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Monoclonal Antibody αIR-3 Inhibits Non-Small Cell Lung Cancer Growth In Vitro and In Vivo

Farah Zia, Steve Jacobs, Frederick Kull Jr., Frank Cuttitta, James L. Mulshine, and Terry W. Moody

Departments of Microbiology, Biochemistry, and Molecular Biology, The George Washington University School of Medicine and Health Sciences, Washington, DC 20037 (F.Z.); Division of Cell Biology, Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709 (S.J., F.K.); National Cancer Institute, Biomarkers and Prevention Research Branch, Rockville, Maryland 20850 (F.C., J.L.M., T.W.M.)

Abstract The ability of monoclonal antibody (mAb) αIR-3 to interact with non-small cell lung cancer (NSCLC) cells was investigated. MAb αIR-3 inhibited specific binding of ^{125}I-IGF-I and ^{125}I-αIR-3 to a panel of 8 NSCLC cell lines with high affinity (IC_{50} = 200 and 50 ng/ml, respectively). ^{125}I-αIR-3 bound with high affinity (Kd = 40 ng/ml) to a single class of sites (Bmax = 8,000/cell) using NCI-H838 cells. ^{125}I-αIR-3 was internalized when exposed to NCI-H838 or H1299 cells at 37°C but not 4°C. αIR-3 immunoprecipitated major 90 and 130 kD proteins. IGF-I stimulated and αIR-3 inhibited the clonal growth of NCI-H1299 cells. αIR-3 slowed the growth of NCI-H157 and H838 xenografts in nude mice. In a biodistribution study ^{125}I-αIR-3 was preferentially localized to the tumor as opposed to other organs. These data suggest that IGF-I may be a regulatory agent in NSCLC.

Key words: IGF-I, non-small cell lung cancer, monoclonal antibodies, growth, receptors

Numerous lung cancer growth factors have been defined. For small cell lung cancer (SCLC) bombesin/gastrin releasing peptide (BN/GRP) is an important autocrine growth factor, whereas transforming growth factor α (TGFα) and vasoactive intestinal peptide (VIP) are important in non-SCLC (NSCLC) [1]. Recently insulin-like growth factor I (IGF-I) was defined as an important growth factor in SCLC. The IGF-I gene contains 5 exons and is alternatively spliced to yield IGF-1A and IGF-IB mRNAs [2]. Each of these mRNAs is translated to a large molecular weight precursor protein (17 kD) which contains IGF-I. IGF-I is cleaved from the precursor protein by proteolytic enzymes and immunoreactive IGF-I is present in conditioned media exposed to SCLC and NSCLC cells [3,4]. Also, the IGF-1B precursor protein contains a novel insulin-like growth factor 1B which stimulates growth [5].

IGF-I, a 70 amino acid growth factor, has approximately 70% sequence homology to IGF-II and 50% sequence homology to insulin [6]. IGF-I can interact with IGF binding proteins which have been detected in elevated levels in the serum of lung cancer patients [7]. Conditioned medium from lung cancer cells contains IGF binding proteins of Mr 24–32 kD [8].

Lung cells secrete IGF-I which binds with high affinity to IGF-I receptors; the IGF-I receptor binds IGF-II and insulin with low affinity. The IGF-I receptor has approximately 84% sequence homology with the insulin receptor [9] and contains two α subunits and two β subunits connected by disulfide bonds to form a heterotetrameric glycoprotein [10]. The 90 kD α subunits bind IGF-I with high affinity whereas the 130 kD β subunits have tyrosine kinase activity [11]. IGF-I binding sites are present on SCLC tumors [12] and cells [13,14]. IGF-I stimulates the growth of SCLC cells whereas monoclonal antibody (mAb) αIR-3 inhibits SCLC growth. Here IGF-I receptors were investigated in NSCLC cells.

Abbreviations used: BN/GRP, bombesin/gastrin releasing peptide; IGF-I, insulin-like growth factor-I; mAb, monoclonal antibody; NSCLC, non-small cell lung cancer; PBS, phosphate buffered saline; TGFα, transforming growth factor α; VIP, vasoactive intestinal peptide.

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Address reprint requests to Dr. Terry W. Moody, NCI, BPRP, Bldg. C, Rm. 300, 9610 Medical Ctr. Dr., Rockville, MD 20850.

METHODS

Cell Culture

Human lung cancer cell lines were cultured in serum supplemented medium at 37°C (RPMI-1640, [Fischer Scientific, Fair Lawn, NJ] containing 10% heat inactivated fetal bovine serum [Gibco, Grand Island, NY]). The NSCLC cell lines were adherent and were split weekly 1:20 using trypsin/EDTA (Gibco). Routinely, the cells had greater than 90% viability and were mycoplasma free [15]. The cells were used when they were in exponential growth phase.

Receptor Binding

The αIR-3 antibody, an IgG1, was prepared as described previously [16,17]. The ability of agents to inhibit ^{125}I-IGF-I or ^{125}I-αIR-3 binding was investigated. The NSCLC cells were harvested after treatment with trypsin/EDTA and 5×10^4 cells were added to 24 well plates which were pretreated with 10 μg of human fibronectin (Sigma Chemical Co., St. Louis, MO). After a monolayer of cells had formed (3 days) receptor binding studies were conducted. The cells were washed 4 times with ST media (RMPI-1640 containing 3×10^{-8} M Na_2SeO_3 and 10 μg/ml transferrin; Sigma Chemical Co.) and incubated with ^{125}I-IGF-I (10^5 cpm) in 200 μl of receptor binding buffer (RPMI-1640 containing 0.1% bovine serum albumin; Calbiochem, La Jolla, CA). αIR-3 was iodinated using the chloramine T procedure and ^{125}I-αIR-3 (20 μCi/μg) purified using gel filtration techniques. After 30 min at 37°C, free radiolabeled protein was removed and the cells that contained bound growth factor dissolved in 0.2 N NaOH and counted in a gamma counter.

Immunoprecipitation

The IGF-I receptors were labeled with ^{35}S-methionine (50 μCi/ml) for 16 h at 37°C using methionine-free RPMI-1640. The cells were washed 3 times in PBS and solubilized in cold 20 mM HEPES · NaOH (pH 7.4), 10% glycerol, 1% Triton X-100, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The cells were centrifuged at 27,000*g* for 30 min at 4°C and the supernatant diluted 5-fold in 20 mM HEPES and incubated for 30 min with 10 mg protein A-Sepharose which was preincubated with MAb αIR-3 (20 μg). The immunoprecipitates were washed twice with 40 mM HEPES · NaOH (pH 8.0), 500 mM NaCl, 0.1% SDS, 0.2% Triton X-100, and 5 mM EGTA followed by washes with 40 mM HEPES, 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100, and 5 mM EGTA as well as 10 mM Tris · HCl (pH 8.0) and 0.1% Triton X-100. The IGF-I receptor was eluted with sample buffer and after heating for 3 min at 95°C, the samples were analyzed by SDS-PAGE techniques. The gels were fixed with 20% isopropanol, 5% acetic acid, treated with AMPLIFY for 30 min, dried and autoradiographed on Kodak XAR2 film [18].

Clonogenic Assay

NSCLC cell lines were harvested and tested in the agarose cloning system described previously [19]. The base layer consisted of 3 ml of 0.5% agarose in ST medium containing 5% fetal bovine serum in 6 well plates (Falcon, Oxnard, CA). The top layer consisted of 3 ml of ST medium in 0.3% agarose, αIR-3 doubly concentrated, and 1×10^4 single viable cells. For each cell line and peptide concentration, triplicate wells were plated. After 2 weeks 1 ml of 0.1% p-iodonitrotetrazolium violet was added and after 16 h at 37°C the plates were screened for colony formation; the number of colonies larger than 120 μm in diameter were counted.

Nude Mouse Assay

The ability of αIR-3 to inhibit xenograft formation in nude mice was investigated. Female athymic Balb/c nude mice, 4–5 weeks old, were housed in a pathogen-free temperature controlled isolation room and the diet consisted of autoclaved rodent chow and autoclaved water given ad libitum. NCI-H157 cells (1×10^7) were injected into the right flank of each mouse by subcutaneous injection [19]. Palpable tumors were observed in approximately 90% of the mice after 1 week. PBS (100 μl) or αIR-3 (100 μg/100 μl) were injected i.p. three times weekly. The tumor volume (height × width × depth) was determined twice weekly by calipers and recorded. When the tumor became necrotic (2,000 mm^3), the growth studies were terminated.

RESULTS

Receptor Binding

The binding of ^{125}I-IGF-I and ^{125}I-αIR-3 to NSCLC cell lines was investigated. Table I shows that ^{125}I-IGF-I bound well to NCI-H460 and H727 (28,546 and 21,000 cpm, respectively) whereas intermediate levels of binding were ob-

TABLE I. Specific Binding of ^{125}I-IGF-I and ^{125}I-αIR-3 to NSCLC Cell Lines*

Cell line	^{125}I-IGF-I	^{125}I-αIR-3
Adenocarcinoma		
NCI-H23	7,557	6,586
NCI-H838	2,688	4,908
NCI-H1264	5,629	6,586
Bronchoalveolar		
NCI-H322	8,227	11,187
Carcinoid		
NCI-H727	21,000	9,564
Large cell carcinoma		
NCI-H1299	6,353	3,374
Neuroendocrine		
NCI-H460	28,564	6,980
Squamous cell carcinoma		
NCI-H157	10,324	10,020

*The mean amount of specific binding ± S.E. of 4 determinations is indicated. NSCLC cells (5×10^5) were used and 10^5 cpm of radiolabeled protein added.

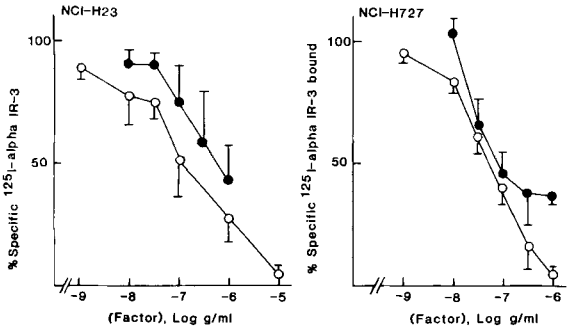

Fig. 1. Pharmacology of αIR-3 binding. The % specific ^{125}I-αIR-3 binding was determined in the presence of increasing concentration of IGF-I (●) or αIR-3 (○) for cell line NCI-H727. The mean value ± S.E. of 3 determinations, each repeated in quadruplicate, is indicated. The lines are drawn point to point.

served using NCI-H23, 157, H322, H838, H1264, and H1299. In contrast, ^{125}I-αIR-3 bound best to NCI-H322 and H157 (11,187 and 10,020 cpm, respectively) whereas intermediate levels of binding were observed using NCI-H23, H460, H727, H1264, and H-1299. These data indicate that ^{125}I-IGF-I and ^{125}I-αIR-3 bound with high affinityto all NSCLC cell lines examined.

The effect of competitors on ^{125}I-IGF-I and ^{125}I-αIR-3 binding was investigated. Specific ^{125}I-αIR-3 binding to NCI-H727 was minimally inhibited by 1 ng/ml αIR-3 whereas 10 μg/ml inhibited almost all ^{125}I-αIR-3 binding (Fig. 1). Similarly ^{125}I-αIR-3 binding to NCI-H727 was inhibited by αIR-3 and IGF-I with IC_{50} values of 50 and 100 ng/ml, respectively. For ^{125}I-αIR-3 binding, αIR-3 was a more potent competitor than was IGF-I. Specific ^{125}I-IGF-I binding to NCI-H23 was minimally inhibited by 1 ng/ml IGF-I whereas 1 μg/ml IGF-I inhibited almost all specific binding (Fig. 2). The IC_{50} for IGF-I was 20 ng/ml whereas αIR-3 was less potent with an IC_{50} value of 150 ng/ml. Similarly, ^{125}I-IGF-I binding to NCI-H157 was inhibited by IGF-I and αIR-3 with IC_{50} values of 25 and 200 ng/ml. For ^{125}I-IGF-I binding IGF-I was a more potent competitor than was αIR-3. As a control, mAb IgG, which did not recognize the IGF-I receptor, had no effect on ^{125}I-IGF-I or ^{125}I-αIR-3 binding (data not shown).

The amount of ^{125}I-αIR-3 bound was determined as a function of αIR-3 concentration (Fig. 3). Using cell line NCI-H838 a Scatchard plot of the specific binding data was linear and ^{125}I-αIR-3 bound with high affinity (Kd = 40 ng/ml) to a single class of sites (Bmax = 8,000/cell).

Ligand Internalization

The internalization of ^{125}I-αIR-3 and ^{125}I-IGF-I was investigated. Figure 4 (left) shows that approximately 9,500 and 6,500 cpm of ^{125}I-αIR-3 bound to cell surface of cell lines NCI-H838 and H1299 and could be dissociated by acetic acid/NaCl at 4°C. In contrast approximately 4,500 and 2,500 cpm of ^{125}I-αIR-3 was internalized by the cells and went into solution when the cells were treated with 0.2 N NaOH. These data indicate that 40 and 30% of the ^{125}I-αIR-3 was internalized by NCI-H838 and H1299 at 4°C. In contrast, at 37°C only approximately 4,500 and 4,000 cpm of ^{125}I-αIR-3 were at the cell surface whereas 9,000 and 5,500 cpm were internalized (Fig. 4, right). These data indicate that 65 and 60% of the ^{125}I-αIR-3 bound was internalized to NCI-H838 and H1299, respectively, at 37°C. Similarly, using ^{125}I-IGF-I and NCI-H1299 cells, 25% was internalized at 4°C whereas 55% was internalized at 37°C. These data indicate that αIR-3 and IGF-I are internalized at 37°C but not 4°C using NSCLC cells.

Immunoprecipitation

NCI-H838 and H1299 cells were labeled with ^{35}S-methionine for 16 h. The cells were then washed with PBS to remove free ^{35}S-methionine and solubilized with HNTG buffer. The cellular extract which contained newly synthesized proteins containing ^{35}S-methionine was immunoprecipitated with protein A-Sepharose containing αIR-3. The immunoprecipitates were washed to remove proteins nonspecifically adhering to the

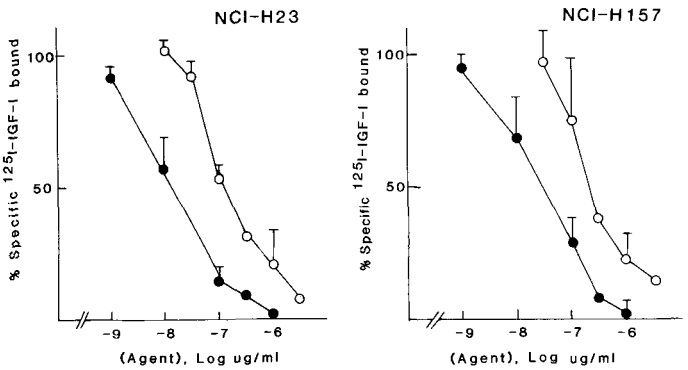

Fig. 2. Pharmacology of IGF-I binding. The % specific ^{125}I-IGF-I binding was determined in the presence of increasing concentration of IGF-I (●) or αIR-3 (○) for cell lines NCI-H23 and NCI-H157. The mean value ± S.E. of 3 determinations, each repeated in quadruplicate, is indicated.

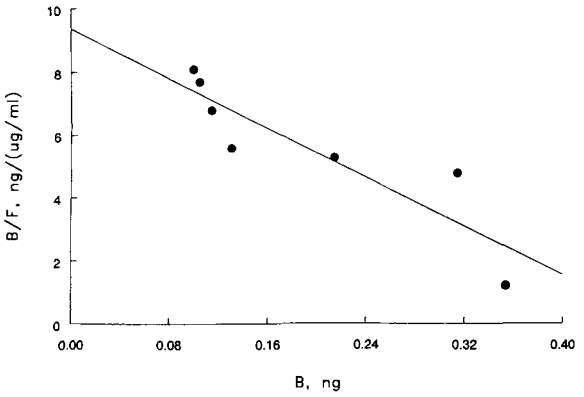

Fig. 3. Dose-response of αIR-3 binding. Scatchard plot of the specific binding data. The best fit line was drawn assuming a single class of sites.

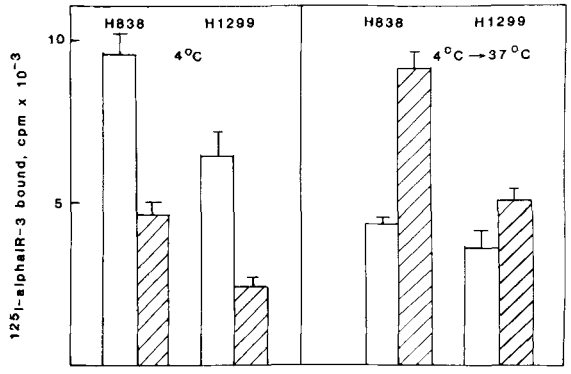

Fig. 4. Internalization of αIR-3. The cpm of ^{125}I-αIR-3 bound to the cell surface (□) and internalized (▨) at 4°C or 37°C is indicated for cell lines NCI-H838 and H1299. The mean value ± S.E. of 4 determinations is indicated.

protein A-Sepharose and the eluted proteins analyzed by SDS-PAGE followed by autoradiography. Figure 5 shows that 2 major bands were detected with molecular weights of 130 and 90 kD and minor bands detected at 70, 65, and 55

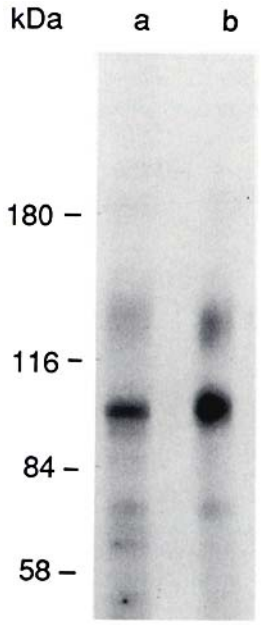

Fig. 5. Immunoprecipitation. Cell lines NCI-H157 (**lane a**) and NCI-H838 (**lane b**) were metabolically labeled with ^{35}S-methionine, solubilized, and immunoprecipitated with mAb αIR-3. The samples were analyzed by SDS gel electrophoresis followed by autoradiography. Molecular weight standards are indicated on the left.

kD. The major bands may represent subunits of the IGF-I receptor.

Growth Assay In Vitro

NCI-H1299 colonies formed in the presence of ST medium (Table II). Addition of 1 ng/ml IGF-I increased the number of NCI-H1299 colonies from 25 to 45. Addition of 10 ng/ml or 100 ng/ml increased the number of NCI-H1299 colonies 5- and 7-fold, respectively. Also, addition of 1 μg/ml αIR-3 significantly decreased the number of NCI-H1299 colonies from 25 to 3. In

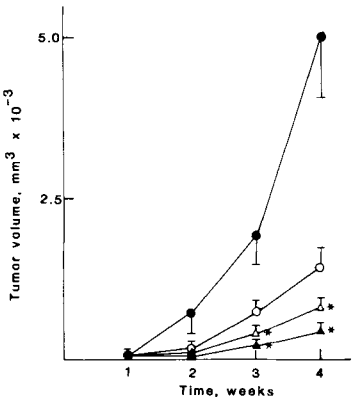

Fig. 6. Effect of αIR-3 on nude mice. NCI-H157 cells (10^7) were implanted into nude mice and xenografts formed after 1 week. Starting at week 1 PBS (●), 30 μg (○), 100 μg (△) or 300 μg (▲) αIR-3 were injected 3 times weekly i.p. The mean tumor volume ± S.E. of 3 determinations is indicated; *P < 0.05.

TABLE II. Effect of IGF-I on NCI-H1299 Growth

Agent	Colonies
None	25 ± 2
IGF-I, 1 ng/ml	45 ± 5*
IGF-I, 10 ng/ml	125 ± 10**
IGF-I, 100 ng/ml	171 ± 8**
αIR-3, 1 μg/ml	3 ± 3**

*P < 0.05, **P < 0.01, using the Student's t-test. The mean value ± S.D. of 3 determinations is shown.

contrast, a control mAb (IgG) had no effect on NCI-H1299 proliferation (data not shown). The data indicate that IGF-I stimulates and αIR-3 inhibits NSCLC growth in vitro.

Growth Assay In Vivo

The effects of αIR-3 were investigated in vivo. NCI-H157 cells (10^7) were injected subcutaneously and after 1 week a palpable mass had formed in approximately 90% of the mice. Figure 6 shows that the tumors grew exponentally and after 4 weeks the tumor volume in the control was 5,086 mm^3. In mice, which were injected 3 times weekly i.p. with 30, 100, or 300 μg of αIR-3, the tumors were significantly smaller at 1,396, 857, and 571 mm^3, respectively. Also, Table III shows that when the tumors were excised and weighed the tumor weight was significantly reduced in animals treated with 30, 100, or 300 μg of αIR-3.

Also, in another set of animals the biodistribution of αIR-3 was determined. Table IV shows that the tumor had the greatest incorporation of

TABLE III. Tumor Weight of NCI-H157 Xenografts

Agent injected	Weight (g)
PBS	8.03 ± 0.35
αIR3, 30 μg	5.05 ± 0.60*
αIR3, 100 μg	3.40 ± 0.90*
αIR3, 300 μg	3.68 ± 1.38*

*P < 0.05. The mean value ± S.E. of 3 determinations is indicated.

TABLE IV. Localization of αIR-3 to NCI-H157 Tumors in Nude Mice*

Organ	Density (cpm/mg)
Tumor	41.0 ± 3.6
Stomach	18.6 ± 2.9
Heart	11.8 ± 0.1
Liver	11.7 ± 2.3
Lung	10.7 ± 4.5
Kidney	8.0 ± 1.9
Spleen	5.7 ± 0.6
Intestine	3.8 ± 0.6

*^{125}I-αIR-3 (10^6 cpm) was injected i.p. and the radioisotope localized to organs after 7 days. The mean value ± S.E. of 3 determinations is shown.

^{125}I-αIR-3 (41 cpm/mg wet weight). Moderate densities of ^{125}I-α-IR-3 were localized to the stomach, heart, liver, lung, and kidney (8–19 cpm/mg) whereas low densities were present in the spleen and intestine (4–6 cpm/mg). These data indicate that αIR-3 inhibits tumor growth in nude mice and that αIR-3 is localized to the tumor.

DISCUSSION

The results presented here indicate the IGF-I receptors are present on NSCLC cells. ^{125}I-IGF-I and ^{125}I-αIR-3 bind with high affinity to NSCLC cells. Most cell lines bind approximately the same amount of ^{125}I-IGF-I and ^{125}I-αIR-3. Cell lines NCI-H727 and H460 may bind more IGF-I than αIR-3 due to IGF-I interaction with binding proteins in addition to IGF-I receptors.

MAb α-IR-3, however, binds only with high affinity to the IGF-I receptor. Here ^{125}I-mAb αIR-3 bound with high affinity (Kd = 40 ng/ml) to a single class of sites (Bmax = 8,000/cell). Similarly for SCLC cells 12,000 ^{125}I-IGF-I binding sites were present per SCLC cell [14]. Specific ^{125}I-mAb αIR-3 binding was inhibited with greater potency by αIR-3 (IC_{50} = 50 ng/ml) than IGF-I (IC_{50} = 100 ng/ml). In contrast, specific ^{125}I-IGF-I binding was inhibited with greater potency by IGF-I (IC_{50} = 20 ng/ml) than αIR-3

(IC_{50} = 150 ng/ml). These data suggest that IGF-I and αIR-3 have partially overlapping binding sites. Using either ^{125}I-IGF-I or ^{125}I-αIR-3 as receptor probes, however, IGF-I was more potent at inhibiting binding than was IGF-II or insulin (F. Zia, unpublished data). Previously αIR-3 was found to inhibit ^{125}I-IGF-I binding to breast cancer cells with IC_{50} values of approximately 150 ng/ml [20].

The αIR-3 interacted with IGF-I receptors. Using immunoprecipitation techniques and the αIR-3 antibody, major bands of 130 and 90 kD were detected. In contrast the insulin receptor is an $\alpha_2\beta_2$ tetramer where the α subunit has 719 and the β subunit 620 amino acid residues [9]. The IGF II receptor is a monomer of Mr 250,000 daltons [21].

Both IGF-I and αIR-3 were internalized at 37°C but not 4°C. IGF-I stimulates tyrosine kinase activity, elevates cytoplasmic Ca^{2+} as well as pH and stimulates growth. In contrast, αIR-3 binds to the IGF-I receptor but antagonizes the biological responses induced by IGF-I.

The clonal growth of NSCLC cells was stimulated 2-fold by 1 ng/ml of IGF-I and 5-fold by 10 ng/ml of IGF-I. In contrast 1 μg/ml of αIR-3 significantly inhibited NSCLC colony formation. In contrast a non-specific IgG kappa monoclonal antibody (MOPC11) had no effect on colony formation [4]. Previously, αIR-3 was found to inhibit MCF-7 and MDA-MB231 colony formation significantly at a 1.5 μg/ml concentration [20]. Also, 15 μg/ml of αIR-3 inhibited cell number and ^{3}H-thymidine uptake stimulated by IGF-I. These results suggest that αIR-3 antagonizes growth induced by exogenous or endogenous IGF-I in vitro.

In vivo αIR-3 inhibited NSCLC xenograft formation. The effects were dose dependent and after a palpable mass had formed, injection of only 30 μg i.p. 3 times weekly significantly inhibited NSCLC tumor volume and weight. These data indicate that mAb αIR-3 strongly inhibits NSCLC tumor proliferation, whereas it was not as potent in breast tumors. After formation of a palpable mass using cell line MDA-MB231, αIR-3 (500 μg, i.p., 3 times weekly) did not slow xenograft growth. If αIR-3 was injected with MDA-MB231 cells into nude mice and subsequently was injected twice weekly i.p. (500 μg), MDA-MB231 tumor growth was slowed [21]. These data suggest that mAb αIR-3 is only potent using MDA-MB231 cells if it was injected prior to formation of a palpable mass. αIR-3 could be more potent at inhibiting NSCLC than breast cancer growth in vivo due to greater IGF-I receptor densities and/or better tumor localization.

αIR-3 is preferentially localized to the tumor and not other organs. Previously we found that mAb 108 against the epidermal growth factor (EGF) receptor was preferentially localized to NSCLC tumors [18]. MAb 108 blocks EGF receptors and for effective inhibition of NSCLC xenografts it was necessary to inject mAb 108 with the NSCLC cells, and subsequently to administer it 100 μg i.p. three times weekly. If mAb 108 was administered after formation of a palpable mass it was ineffective. These data suggest that mAb αIR-3 is more potent at inhibiting NSCLC cell growth than is mAb 108. Because NSCLC cells have 100,000 EGF receptors and only 10,000 IGF receptors this difference in potency is not a function of receptor number. It is possible IGF-I is a more important growth factor in NSCLC cells than EGF or TGFα.

Another growth factor for NSCLC is VIP [22]. NSCLC cells have mRNA for VIP and immunoreactive VIP is present in NSCLC conditioned media. Also approximately 100,000 VIP receptors are present on NSCLC cells. VIP elevates cAMP and stimulates NSCLC colony formation. The growth of VIP in vitro and in vivo is inhibited by a synthetic VIP receptor antagonist named VIPhybrid. On a molar basis VIPhybrid is as potent at slowing NSCLC xenograft formation as is αIR-3. These data indicate that NSCLC cells have multiple growth factors and it may be best to use a combination of agents such as VIPhybrid and αIR-3 to greatly slow NSCLC growth.

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