Inhibition of insulin-like growth factor-I receptor (IGF-IR) signaling and tumor cell growth by a fully human neutralizing anti–IGF-IR antibody

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Abstract

Insulin-like growth factor-I receptor (IGF-IR) plays an important role in tumor cell growth and survival. On ligand stimulation, IGF-IR, a receptor tyrosine kinase, phosphorylates tyrosine residues on two major substrates, IRS-1 and Shc, which subsequently signal through the Ras/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKT pathways. Here, we describe the characterization of a fully human anti–IGF-IR monoclonal antibody 19D12 that inhibits IGF binding and autophosphorylation of both IGF-IR/IGF-IR homodimers and IGF-IR/insulin receptor heterodimers. 19D12 does not recognize insulin receptor homodimers. In addition to inhibiting IGF-IR autophosphorylation, 19D12 also inhibits IRS-1 phosphorylation and activation of the major downstream signaling molecules AKT and extracellular signal-regulated kinase 1/2. Furthermore, the antibody down-regulates the total IGF-IR protein level and can exhibit antibody-dependent cellular cytotoxicity activity against a non–small cell adenocarcinoma cell line *in vitro* in the presence of isolated human natural killer cells. 19D12 binds tightly to the receptor, with an affinity of 3.8 pmol/L as measured by KinExA. In cell culture, 19D12 inhibits proliferation and soft agar growth of various tumor cell lines. *In vivo*, 19D12 inhibits the tumor growth of a very aggressive human ovarian tumor xenograft model A2780. These data support the development of this anti–IGF-IR monoclonal antibody as a promising anticancer agent. [Mol Cancer Ther 2005;4(8):1214–21]

Introduction

Insulin-like growth factor-I receptor (IGF-IR) plays an important role in cell growth and differentiation (1). Overexpression of IGF-IR results in aberrant cell proliferation and malignant transformation (2–4). Cells with IGF-IR deleted are resistant to oncogenic transformation (5). In addition to its role in cell proliferation, IGF-IR plays an important role in tumor cell survival. Overexpressed IGF-IR can protect cells from apoptosis induced by serum withdrawal, tumor necrosis factor, etoposide, or interleukin-3 withdrawal *in vitro* (6–10). Epidemiologic data have linked elevated plasma IGF-I level with prostate, breast, lung, and colon cancer risk (11–14). IGF-II is frequently overexpressed in colorectal, neuroblastoma, liver, adrenocortical, and Wilms' tumors (15–20). IGF-IR is the signaling receptor for both IGF-I and IGF-II ligands and is overexpressed in some human cancers as well (21–26). Therefore, IGF-IR is an attractive antitumor target.

Inhibition of IGF-IR signaling by various methods, including antisense, dominant-negative truncation, small-molecule kinase inhibitors or neutralizing antibodies, has resulted in growth inhibition and apoptosis of tumor cells (27–40). We show here that a fully human anti–IGF-IR neutralizing antibody inhibits IGF-IR signaling in cell culture and inhibits tumor growth in xenograft models. The antibody was obtained using the Medarex's HuMab transgenic mouse technology (41). Therefore, it is a fully human antibody and is expected to show minimal immunogenicity in humans. Expression of IGF-IR on many tumor cell types suggests that this anti–IGF-IR monoclonal antibody has the potential to treat patients for many different types of cancer.

Materials and Methods

Cells and Reagents

Rat1/hIRβ cells are a generous gift from Dr. Byung-Hak Jhun (Pusan National University, Busan, Korea). Anti–IGF-IR antibody C-20 and anti–insulin receptor antibody C-19 are purchased from Santa Cruz (Santa Cruz, CA). Anti-phosphotyrosine antibody 4G10 is from Upstate Biotechnology (Charlottesville, VA). Antibodies for detecting AKT, phospho-AKT, mitogen-activated protein kinase (MAPK), phospho-MAPK, and phospho-IRS-1 are from Cell Signaling Technology (Beverly, MA). CellTiter-Glo reagents are

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purchased from Promega (Madison, WI). Human natural killer cells were purified with antibody-coupled magnetic bead kit from Miltenyi Biotech (Auburn, CA). Antibody-dependent cellular cytotoxicity was determined with a cytotoxicity detection kit for lactate dehydrogenase from Roche (Indianapolis, IN). [^{125}I]IGF-I is from Amersham (Piscataway, NJ).

Receptor Autophosphorylation Assay

Antibodies were added to cells for 30 minutes. Cells were then stimulated with 20 ng/mL IGF-I for 5 to 10 minutes at 37°C, washed twice with cold PBS containing 0.1 mmol/L sodium vanadate, and lysed in lysis buffer [50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, protease inhibitors, 2 mmol/L sodium vanadate]. Lysates were incubated on ice for 30 minutes and then centrifuged at 13,000 rpm for 10 minutes at 4°C. Protein concentrations of the lysates were measured with a Coomassie Plus Protein Assay (Pierce, Rockford, IL). Lysates were then subjected to immunoprecipitation and Western blot analysis.

Immunoprecipitation and Western Blot Analysis

IGF-IR was immunoprecipitated using 1 μg antibody (C-20) and 15 μL of 50% protein G agarose. The precipitates were washed four times with lysis buffer. Precipitated proteins were separated on 10% SDS-PAGE, transferred to a nitrocellulose filter, probed with either anti–IGF-IR or anti-phosphotyrosine (4G10) antibodies, and visualized by enhanced chemiluminescence (Amersham).

For direct Western blot analysis, equal amounts of cell lysates were separated on 10% SDS-PAGE, transferred to nitrocellulose filters, probed with a desired antibody, and visualized by enhanced chemiluminescence.

Ligand and Receptor Binding

Millipore 96-well filter plates (1.2 μm Durapore, Bedford, MA) were prewet with 0.5% bovine serum albumin/PBS for 2 hours at 4°C. The buffer was then removed with a vacuum manifold. Various concentrations of 6× control or test antibody were added to the wells (25 μL). The [^{125}I]IGF-I ligand was then added to the wells at a final concentration of 0.375 nmol/L in bovine serum albumin/PBS. Cells were harvested with cell dissociation solution, counted with trypan blue, and resuspended in 0.5% bovine serum albumin/PBS to a cell density of 1×10^5 to 3×10^5/mL. Cells (10,000-30,000; 100 μL) were added to each well. The plate was shaken at 4°C for 1 hour. The plate was then aspirated and washed thrice with ice-cold PBS. The filters were punched out and counted on an ICN Micromedex gamma counter (Aurora, OH).

Antibody-Dependent Cellular Cytotoxicity

NCI-H322 cells were trypsinized for 3 minutes, resuspended in assay medium (RPMI 1640 containing 1% fetal bovine serum) at 4×10^5/mL, and transferred to 96-well round-bottomed plate containing 100 ng/mL 19D12. Cells were opsonized for 30 minutes at room temperature. Serial 2-fold dilutions of purified natural killer cells were prepared in a separate plate in assay medium and transferred to antibody-treated tumor cell targets. Cells were incubated at 37°C for 4 hours. Supernatants were harvested and lactate dehydrogenase content was measured. Percent lactate dehydrogenase release by antibody-dependent cellular cytotoxicity was calculated as a percentage of total release determined by Triton X-100 lysis.

Proliferation Assay

Cells were trypsinized, counted, and resuspended at 25,000 cells/mL in 10% HI-FBS RPMI 1640 containing nonessential amino acids, L-glutamine, MEM vitamins, and PS. Cell suspension (2,500 cells; 100 μL) was added to each well of BD Falcon 96-well black, clear-bottomed TC-treated plate (Bedford, MA). Cells were allowed to attach and spread overnight at 37°C. The 10% RPMI 1640 was replaced with 100 μL of 2% RPMI 1640 the following day. All treatments were prepared in 2% RPMI 1640 at 20× concentration and serial diluted. Every test point was prepared in triplicate on separate assay plates. Cell proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay 96 hours after treatment. Luminescence was detected on a Wallac (Boston, MA) 420 plate reader with stacker.

Xenograft Model in Mice

Four million A2780 human ovarian carcinoma cells in 50% growth factor–reduced Matrigel were inoculated s.c. into each nude mouse. 19D12 treatment was initiated when the tumor size reached ~50 mm^3 (defined as day 0). Two additional doses were given at days 3 and 7. Tumor volumes were measured by Labcat.

Results

19D12 Inhibits IGF-IR Autophosphorylation and Competes with Ligand Binding to the Receptor

Medarex's HuMab mice (41) were alternately immunized with two different human IGF-IR antigens: (*a*) HEK-293 cells stably overexpressing full-length IGF-IR and (*b*) purified soluble IGF-IR receptor. Mouse serum was titred based on the presence of antigen-specific activity measured by ELISA. Spleens from high-titer mice were fused and the resulting hybridomas were screened for anti–IGF-IR activity by both a cell-based ELISA and flow cytometric analysis. Antigen-positive supernatants were further evaluated in a cell-based autophosphorylation assay to select neutralizing antibodies. Among the 13 neutralizing antibodies identified, 19D12 is the most potent and has a binding affinity of 3.8 pmol/L determined by KinExA (data not shown).

Figure 1A shows the effect of 19D12 on IGF-IR autophosphorylation in the human ovarian tumor cell line A2780. After preincubation with 19D12 for 30 minutes, cells were stimulated with IGF-I (20 ng/mL) for 5 minutes. Cell lysates were made, and IGF-IR was immunoprecipitated with an anti–IGF-IR–specific antibody. The blot was probed with the anti-phosphotyrosine antibody 4G10. Therefore, the band on *top* represents the level of the phospho–IGF-IR in cell lysates. 19D12 completely inhibited the phosphorylation of IGF-IR on ligand stimulation in a dose-dependent manner. The blot was then stripped and reprobed with anti–IGF-IR antibody to ensure equivalent loading of IGF-IR in all lanes (*bottom*).

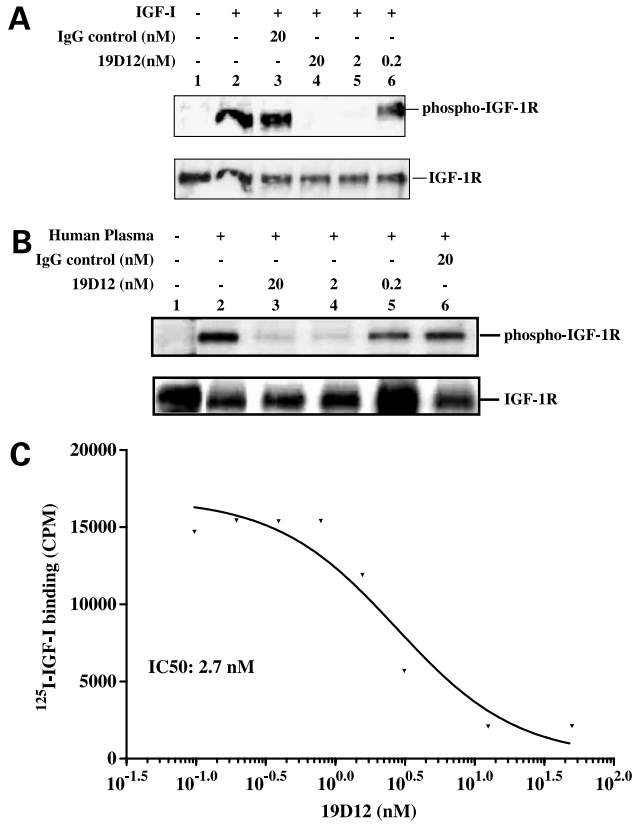

Figure 1. Inhibition of receptor autophosphorylation and ligand binding to the receptor. **A,** A2780 cells were treated with various concentrations of 19D12 antibody (*lane 4,* 20 nmol/L; *lane 5,* 2 nmol/L; *lane 6,* 0.2 nmol/L) or control IgG (*lane 3,* 20 nmol/L) for 0.5 h before stimulation with IGF-I (20 ng/mL). Each cell lysate (400 μg) was subjected to immunoprecipitation with an IGF-IR–specific antibody. The precipitated cell lysates were separated on 10% SDS-PAGE and transferred to a nitrocellulose blot. The filter was probed with anti-phosphotyrosine antibody 4G10. *Lane 1,* untreated cells; *lanes 2* to *6,* IGF-I (20 ng/mL)–treated cells. The blot was then stripped and reprobed with anti–IGF-IR antibody (*bottom*). **B,** A2780 cells were treated with various concentrations of 19D12 antibody (*lane 3,* 20 nmol/L; *lane 4,* 2 nmol/L; *lane 5,* 0.2 nmol/L) or control IgG (*lane 6,* 20 nmol/L) for 0.5 h before stimulation with human plasma. Each cell lysate (400 μg) was subjected to immunoprecipitation with an IGF-IR–specific antibody. The precipitated cell lysates were separated on 10% SDS-PAGE and transferred to a nitrocellulose blot. The filter was probed with the anti-phosphotyrosine antibody 4G10. *Lane 1,* untreated cells; *lanes 2* to *6,* IGF-I (20 ng/mL)–treated cells. The blot was then stripped and reprobed with anti–IGF-IR antibody (*bottom*). **C,** various concentrations of 19D12 and [^{125}I]IGF-I were added to a 96-well filter plate. IGF-IR-transfected HEK-293 cells (20,000 per well) were added to each well to initiate binding. The plate was shaken for 1 h, aspirated, washed, and counted on a gamma counter.

In addition to the inhibition of IGF-IR autophosphorylation stimulated by IGF-I ligand, 19D12 also inhibited IGF-IR autophosphorylation stimulated by human plasma (Fig. 1B). Human plasma has a natural balance of IGF-I, IGF-II, and IGF-binding proteins.

To determine whether 19D12 inhibits IGF-I binding to the receptor, ligand-receptor binding was measured using [^{125}I]IGF-I as the ligand. Data in Fig. 1C show that 19D12 displaces IGF-I binding to the IGF-IR receptor with an IC_{50} value of 2.7 nmol/L.

19D12 Binds and Inhibits the IGF-IR/Insulin Receptor Heterodimeric Receptors but not the Insulin Receptor Homodimeric Receptors

Insulin receptor is a closely related receptor tyrosine kinase and shares high sequence homology with IGF-IR (42, 43). To test the selectivity of the anti–IGF-IR antibody 19D12, direct Western blotting experiments were done with 19D12 using lysates from cells expressing a high endogenous level of human IGF-IR as well as lysates from stably transfected Rat1 cells that overexpress a high level of human insulin receptor (hIRβ cells). 19D12 only recognized a protein of ~135,000 dalton molecular weight (α-subunit of IGF-IR) in cells containing human IGF-IR but did not recognize the overexpressed human insulin receptor (data not shown). To further show the functional selectivity of 19D12, an autophosphorylation assay in hIRβ cells was done. Cells were stimulated with insulin (20 ng/mL) after preincubation with 19D12 or an anti–insulin receptor neutralizing antibody for 30 minutes. Cell lysates were made, and insulin receptor was immunoprecipitated with an anti–insulin receptor–specific antibody. The blot was probed with the anti-phosphotyrosine antibody 4G10. As shown in Fig. 2A, 19D12 did not inhibit the phosphorylation of insulin receptor on insulin stimulation, whereas the anti–insulin receptor–positive control neutralizing antibody inhibited insulin receptor autophosphorylation.

Both the IGF-IR and the insulin receptor are tetrameric complexes consisting of two extracellular α-subunits that bind the ligands and two β-subunits that have ligand-dependent tyrosine kinase activity. In cells that coexpress IGF-IR and insulin receptor, heterodimeric receptors can form between one half of the receptor consisting of one IGF-IR α-subunit and one IGF-IR β-subunit and the other half of the receptor consisting of one insulin receptor α-subunit and one insulin receptor β-subunit (44). The IGF-IR/insulin receptor heterodimeric receptor behaves more like IGF-IR than insulin receptor, exhibiting affinities for IGF-I and IGF-II similar to the IGF-IR homodimer. The heterodimeric receptor binds insulin with much lower affinity (44). To examine whether 19D12 can bind the IGF-IR/insulin receptor heterodimeric receptor and inhibit its signaling, we did immunoprecipitation experiments using 19D12 as the immunoprecipitating antibody in A2780 cells that endogenously express both IGF-IR (150,000 IGF-IR/cell) and insulin receptor (30,000 insulin receptor/cell). As shown in Fig. 2B, both insulin receptor (*left*) and IGF-IR (*right*) were detected in 19D12 immunoprecipitates, suggesting that 19D12 can bind to both the IGF-IR homodimer and the IGF-IR/insulin receptor heterodimer. The phosphorylation status of the heterodimeric receptor was also examined in the autophosphorylation assay. As shown in Fig. 2C, 19D12 was able to completely inhibit IGF-I-stimulated autophosphorylation of the heterodimeric receptors immunoprecipitated with an anti–insulin receptor antibody.

19D12 Down-Regulates IGF-IR Level and Blocks IGF-IR Signaling

In addition to inhibiting receptor autophosphorylation, we have also found that 19D12 down-regulates the IGF-IR

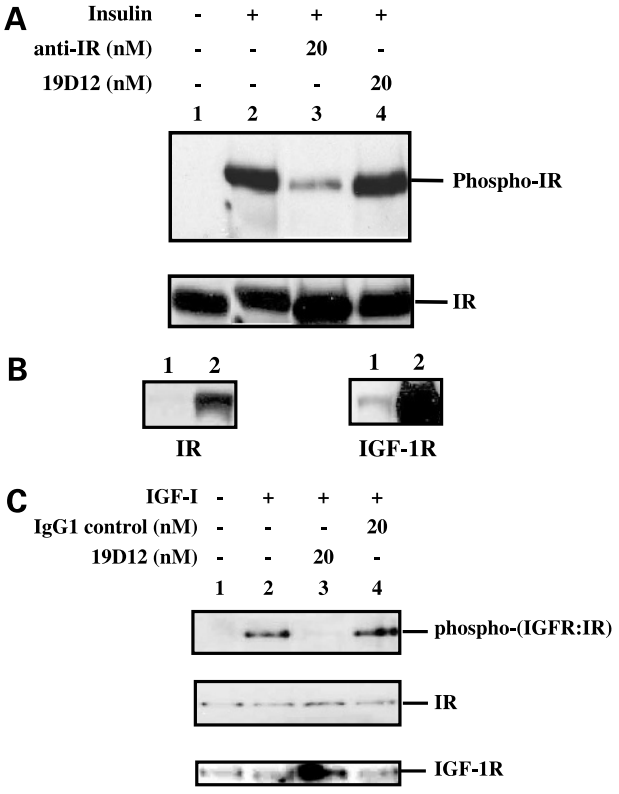

Figure 2. 19D12 activity against insulin receptor (*IR*) receptors and IGF-IR/insulin receptor hybrid receptors. **A,** Rat1/hIRβ cells were treated 20 nmol/L 19D12 (*lane 4*) or neutralizing antibody against insulin receptor (*lane 3*) for 0.5 h before stimulation with insulin (20 ng/mL) for 5 min. Each cell lysate (400 μg) was subjected to immunoprecipitation with an insulin receptor – specific antibody. The precipitated cell lysates were separated on 10% SDS-PAGE and transferred to a nitrocellulose blot. The filter was probed with the anti-phosphotyrosine antibody 4G10. *Lane 1,* untreated cells; *lane 2,* insulin (20 ng/mL) – treated cells. The blot was then stripped and reprobed with anti – insulin receptor antibody (*bottom*). **B,** 19D12 (*lane 2*) or protein G agarose alone (*lane 1*) was used to immunoprecipitate 400 μg each of A2780 cell lysate. The precipitated cell lysates were separated on 10% SDS-PAGE and transferred to a nitrocellulose blot. The filter was probed with an anti – insulin receptor antibody (*left*). The blot was then stripped and reprobed with anti – IGF-IR antibody (*right*). **C,** A2780 cells were treated with 20 nmol/L 19D12 (*lane 3*) or control IgG antibody (*lane 4*) for 0.5 h before stimulation with IGF-I (20 ng/mL) for 5 min. Each cell lysate (400 μg) was subjected to immunoprecipitation with an insulin receptor – specific antibody. The precipitated cell lysates were separated on 10% SDS-PAGE and transferred to a nitrocellulose blot. The filter was probed with the anti-phosphotyrosine antibody, 4G10. *Lane 1,* untreated cells; *lane 2,* IGF-I (20 ng/mL) – only treated cells. The blot was stripped and reprobed with anti-insulin receptor antibody (*middle*) and subsequently stripped again and reprobed with anti – IGF-IR (*bottom*).

protein level. As shown in Fig. 3A, 19D12 down-regulated IGF-IR protein expression on 4 hours of treatment in a dose-dependent manner. The down-regulation was observed starting at 1-hour incubation, and the extent of down-regulation was maximal at 4 hours (data not shown). The isotype control antibody or IGF-I ligand did not affect the IGF-IR level with up to overnight incubation (data not shown).

To further evaluate the inhibitory effects of 19D12 in the IGF signaling pathway, the phosphorylation status of downstream signaling molecules of the IGF pathway was investigated. Activation status of IRS-1, the key immediate downstream signaling molecule of IGF-IR, and activation status of AKT and MAPK, key effector molecules of the two major IGF-IR signaling pathways, were examined using antibodies that are specific for the phosphorylated forms of the respective target molecules after 19D12 treatment. As shown in Fig. 3B and C, 19D12 potently inhibited IGF-I-stimulated phosphorylation of IRS-1, AKT, and MAPK.

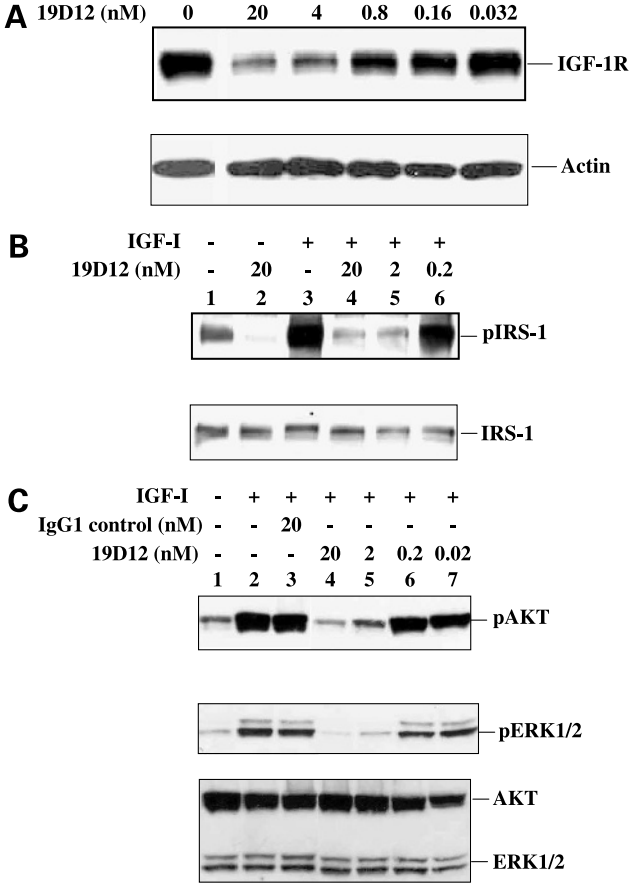

Figure 3. Down-modulation of IGF-IR and inhibition of IRS-1, AKT, and MAPK activation. **A,** MCF7 cells were treated with various concentrations of 19D12 antibody (*lane 2,* 20 nmol/L; *lane 3,* 4 nmol/L; *lane 4,* 0.8 nmol/L; *lane 5,* 0.16 nmol/L; *lane 6,* 0.032 nmol/L) for 4 h. Each cell lysate (50 μg) was separated by 10% SDS-PAGE and transferred to a nitrocellulose blot. The blot was probed with anti – IGF-IR antibody (*top*), stripped, and reprobed with anti-actin antibody (*bottom*). **B,** A2780 cells were treated with various concentrations of 19D12 antibody (*lanes 2* and *4,* 20 nmol/L; *lane 5,* 2 nmol/L; *lane 6,* 0.2 nmol/L) for 4 h. Each cell lysate (50 μg) was separated by a 10% SDS-PAGE and transferred to a nitrocellulose blot. The blot was probed with anti-phospho-IRS-1 antibody (*top*). *Bottom,* loading control where the blot was probed with anti-IRS-1 antibody. *Lane 1,* untreated cells; *lane 3,* IGF-I (20 ng/mL) – treated cells. **C,** A2780 cells were treated with various concentrations of 19D12 antibody (*lane 4,* 20 nmol/L; *lane 5,* 2 nmol/L; *lane 6,* 0.2 nmol/L; *lane 7,* 0.02 nmol/L) for 4 h. Each cell lysate (50 μg) was separated by a 10% SDS-PAGE and transferred to a nitrocellulose blot. The blot was probed with anti-phospho-AKT antibody first (*top*), then reprobed with anti-phospho – extracellular signal-regulated kinase 1/2 (*ERK1/2*) antibody (*middle*). The blot was subsequently stripped and probed with anti-AKT and anti-ERk1/2 antibodies (*bottom*). *Lane 1,* untreated cells; *lanes 2* to *7,* IGF-I (20 ng/mL) treated cells; *lane 3,* cells treated with 20 nmol/L control IgG.

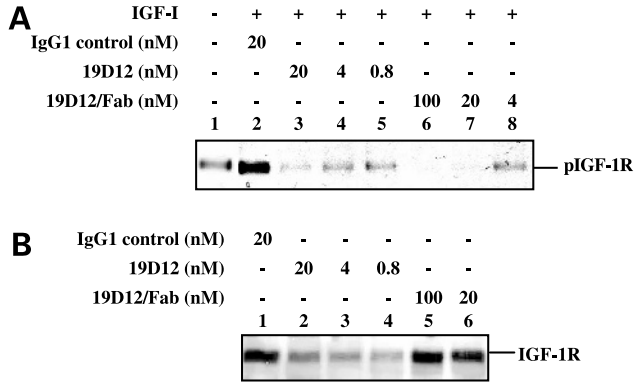

Figure 4. Activities of F(ab) fragment of 19D12. **A**, MCF7 cells were treated with various concentrations of full-length 19D12 antibody (*lane 3,* 20 nmol/L; *lane 4,* 4 nmol/L; *lane 5,* 0.8 nmol/L) or F(ab) fragment of 19D12 (*lane 6,* 100 nmol/L; *lane 7,* 20 nmol/L; *lane 8,* 4 nmol/L) for 0.5 h before stimulation with IGF-I (20 ng/mL). Each cell lysate (400 μg) was subjected to immunoprecipitation with an IGF-IR–specific antibody. The precipitated cell lysates were separated on 10% SDS-PAGE and transferred to a nitrocellulose blot. The filter was probed with anti-phosphotyrosine antibody 4G10. *Lane 1,* untreated cells; *lane 2,* cells treated with 20 nmol/L control IgG. **B**, MCF7 cells were treated with various concentrations of full-length 19D12 antibody (*lane 2,* 20 nmol/L; *lane 3,* 4 nmol/L; *lane 4,* 0.8 nmol/L) or F(ab) fragment of 19D12 (*lane 5,* 100 nmol/L; *lane 6,* 20 nmol/L) for 4 h. Each cell lysate (50 μg) was separated by a 10% SDS-PAGE and transferred to a nitrocellulose blot. The blot was probed with anti–IGF-IR antibody. *Lane 1,* cells treated with 20 nmol/L control IgG.

F(ab) Fragment of 19D12 Inhibits Receptor Autophosphorylation but Does Not Down-Regulate Receptor Expression

The full-length 19D12 antibody is a bivalent molecule that consists of two identical heavy chains and two identical light chains. Functionally, the IgG molecule is divided into three domains: F(ab), the antigen-binding domains; the flexible hinge domain; and the Fc effector domain that interacts with complement and immune effector cells. We were interested to determine whether a single F(ab) fragment of 19D12 could induce both antagonist activity and receptor down-regulation as observed with the full-length antibody. A single F(ab) of 19D12 was made, and it was able to inhibit IGF-IR autophosphorylation in response to IGF-I as shown in Fig. 4A. However, the F(ab) fragment failed to down-regulate IGF-IR expression, suggesting that the receptor down-regulation requires a bivalent IgG molecule (Fig. 4B). The F(ab) fragment of 19D12 exhibited very similar IGF-IR affinity to the full-length 19D12 (data not shown).

Induction of Antibody-Dependent Cellular Cytotoxicity *In vitro*

The Fc portion of an IgG1 isotype monoclonal antibody can potentially mediate effector functions by selective binding to FcγRIII (CD16) expressed by natural killer cells to mediate antibody-dependent cellular cytotoxicity of tumor cells. Some therapeutic monoclonal antibody agents in the clinic, such as Rituxan and Herceptin, may owe part of their antitumor efficacy to their ability to induce antibody-dependent cellular cytotoxicity. We investigated the ability of 19D12/IgG1 to induce antibody-dependent cellular cytotoxicity *in vitro*. Data in Fig. 5 show that 19D12/IgG1 can augment killing of NCI-H322 non–small cell lung carcinoma tumor cells in the presence of isolated human natural killer cells. In contrast, the IgG4 isotype of 19D12 did not induce antibody-dependent cellular cytotoxicity above background as expected.

Inhibition of Tumor Cell Proliferation by 19D12

To further investigate the effect of IGF-IR signaling inhibition by 19D12 on cell growth, the CellTiter-Glo Luminescent Cell Viability Assay was used to measure tumor cell proliferation in culture after 19D12 treatment. As shown in Fig. 6, 19D12 dose-dependently inhibited growth of A2780 ovarian tumor cells. A [^{14}C]thymidine incorporation assay was also used to measure proliferation and similar results were obtained (data not shown).

Inhibition of Human Ovarian Xenograft Tumor Growth by 19D12

We tested the antitumor activity of 19D12 in a human tumor xenograft model. Human A2780 ovarian tumor cells were implanted s.c. in nude mice in the presence of Matrigel. Treatment with 19D12 (twice weekly) was initiated after the tumors reached 50 mm^3. The study was terminated after the tumors in the control group reached >1,000 mm^3. As shown in Fig. 7A, 19D12 dose-dependently inhibited the tumor growth of this aggressive human ovarian cancer line in mice. Only three injections of 19D12 were given during the study. At the end of the study, tumors were removed and tumor lysates were prepared. Total IGF-IR levels in tumors were measured by Western analysis. As shown in Fig. 7B, the IGF-IR level in treated animals was lower than the IGF-IR in control IgG-treated animals. This shows that the receptor down-regulation by 19D12 occurs both in cell culture and *in vivo*. This feature can potentially be used as a surrogate marker for anti–IGF-IR monoclonal antibody therapy.

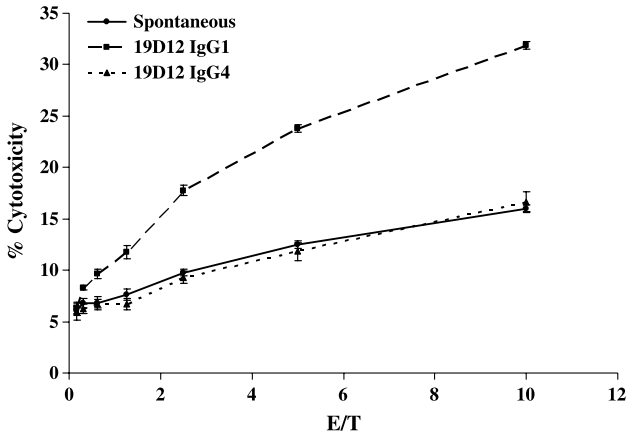

Figure 5. Induction of antibody-dependent cellular cytotoxicity by 19D12. NCI-H322 cells and various antibodies were added to a 96-well plate and opsonized with 100 ng/mL 19D12 for 30 min at room temperature. Purified human natural killer cells were then added at various ratios to numbers of target H322 cells. Cells were incubated at 37°C for 9 h. Lactate dehydrogenase released was then measured as an indicator of cell cytotoxicity. Each data point was assayed in quadruple in the experiment.

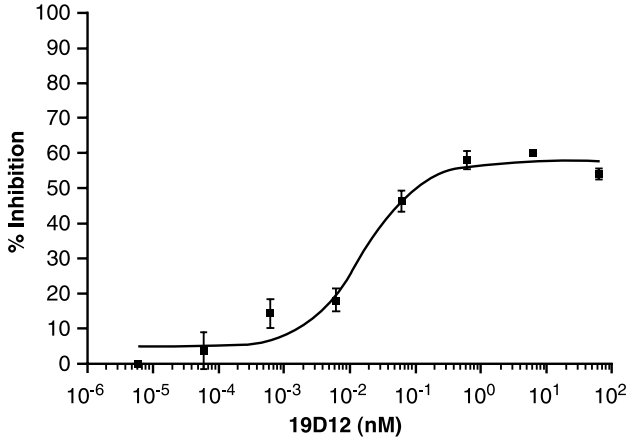

Figure 6. Inhibition of tumor cell proliferation by 19D12. Various concentrations of 19D12 were added to A2780 human ovarian carcinoma cells (2,500 cells per well) for 4 d in RPMI 1640 with 2% fetal bovine serum. Cell proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay.

Discussion

IGF-IR plays an important role in both transformation and survival. Cells from IGF-IR knockout mice are resistant to various viral and cellular oncogenes, including SV40 T Ag and Ras (5). Inhibition of the IGF/IGF-IR axis may contribute in part to the efficacies observed with some clinically effective anticancer therapeutics, such as tamoxifen (45) and 17-allylamino geldanamycin (46). In addition to its roles in transformation and survival, the IGF/IGF-IR system also regulates vascular endothelial growth factor production, suggesting a role in angiogenesis (47). IGF-IR is therefore an attractive anticancer target. We described here the generation and characterization of a fully human neutralizing anti–IGF-IR antibody, 19D12. 19D12 specifically recognizes both IGF-IR homodimers and IGF-IR/insulin receptor heterodimers. However, it is specific and does not recognize insulin receptor homodimers (Fig. 2). This selectivity relative to insulin receptor homodimers may be an advantage over potential IGF-IR kinase inhibitors, because this antibody might be less likely to induce hyperglycemia. 19D12 has a high affinity for IGF-IR and inhibits the receptor activation stimulated by both purified IGFs and human plasma (Fig. 1). In addition to inhibiting receptor activation, 19D12 down-regulates the IGF-IR receptor at the protein level, shuts down the signaling of the receptor as measured by phosphorylation of IRS-1, AKT, and MAPK, and has the ability to induce antibody-dependent cellular cytotoxicity (Figs. 3 and 5).

To further understand the mechanism of the down-regulation, the F(ab) fragment of 19D12 was made. The data in Fig. 4 show that receptor down-regulation requires the bivalency of the full-length antibody or minimally an $F(ab')_2$ fragment. Both neutralization and down-regulation activities could contribute to the antitumor activities observed *in vivo*. It would be interesting to examine the efficacy of the F(ab) fragment *in vivo* to further understand the functional importance of IGF-IR receptor down-regulation.

The observations that the single F(ab) is able to inhibit receptor autophosphorylation and that it has similar affinity to the full-length antibody suggest that 19D12 interacts with

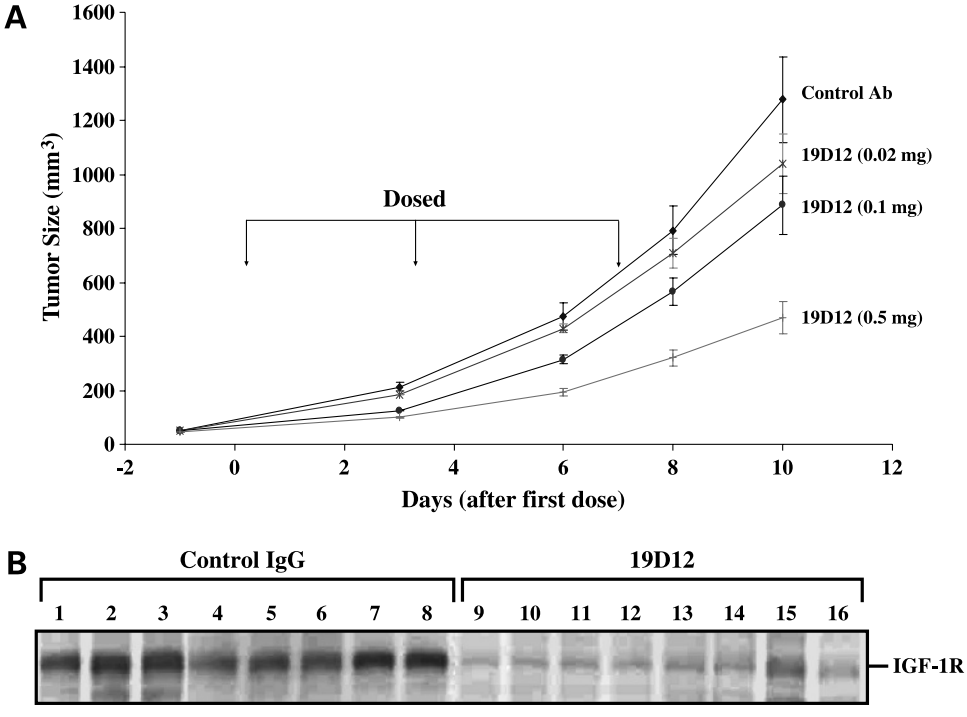

Figure 7. Inhibition of A2780 tumor growth in mice. **A,** four million A2780 human ovarian carcinoma cells in 50% growth factor–reduced Matrigel were inoculated s.c. into nude mice. 19D12 treatment was initiated when the tumor size reached ~50 mm^3 (day 0). Two additional doses were given at days 3 and 7. Tumor volumes were measured by Labcat. **B,** each tumor lysate (1 mg) was separated by a 10% SDS-PAGE (*lanes 1-8,* tumor lysates from control animals; *lanes 9-16,* tumor lysates from 19D12-treated animals) and transferred to nitrocellulose blots. The blots were probed with anti–IGF-IR antibody.

an epitope on one arm of the IGF-IR homodimer. Consistent with this hypothesis, 19D12 is able to bind and inhibit the signaling of IGF-IR/insulin receptor heterodimers (Fig. 2B and C). This activity is important because the presence of IGF-IR/insulin receptor heterodimers has been reported in human tumor samples and these heterodimers behave functionally similar to IGF-IR homodimers (44).

In recent years, IGF-IR, as an anticancer target, has been pursued actively due to its importance in tumor cell growth and transformation. Because it is a receptor tyrosine kinase, two strategies have been employed: making a neutralizing antibody against its extracellular domain and developing a small-molecule kinase inhibitor against its cytoplasmic kinase domain (36–40, 48–52). The potential advantages of an antibody approach over the small-molecule approach include selectivity against the insulin receptor and potential for increased antitumor efficacy through receptor down-regulation and antibody-dependent cellular cytotoxicity. In a xenograft model, 19D12 inhibited tumor growth of a very aggressive human ovarian cancer line A2780. In addition to the activities described here with A2780 ovarian cancer, 19D12 also has shown potent activities against a wide variety of other human tumor cell lines *in vivo*, including MCF7 breast cancer cells and NCI-H322 non–small cell lung carcinoma cells (data not shown).

In addition to potent antitumor activity as a single agent, 19D12 also displays an additive effect with several approved chemotherapeutic agents and with some targeted antitumor agents (data not shown). Some experiments conducted recently with both cultured cells and primary tumor cells have suggested that IGF-IR can confer resistance to both anti-HER-2 and anti–epidermal growth factor receptor therapies (53, 54). This anti–IGF-IR neutralizing antibody 19D12 is therefore a promising agent both as monotherapy and for use in combination therapy for a wide range of cancer patients.

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