preparation of PCR mixture for the temperature cyclor was performed in a designated PCR room in a sterile laminar flow hood.

The PCR cDNA product was detected by electrophoresis on a 2% agarose gel (Life Technologies Inc) and visualized by ethidium bromide staining under UV light. A 100-bp DNA ladder (Life Technologies Inc) was used as a reference marker for all assays.

Southern blot analyses. After electrophoresis of PCR cDNA products, agarose gels were transferred overnight onto nitrocellulose membranes (Scheicher and Schull, Keene, NH) with 20 $\times$ standard sodium citrate (SSC) buffer (1 $\times$ SSC = 0.15 mol/L sodium chloride plus 0.015 mol/L sodium citrate), as previously described,¹⁴ and UV cross-linked at 1200 mJ/cm² by using a UV Crosslinker (Fisher Scientific, Pittsburgh, Pa). The cDNA probe was prepared from PCR cDNA product of the IL-2R γ -chain sequence inside the PCR primers. The probe was prepared and labeled by using the DIG DNA Labeling Kit (Boehringer Mannheim, Indianapolis, Ind) according to the manufacture's protocol. The template cDNA was heat denatured before random primer labeling. The labeling reaction resulted in incorporation of DIG-dUTP (uracil triphosphate) every 20 to 25 nucleotides in the newly synthesized DNA probe. Labeled DNA was precipitated with 4 mol/L lithium chloride and 70% ethanol. The blot was prehybridized, and the labeled cDNA was added to the blot in hybridization solution (5 $\times$ SSC, blocking reagent, 0.1% N-lauroylsarcosine, and 0.02% sodium dodecylsulfate [SDS]) at the concentration of 26 ng/mL and hybridized overnight at 68°C. Specific binding was detected by using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim). After hybridization to target DNA, the hybrids were detected by using an enzyme-linked immunoassay with the antidigoxigenin-alkaline phosphatase conjugate and subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphatase and nitro blue tetrazolium salt at room temperature until the desired bands were detected. The reaction was terminated by washing the blot for 5 minutes in 10 mmol/L TRIS-HCl and 1 mmol/L EDTA (pH 8.0). The results were documented by photograph.

Flow cytometry. Flow cytometric examination of IL-4R α expression on the cell surface was performed with biotin-labeled IL-4, as described previously.⁵ Briefly, biotin-N-hydroxysuccinimide (Calbiochem, La Jolla, Calif) and purified rhIL-4 were mixed at molar ratios. This mixture was incubated at room temperature for 30 minutes and mixed every 10 minutes during incubation. The reaction mixture was then dialyzed in PBS and adjusted to 10 μ g/mL in PBS plus 0.01% NaN₃.

Tumor cells (5×10^5) were incubated with 50 ng of biotinylated IL-4 in 100 μ L of PBS plus 0.02% NaN₃ plus 2% heat-inactivated fetal calf serum for 30 minutes at 4°C. After 3 washes with PBS-NaN₃, the cells were further incubated in 50 μ L of diluted streptavidin-fluorescein isothiocyanate for 30 minutes at 4°C. Cells were washed 3 times and analyzed by FACSscan (Becton-Dickinson, Mountain View, Calif).

Peripheral blood lymphocytes (PBLs) were obtained from normal healthy donors and activated with 0.5 μ g/mL phytohemagglutinin (Sigma Chemical Corp, St Louis, Mo) plus IL-2 for 48 hours in culture. These activated PBL cultures were used as a positive control for IL-4R α analysis.

Cell proliferation. A standard tritiated-thymidine (New England Nuclear, Boston, Mass) incorporation proliferation assay was set up in 96-well, round-bottom microplates (Costar, Cambridge, Mass).¹⁵ NSCLC cell lines were seeded in the wells at a concentration of 10^4 cells per well in 200 μ L of culture medium in the presence or absence of IL-4. Each test was performed in triplicate. Cells were cultured for 48 hours and pulsed with tritiated thymidine for 6 hours before harvesting. Microplate wells were harvested by using a Brandel harvester (Brandel, Gaithersburg, Md) and counted in a Beckman scintillation counter (Beckman, Fullerton, Calif), as previously described.^{5,15}

Immunoprecipitation and immunoblot analysis. Cell lines were washed and then incubated in serum-free medium 1 night before adding cytokine. After stimulation with IL-4 (500 U/mL) for approximately 10 minutes at 37°C, the cells were lysed in lysis buffer (10 mmol/L TRIS-HCl [pH 7.2], 150 mmol/L sodium chloride, 1% sodium deoxycholate, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L sodium fluoride, 100 μ mol/L sodium vanadate, 10 μ g/mL leupetin, and aprotinin; Sigma Chemical Co). The lysates were centrifuged at 12,000g for 20 minutes at 4°C to remove insoluble material. The total protein content of the lysates was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif). Equal amounts of clarified cell lysates (approximately 2 mg) were immunoprecipitated with 3 μ g of anti-IL-2R γ -chain (Santa Cruz Biotechnology) or monoclonal antiphosphotyrosine antibody (4G10; Upstate Biotechnology) by using 20 μ L of protein A insolubilized on Sepharose 4B fast flow (Sigma Chemical Co). The immunoprecipitates were washed twice in dilution buffer (0.1% Triton X-100 and bovine hemoglobin in TSA solution [0.01 mol/L TRIS-Cl, pH 8.0; 0.14 mol/L sodium chloride; and 0.025% sodium azide]) one time in TSA solution and another in 0.05 mol/L TRIS-Cl (pH 6.8) solution solubilized with Laemmli buffer, boiled, and resolved by TRIS-glycine 4% to 12% SDS-polyacrylamide gel electrophoresis

Fig 1. Effects of IL-4 on growth of 2 NSCLC lines, LUST and SK-MES-1. Cells were cultured for 48 hours in the presence or absence of IL-4. Growth inhibition was determined by percentage inhibition of tritiated thymidine incorporation. Values are means $\pm$ SDs and demonstrate significant ($P = .0001$; sign test, nonparametric t test) growth inhibition induced by IL-4 for cell line LUST versus untreated cell line (untreated LUST, $11,204 \pm 175$ cpm). IL-4 had no effect on tritiated thymidine incorporation of SK-MES-1 (untreated SK-MES-1, $34,299 \pm 5702$ cpm). Representative experiments were performed in triplicate.

(Novex, San Diego, Calif). In some experiments proteins (75 μ g) were directly resolved by SDS-polyacrylamide gel electrophoresis without prior immunoprecipitation.

Western blot analyses were performed by transferring proteins from polyacrylamide gels onto Hybond-ECL nitrocellulose membranes (Amersham Corp, Arlington Heights, Ill) at 25 V for 2 hours in TRIS-glycine buffer containing 25 mmol/L TRIS, 192 mmol/L glycine, 0.1% SDS, 100 μ mol/L sodium vanadate, and 20% methanol. The blots were treated for 1 hour with blocking buffer (2.5% nonfat dry milk, 10 mmol/L TRIS-Cl [pH 7.5], 100 mmol/L sodium chloride, and 0.1% Tween 20) and then incubated with 2 μ g/mL antibody in blocking buffer for another hour. Antibody binding was detected by incubating the blots for 1 hour with sheep anti-mouse or anti-rabbit immunoglobulin conjugated with horseradish peroxidase, followed by a 1-minute incubation with iodinated substrate and then enhanced chemiluminescence detection (ECL; Amersham Corp, Arlington Heights, Ill). The blots were exposed to autoradiography film (Hyperfilm-ECL). Some blots were reprobed after being stripped with 2% SDS, 6.25 mmol/L TRIS (pH 6.7), and 100 mmol/L 2-mercaptoethanol at 55°C for 30 minutes with gentle agitation. Relative differences in protein expression were determined

Fig 2. Western blot analysis demonstrating specific binding of the 140-kd IL-4R α . The 2 NSCLC cell lines, SK-MES-1 and LUST, were treated for up to 30 minutes in the presence of 500 U/mL of rhIL-4. Western blots were performed from cell lysates by using antibody to IL-4R α (see "Methods" section). The human colon carcinoma cell line Spiro (CoSp) established in our laboratory has previously been found to express a high level of IL-4R α and served as positive control for this experiment.²⁵

through densitometry by using a dual-wavelength flying-spot scanner (Shimadzu Corp, Kyoto, Japan).

Statistics. Differences in means and SDs for growth analyses were performed by using the sign test with the nonparametric t test.

Results

Effect of IL-4 on cell growth. Because IL-4 has been implicated as having growth-inhibitory effects on human nonhematopoietic cell lines and, in particular, lung carcinoma cells, we examined the mechanisms of its action on the 2 NSCLC lines, LUST and SK-MES-1. IL-4 (100-500 U/mL) significantly ($P = .0001$) inhibited the growth of LUST in 48-hour culture compared with untreated LUST (Fig 1). Growth inhibition was dose dependent and was seen with as little as 100 U/mL IL-4. Maximal inhibition of growth ($68\% \pm 5\%$) was

Fig 3. Flow cytometric analysis of IL-4R α expression. Both LUst and SK-MES-1 cell lines were subjected to flow cytometric analysis by using biotin-labeled human rhIL-4 (see "Methods" section). SK-MES-1 was found not to express IL-4R α (A), whereas 70% of LUst cells were found to express IL-4R α (B). PHA- and IL-2-stimulated PBLs served as positive controls for IL-4R α expression in this experiment.

Fig 4. IL-4R α and IL-2R γ -chain mRNA expression from LUst and SK-MES-1 cell lines. Both LUst and SK-MES-1 cell lines were assessed by RT-PCR to determine expression of IL-4R α and IL-2R γ -chain. The 345-bp IL-4R α was expressed by both LUst and SK-MES-1 cell lines. The 492-bp IL-2R γ -chain product was expressed by the LUst but not by the SK-MES-1 cell line. Southern blotting confirmed the RT-PCR product was IL-2R γ -chain (see "Methods" section).

observed with 500 U/mL IL-4. Higher concentrations of IL-4 (>500 U/mL) had no further effect on growth inhibition in LUst (data not shown). These findings of a maximal dose effect by IL-4 have been observed by other investigators. Cultured cells were noted to become wider and fattened but remained adherent to plastic plates in response to IL-4. Exchanging the culture supernatant with IL-4-free culture medium 24 hours after the experiment was initiated did not reverse

the effect of IL-4. Previous work by our laboratory suggests IL-4 causes G0/G1 cell cycle-phase arrest of IL-4-responsive lines.^{5,9} Trypan blue exclusion demonstrated that IL-4 was not toxic to the cell lines, and the concentrations were accordingly well below the reported level of toxicity for B and T cells in vitro.¹⁶ The SK-MES-1 cell line was nonresponsive to the growth-inhibitory effects of IL-4 at all of the concentrations used for LUst.

Fig 5. Tyrosine phosphorylation of IL-4-treated and -untreated NSCLC cell lines. Both human NSCLC cell lines were cultured in serum-free medium overnight and then treated with 500 U/mL IL-4 for 10 minutes. **A**, Western blotting with antiphosphotyrosine antibody (4G10) demonstrated 140-kd and 65-kd phosphoproteins in IL-4-treated LUSt not present in SK-MES-1. **B**, Immunoprecipitation with antiphosphotyrosine (4G10) and blotting with anti-IRS-1 demonstrated specific expression of IRS-1 from both cell lines. *PTyr*, Phosphotyrosine.

Expression of IL-4R α . Western blot and flow cytometry analyses were performed to determine the expression of IL-4R α on our 2 cell lines. Western blot analyses with specific antibodies to IL-4R α demonstrated evidence of the 140-kd IL-4R α in LUSt and in a colon carcinoma cell line, Spiro (CoSp), we established in our laboratory,¹⁷ whereas SK-MES-1 had only minimal expression of the IL-4R α (Fig 2). Flow cytometry experiments demonstrated binding of biotinylated rhIL-4 to IL4R α -positive LUSt and no binding to SK-MES-1 (Fig 3). The discrepancy between the Western blot and flow cytometric results may be explained by the difference in sensitivity of the 2 assays and the possibility of differences in function of the cell surface IL-4R α and expression from cell lysates on the responsive and nonresponsive cell lines. Human PBLs stimulated with IL-2 served as positive controls for the flow cytometry experiments (data not shown).

Expression of IL-2 R γ -chain. We performed RT-PCR analyses of the 2 cell lines to determine whether

IL-4 binding to our 2 cell lines related to messenger (m)RNA expression of either IL-4R α or IL-2R γ -chain. The 345-bp IL-4R α product was present in both LUSt and SK-MES-1, confirming the findings obtained by Western blotting. The lack of IL-4 binding by SK-MES-1 may be indicative of a quantitative difference in cell surface expression or posttranslational modification of the IL-4R α product (Fig 4).

The IL-2R γ -chain has been found to be an important component of IL-2 binding in hematopoietic cells and recently has been shown to be essential for signal transduction of a variety of cytokines, including IL-4.¹³⁻¹⁸ We assessed IL-2R γ -chain mRNA expression by RT-PCR to determine its relationship to IL-4 binding in our 2 NSCLC cell lines. The 492-bp IL-2R γ -chain was found in untreated LUSt and human PBL control cells but was absent in SK-MES-1. Southern blot analyses confirmed that the 492-bp RT-PCR cDNA products found from both the human PBLs and LUSt cell line were IL-2R γ -chain products.

Fig 6. IL-2R γ -chain expression in NSCLC cell line LUST. The cell line LUST was cultured in serum-free medium overnight and then treated with 500 U/mL IL-4 for 10 minutes. Cell lysates were immunoprecipitated with anti-IL-2R γ -chain antibody and probed with specific antibodies to antiphosphotyrosine, IL-2R γ -chain (65 kd), IL-4R α (140 kd), JAK1 (130 kd), and JAK3 (116 kd). PTyr; Phosphotyrosine.

Tyrosine phosphorylation response to IL-4. Because the LUST cell line was found to express both IL-4R α and IL-2R γ -chain, we sought to determine whether the signal transduction pathways known to be essential for hematopoietic cells (by means of IRS-1 or JAK-STAT) would be important for IL-4-induced growth inhibition in the LUST cell line. Western blot of the IL-4-treated LUST cell line with the mouse monoclonal antiphosphotyrosine (4G10) demonstrated tyrosine phosphorylation of 140-kd and 65-kd proteins in the IL-4-treated LUST cell line that were not present from untreated cells (Fig 5, A). Analysis of SK-MES-1 cell lysates from IL-4-treated and -untreated lines demonstrated no specific protein patterns in the presence of IL-4.

The IL-4R α has been found to signal through IRS-1-dependent pathways in both hematopoietic and non-hematopoietic cell lines.¹⁹ We observed no evidence of the 170-kd IRS-1 phosphoprotein by using Western blotting with 4G10 antibody or specific binding with anti-IRS-1 antibody (data not shown). When the cell lines were immunoprecipitated with 4G10 and blotted with anti-IRS-1 antibody, we found that both cell lines expressed almost equal quantities of IRS-1 protein (Fig 5, B). There was no enhancement in IRS-1 protein expression (gel films assessed by densitometry) after treatment with IL-4 for 10 minutes or up to 1 hour, sug-

gesting that IRS-1 is likely not involved in IL-4-induced growth inhibition of the LUST cell line.

Phosphorylation of the IL-2R γ -chain. Because our earlier experiments demonstrated the presence of IL-2R γ -chain in the LUST cell line, we examined for the presence of the secondary messengers known to be associated with IL-2R γ -chain binding. Immunoprecipitation with anti-IL-2R γ -chain antibody and blotting with 4G10 antibody demonstrated multiple (65 kd, 140 kd, 130 kd, and 116 kd) phosphoproteins from the IL-4-treated LUST cell line (Fig 6). The 130- and 116-kd phosphoproteins were not observed by using Western blotting with antibody 4G10 alone, which we suspect to be because of the high level of background phosphoproteins seen by blotting with 4G10 alone (Fig 2). SK-MES-1 failed to demonstrate any significant binding of the IL-2R γ -chain antibodies, suggesting that the IL-4R α /IL-2R γ -chain complex was necessary for phosphorylation of the multiple secondary messengers. We reblotted the membranes with anti-IL-2R γ -chain and anti-IL-4R α antibodies, demonstrating that both the IL-4R α and IL-2R γ -chain products are present and interact in the IL-4-treated LUST cell line.

Phosphorylation of JAK. JAK3 and JAK1 have been shown to be an important part of IL-4 signal transduction in hematopoietic cells.²⁰ The 116-kd JAK3

protein has been found to associate with IL-2R γ -chain in nonhematopoietic cells and serves as an activator of the JAK-STAT pathway. We performed immunoprecipitation of cell lysates with anti-IL-2R γ -chain antibody and probed with anti-JAK3 antibodies. Western blotting with anti-JAK3 antibody demonstrated slight enhancement in quantitative binding (determined by densitometry of the blot films) between IL-4-treated and -untreated LUst cell lines (and control cell lines CRL1739 and Spiro; Fig 7). Similar results were observed when blots were reprobed for the 130-kd JAK1. JAK1 binds to the IL-4 receptor and complexes with the IL-2R γ -chain. The IL-4-responsive LUst cell line demonstrated activation of JAK1 (Fig 8). We attempted to examine the response of the STAT6 protein in the IL-4-sensitive LUst cell line. Western blots demonstrated the presence of the 100-kd STAT6 (IL-4 Stat), but no quantitative differences were seen in the presence of IL-4, whereas SK-MES-1 cell lysates demonstrated no binding to the anti-STAT6 antibody (data not shown). These results suggest that the IL-4R α /IL-2R γ -chain complex binds to JAK-dependent pathways after activation with IL-4.^{21,22}

Discussion

Our results demonstrate that rhIL-4 inhibited the growth of a human NSCLC cell line, and the effect was directly related to the expression of IL-4R α . A second NSCLC cell line, SK-MES-1, demonstrated only minimal expression of IL-4R α and was unresponsive to the growth-inhibitory effect of IL-4. The 140-kd IL-4R α has been well characterized and has been found on a number of human nonhematopoietic cell lines. Although we did not quantitate the number of IL-4R α molecules present on these 2 cell lines, other investigators have found anywhere from 2000 to 6000 IL-4R α molecules per cell on responsive cell lines.⁴⁻⁶ The minimum number of IL-4R α molecules for IL-4 to inhibit cell growth is unknown.

We believe that IL-4-induced growth inhibition of the LUst cell line is directly mediated by IL-4R α . LUst cells were sensitive to the effects of IL-4 and had a high level (>60%) of receptor expression, as demonstrated by flow cytometry binding with a biotinylated rhIL-4. SK-MES-1 expressed minimal levels of IL-4R α by Western blotting (using specific antibody to IL-4R α), but our flow cytometry data indicate no functional binding with biotinylated IL-4. IL-4 had no effect on cell proliferation of SK-MES-1. We performed RT-PCR analyses of these 2 cell lines by using specific oligonucleotide primers that verify the presence of IL-4R α mRNA in the LUst and SK-MES-1 cell lines. The

Fig 7. JAK3 expression from NSCLC cell line LUst. Western blotting of the LUst cell line after immunoprecipitation by anti-IL-2R γ -chain antibody and blotting with specific anti-JAK3 antibody demonstrated JAK3 expression from untreated and IL-4-treated LUst cell line. The IL-4-responsive cell lines CRL1739 and Spiro served as controls.

SK-MES-1 cell line constitutively expressed low levels of IL-4R α mRNA, suggesting that possible posttranscriptional modification of the receptor or poor protein expression may be responsible for the inactivity of the receptor. Monoclonal antibody (M57) to IL-4R α blocks the growth inhibition induced by IL-4 on the LUst cell line, suggesting that the effect of IL-4 is mediated through IL-4R α but does not exclude the possibility that IL-4 works through the activation of other secondary cytostatic factors.⁴

We identified a 65-kd phosphoprotein that is present in the rhIL-4-treated LUst cell line but not present in the SK-MES-1 cell line. Our data suggests that this 65-kd species is the IL-2R γ -chain. Although the IL-2R γ -chain is more commonly associated with IL-4 (and IL-2, IL-7, and IL-13) binding in hematopoietic cells, these results suggest that the IL-2R γ -chain is present in an established IL-4R α ⁺ human NSCLC cell line. Our studies (unpublished observation, 1998) and a report from another group of investigators²³ have also observed the presence of IL-2R γ -chain in other human nonhematopoietic cell lines (gastric and colon cancers) and tumor biopsy specimens. Other investigators have observed a 70-kd product from IL-4-treated nonhematopoietic cells and have suggested that this protein may represent degradation products of the 140-kd IL-4R α ²⁴ or may represent an alternative binding protein, as seen in IL-13 binding.^{19,25} IL-13 had no effect on the growth of either of our cell lines. Specific binding demonstrated by immunoprecipitation studies and confirmation by Southern blotting has found that the 65-kd protein in the NSCLC cell line, LUst, is the IL-2R γ -chain.

To understand the mechanisms of IL-4-induced inhi-

Fig 8. JAK1 expression from NSCLC cell line LUst. Western blotting of untreated (—) and 500 U/mL IL-4-treated (+) LUst cell line demonstrated expression of the 130-kd JAK1 after immunoprecipitation with anti-IL-2R γ -chain antibody. SK-MES-1 cell line demonstrated no evidence of JAK activation by means of IL-2R γ -chain-induced binding.

bition of tumor cell growth inhibition, we investigated the signal transduction pathways used by IL-4 in the NSCLC cell line LUst. IL-4 signaling events appear to act through the IL-4R α and JAK-STAT pathways used by hematopoietic cells. Several studies have shown that an alternative pathway exists in which the secondary messenger IRS-1 is phosphorylated in response to IL-4.²⁶ Yet it has been suggested that the phosphorylation of IRS-1 may occur only in the presence of IL-2R γ -chain.²⁷ Our results suggest that although IRS-1 was expressed in both IL-4-responsive and -nonresponsive cell lines, the level of expression was no different in the 2 cell lines and likely excludes the possibility of IRS-1 contributing to IL-4-induced growth arrest.²⁴ Because JAK and IRS-1 have been found to associate with the 140-kd IL-4R α in murine T cells, it is possible that JAK phosphorylates IRS-1 without involving the IL-2R γ -chain.²⁸

After complexing of IL-4R α and IL-2R γ -chain in hematopoietic cells, several investigators have demonstrated activation of both JAK1 and JAK3.^{21,22} Ligand binding to IL-4R α has been shown to activate associated JAK tyrosine kinases, which phosphorylate each other, as well as receptor tyrosine phosphoproteins. Several investigators have demonstrated that IL-2R γ -chain complexes with JAK3 after activation with IL-2 or IL-4.^{20,21,29} Our results confirm these results in the

NSCLC cell line LUst, and the lack of growth inhibition observed in other nonhematopoietic cell lines may result from the absence of IL-2R γ -chain and JAK interactions. Recent studies demonstrate that STAT6 is recruited to the activated IL-4R α by docking of the SH2 domain where they are phosphorylated by JAKs and then dimerize and translocate to the cell nucleus where gene activation occurs.^{21,22,24} Our work is the first to our knowledge to describe complexing of IL-4R α and IL-2R γ -chain in nonhematopoietic cells along with expression of JAK1, JAK3, and STAT6.

It is clear that IL-4 has contrasting effects on different tissue types. In immune cells IL-4 has both potent growth-stimulating and growth-inhibitory effects; however, in nonhematopoietic cells IL-4 has only growth-inhibitory effects. The reason for the dichotomy is unclear. Some of the differential effects may relate to the signaling pathways used by IL-4R α in different cell types. It is possible that the presence of the IL-2R γ -chain may be responsible for growth inhibition in some nonhematopoietic cell lines by complexing through JAK-1 and JAK-3/STAT (type I IL-4R) pathways, whereas some other cells appear to respond even in the absence of IL-2R γ -chain (type II IL-4R).²⁰

The presence of IL-4R α on NSCLC may relate to the mechanism of development of lung cancers. Although some studies suggest lung carcinomas arise from a sin-

gle common stem cell, the lack of IL-4R α (and IL-2R γ -chain) may represent an adaptive property of these tumors to become autonomous from host growth-regulatory mechanisms. Often in the lung, mast cell and CD4⁺ T-cell infiltrates are present adjacent to the tumor infiltrate. Both of these cell types are known to produce IL-4. The presence of IL-4R α on NSCLC tumors may represent a host defense mechanism that is lost during tumor development.

The demonstration of functional IL-4R α on tumor cells suggests that a direct cytostatic effect may be possible. Either IL-4-directed treatment alone or treatment conjugated to toxins may have a role in the treatment of IL-4R α ⁺ lung carcinoma.³⁰ On the other hand, the ability to induce G1-phase cell-cycle arrest in lung cancer may be possible through replacement or reactivation of the JAK-STAT pathway. Further understanding of the mechanism of IL-4R α signal transduction related to cell growth may be beneficial in developing more effective therapies for lung cancer.

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Discussion

Dr Valerie W. Rusch (*New York, NY*). Can you tell us a little more about the cell lines that you used in terms of their other molecular characteristics and how these are or are not relevant to human tumors?

Dr Essner. We used the LUsT cell line, which is an adenocarcinoma established at UCLA. We found it to be the most responsive to the effects of IL-4, and that is why we used it. Our impression is that as cell lines undergo more and more passages in culture, they lose the expression of the IL-4R and become unresponsive to IL-4. LUsT appears to retain expression of the IL-4R even after multiple passages in cell culture. There have been some studies in the literature suggesting that

about 35% of cell lines and tumor biopsy specimens expressed the IL-4R. We have seen upregulation of HLA-DR expression by IL-4, but we did not further characterize this particular cell line. In other words, we did not attempt to determine what other tumor-associated markers are present on it.

Dr Rusch. It would be interesting to know what the relationship is between the IL-4 regulation in these cell lines and other factors that are known to control the G1 to S transition in most lung cancers, such as p53.

Dr Essner. We did not look specifically for p53 or cyclin D1 regulation and so on, but certainly we could look at that. We previously did some work with gastrointestinal cancers and found that IL-4 upregulated unphosphorylated Rb protein and downregulated cyclin D₁, c-myc protein, and phosphorylated Rb protein.

Dr Rusch. How are you going to extend this work?

Dr Essner. The next question is whether we could in fact take the SK-MES cell line and transfect it with the γ -chain and see whether that reactivates cell cycle arrest. And the other option, obviously, is to look at the LUsT cell line and see whether we can block the γ -chain and see whether this prevents cell-cycle arrest induced by IL-4. We need to extend our studies to other cell lines to see whether the same mechanism is active and could be used for development of new therapies for lung cancer.