Down-regulation of Insulin Receptor by Antibodies against the Type I Insulin-Like Growth Factor Receptor: Implications for Anti–Insulin-Like Growth Factor Therapy in Breast Cancer

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Abstract
Insulin-like growth factor-I (IGF-I), IGF-II, and insulin have all been implicated in regulating several aspects of the malignant phenotype via the type I IGF receptor (IGF1R) and insulin receptor (IR). We have previously shown that a chimeric single-chain antibody against IGF1R (scFv-Fc) and a murine antibody EM164 down-regulate IGF1R, making breast cancer cells unresponsive to IGF-I. To determine if IR signaling is affected, we examined regulation of IR in MCF-7 cells after exposure to these antibodies. Surprisingly, both scFv-Fc and EM164 resulted in decreased levels of IR in vitro and in vivo despite their lack of reactivity against IR. Twenty-four-hour pretreatment with EM164 also inhibited insulin-mediated phosphorylation of IR and insulin-stimulated proliferation of MCF-7 cells. Neither scFv-Fc nor EM164 caused down-regulation of IR in cells that express very low levels of IGF1R or no IGF1R. Expression of IGF1R was required for IR down-regulation, which was specific as neither antibody caused down-regulation of β1 integrin or epidermal growth factor receptor. Reagents that disrupt lipid rafts inhibited IR down-regulation by the antibodies, suggesting that IR in close physical proximity to IGF1R in lipid rafts was being endocytosed. Our data show that down-regulation of IR by monoclonal antibodies against IGF1R requires the coexpression of IGF1R and may be due to endocytosis of hybrid IR/IGF1R or holo-IR. Thus, antibodies against IGF1R provide inhibition of both IGF and insulin signaling in cancer cells. (Cancer Res 2006; 66(4): 2391-402)

Introduction
Abundant data from cell culture, animal, and human studies have suggested that insulin-like growth factors (IGF) regulate the malignant phenotype (1). The tyrosine kinase type I IGF receptor (IGF1R) has been implicated in several different cancers, although its role has not yet been established in any clinical trial.

Several approaches have been used to inhibit signaling via IGF1R. Antisense IGF1R oligodeoxynucleotide-based therapy has been reported in patients with astrocytomas (2). A second way to inhibit IGF1R is the use of small-molecule tyrosine kinase inhibitors. Several small-molecule inhibitors of IGF1R have been developed; however, to date, there are no reported inhibitors completely specific for IGF1R (3–5). Finally, antibodies directed against IGF1R have been used to inhibit activation of IGF1R.

The widely studied monoclonal antibody oIR3 (6) blocks binding of IGF-I to IGF1R. oIR3 inhibits proliferation of MCF-7 cells in vitro (7). It also inhibits the growth of some (MDA-MB-231 and T61) but not all (MCF-7) breast cancer xenografts in vivo (8, 9). Additional monoclonal antibodies directed against IGF1R have been reported (10–16). A chimeric antibody directed against IGF1R, scFv-Fc, partially inhibits the xenograft growth of MCF-7 cells (10). We have previously shown that scFv-Fc is an agonistic antibody but then leads to rapid down-regulation of IGF1R via the endocytic pathway, making cells refractory to further mitogenic effects of IGF-I (11). EM164 is a murine antibody against IGF1R and also does not bind IR (12). EM164 inhibits activation of IGF1R by IGF-I and IGF-II and also causes down-regulation of IGF1R. Other reports confirm that down-regulation of IGF1R levels is a common mechanism of action for all monoclonal antibodies directed against IGF1R studied to date (13–15).

Several lines of evidence show that insulin also regulates breast cancer biology (17–19). IGFs and insulin act via a family of receptors that includes IGF1R and the insulin receptor (IR). IR exists in two isoforms generated by alternative splicing of exon 11: exon 11+ or B isoform (IR-B) and exon 11 or A isoform (IR-A; ref. 20). Although IR-A and IR-B have similar affinities for insulin, IR-A exhibits higher affinity for IGF-II than IR-B (21). Furthermore, human breast cancer specimens have higher IR content than normal breast tissue and fibroadenoma specimens (22). Breast cancer cells express high levels of IR-A compared with IR-B (23, 24). Many cells and tissues have hybrid receptors assembled with one chain of the IGF1R and one of IR (25, 26). As IGF1R expression levels are also elevated in primary breast cancer tissues (27), increased levels of hybrid IGF1R/IR-A receptors likely exist in breast cancer cells and targeting of both IR and IGF1R may be necessary to completely inhibit IGF action. Thus, a strategy that targets only IGF1R may not be sufficient to block all of the receptors important in regulating IGF action. However, due to the importance of IR in glucose homeostasis, an ideal anti-IGF strategy would target IGF1R and IR only in tumor cells and leave host IR in insulin target organs unaffected.

In this study, we assessed the specificity of scFv-Fc and EM164 by determining their effect on IR. Our results show that both scFv-Fc and EM164 down-regulated IR. Furthermore, long-term exposure in vitro and in vivo to EM164 inhibited insulin signaling and insulin-stimulated proliferation of MCF-7 cells. Disruption of membrane lipid rafts inhibited down-regulation of IR by scFv-Fc and EM164. Moreover, antibody-mediated down-regulation of IR requires coexpression of IGF1R, suggesting that
cells with only IR expression may be unaffected by monoclonal antibody strategies.

Materials and Methods

Reagents. All reagents and chemicals were purchased from Sigma (St. Louis, MO) and cell culture reagents were from Invitrogen/ Life Technologies (Rockville, MD). IGF-I and IGF-II were purchased from Neurobiotech (Adelaide, Australia) and human insulin was from Eli Lilly (Indianapolis, IN). Antibodies against the β-subunits of IGF1R and IR and the monoclonal antibody against Ga1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antiphosphotyrosine antibody (PY20-HRP) was from BD Transduction Laboratories (Lexington, KY). Antibodies against p44/p42 extracellular signal-regulated kinase (ERK) 1/2 (phosphospecific and total) and Akt (phosphospecific and total) were purchased from Cell Signaling (Beverly, MA).

Cell lines and culture. MCF-7L cells were obtained from Dr. C. Kent Osborne (Baylor College of Medicine, Houston, TX) and were maintained as described previously. R- cells (28) were obtained from Dr. Renato Baserga (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA) and were maintained in DMEM with 10% fetal bovine serum and 50 μg/mL G418. R-C0 cells (29) were obtained from Dr. Renato Baserga (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA) and were maintained as described previously. R-C0 cells (29) were obtained from Dr. Renato Baserga (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA) and were maintained as described previously. R-C2 cells (28) were obtained from Dr. Renato Baserga (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA) and were maintained as described previously. R-C0 cells (29) were obtained from Dr. Renato Baserga (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA) and were maintained as described previously. R-C0 cells (29) were obtained from Dr. Renato Baserga (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA) and were maintained as described previously. R-C0 cells (29) were obtained from Dr. Renato Baserga (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA) and were maintained as described previously.

Cell stimulation. Seventy percent confluent cells in 6 cm dishes in regular growth medium were washed twice with PBS and serum deprived for 24 hours in serum-free medium as described previously (29). For treatment, medium was replaced with serum-free medium containing 5 nmol/L IGF-I, 10 nmol/L IGF-II, 10 nmol/L insulin, 60 nmol/L scFv-Fc, or 120 nmol/L EM164 for various times as indicated in the figure captions. To determine if EM164 inhibited IGF-I, IGF-II, or insulin-mediated activation of their cognate receptors or other

Figure 1. Antibodies directed against IGF1R down-regulated IR in MCF-7 cells. A, MCF-7 cells were grown in 6 cm dishes in regular growth medium. Confluent cells (70%) were washed twice with PBS and serum deprived for 24 hours in serum-free medium (SFM). MCF-7 cells were either untreated (lanes SFM), treated with 60 nmol/L scFv-Fc, 250 nmol/L scFv-Fc, or 120 nmol/L EM164 for various times as indicated below the lane number. Cells were washed thrice with ice-cold PBS on ice and lysed with 300 μL/6 cm dish lysis buffer TNESV. Lysates were clarified by centrifugation at 12,000 × g for 20 minutes at 4°C and soluble cellular proteins were used for experiments. Fourty micrograms of cellular proteins were subjected to reducing SDS-PAGE on 8% polyacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose membrane. Cellular proteins were then immunoblotted (IB) for total levels of IRβ and chemiluminescence was done using SuperSignal West Pico substrate (Pierce). The 95 kDa β-subunit of IR is shown in the figure. Cellular proteins were also immunoblotted for total levels of p44/p42 ERK1/2 as loading control. Experiments were repeated thrice with similar results and a representative experiment is shown. B, top, MCF-7 cells were untreated or treated with 5 nmol/L IGF-I or 250 nmol/L scFv-Fc for 24 hours. Cellular proteins (500 μg) were immunoprecipitated (IP) with an antibody against IGF1R and immunoblotted for IGF1R to assay for holo-IGF1R (lanes 1-3), immunoprecipitated with an antibody against IR and immunoblotted for IR to assay for hybrid IGF1R/IR (lanes 4-6) or immunoprecipitated with an antibody against IR and immunoblotted for IR to measure holo-IR (lanes 7-9). Lanes 10 to 12, cellular proteins immunoblotted for IR; lanes 13 to 15, total ERK1/2 levels as loading control to indicate equal loading of samples. Bottom, MCF-7 cells were either untreated (lanes 1 and 4) or treated with 10 nmol/L insulin (Ins, lanes 2 and 5) or 120 nmol/L EM164 (lanes 3 and 6) for 15 minutes (lanes 1-3) or 24 hours (lanes 4-6) and assayed for holo-IR as described above. Experiments were repeated thrice with similar results and a representative experiment is shown.
downstream pathways, cells were first pretreated with antibodies for 15 minutes or 24 hours and then with the various ligands for additional 10 minutes or 5 minutes for insulin. All experiments were repeated a minimum of thrice.

**Cell lysis.** Cells were washed thrice with ice-cold PBS on ice and lysed with 300 μL/6 cm dish lysis buffer TNE5 (50 mmol/L Tris-CI (pH 7.4), 1% NP-40, 2 mmol/L EDTA (pH 8.0), 100 mmol/L NaCl, 10 mmol/L Na orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 20 μg/mL leupeptin and 20 μg/mL aprotinin).

**Proliferation assays.** Cells were plated in 24-well plates with 20,000 cells per well in regular growth medium. Cells were switched to serum-free medium for 24 hours and then treated as indicated in the figure captions. All treatments were done in triplicates. Growth was measured 4 to 5 days after treatment by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (11, 30).

**Isolation of plasma membrane lipid rafts.** Lipid rafts were isolated using sequential detergent extraction of membranes as described (31). Cells were resuspended in ice-cold buffer A [25 mmol/L MES and 150 mmol/L NaCl (pH 6.5)] and lysed with an equal volume of ice-cold buffer A with 2% Triton X-100, 2 mmol/L Na orthovanadate, and 2 mmol/L PMSF. Lysates were incubated for 60 minutes on ice with gentle inversion and centrifuged for 30 minutes and the supernatants were saved as Triton-soluble cytoplasmic and nonraft membrane proteins. Triton-insoluble pellets were extracted with buffer B [10 mmol/L Tris-CI (pH 7.6), 500 mmol/L NaCl, 1% Triton X-100, 2 mmol/L Na orthovanadate, 1 mmol/L PMSF, and 60 mmol/L β-octylglucoside] on ice for 30 minutes with frequent mixing. Triton-insoluble and β-octylglucoside–soluble supernatants containing lipid raft proteins were obtained after centrifugation at 15,000 × g for 20 minutes.

**Disruption of rafts.** Lipid rafts were disrupted by treating cells with two different classes of cholesterol-depleting reagents—filipin III and methyl-β-cyclodextrin for 24 hours.

**Immunoblotting.** For immunoblotting, 40 μg of cellular proteins were subjected to reducing SDS-PAGE. Immunoblotting was done as described previously (11). To detect levels of receptors, membranes were blotted with a 1:1,000 dilution of rabbit polyclonal antibody against IRβ or IGF1Rβ. Chemiluminescence was done using SuperSignal West Pico substrate (Pierce, Rockford, IL). For detecting phosphorylated proteins, membranes were incubated with 1:2,000 dilution of PY20-HP2 antiphosphotyrosine antibody in TBST for 1 hour at room temperature. All other antibodies were used as per instructions of the manufacturer. In all immunoblot analyses, total levels of ERK1/2 were used as loading control to indicate equal loading of samples.

**Immunoprecipitations.** Five hundred micrograms of total cellular proteins were immunoprecipitated with 2 μg polyclonal antibody against IGF1Rβ or IRβ as described previously (11).

**Animal studies and xenograft tumor extract analyses.** All animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. MCF-7 xenografts were grown in 4-week-old female athymic mice implanted with 60-day release pellets of 0.5 mg 17β-estradiol (Innovative Research of America, Sarasota, FL) as described previously (11). Tumor volume was estimated from bidirectional measurements using the formula length × breadth 2 / 2. When tumors entered the exponential growth phase, mice were treated with 800 μg EM164 or an isotope-matched control antibody i.p. every 3 days. Treatments were continued for 4 weeks and at the end of the experiment, mice were sacrificed and tumors were harvested. The tumors were snap frozen in liquid nitrogen. Frozen tumor samples were homogenized in a tissue pulverizer in a dry ice/ethanol bath as described previously.

To determine the effect of scFv-Fc on receptor levels, mice with MCF-7 xenografts on either side were used. Tumors from the left side of all mice were resected before treatment. Twenty-four hours after resection, mice were injected i.p. with 500 μg scFv-Fc or PBS as control. Twenty-four hours after treatment, mice were sacrificed and the remaining tumors were harvested.

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**Results**

**Antibodies against IGF1R down-regulated IR in breast cancer cells.** We and others have shown that antibodies against IGF1R inhibit growth of breast cancer cells and cause efficient down-regulation of IGF1R (10, 11, 13). To determine if IR was also affected, we examined the effect of three different antibodies on IR levels in MCF-7 cells. None of the antibodies caused significant changes in the level of IR after 30 minutes of exposure (Fig. 1A). However, scFv-Fc down-regulation of IR was seen within 6 hours (Fig. 1A, lane 4) and by 24 hours levels of IR were markedly decreased (lane 10) when compared with untreated cells. αIR3 and EM164 also caused a decrease in IR levels after 6 hours and by 24 hours IR levels were decreased by ~50%. The down-regulation of IR by these antibodies was via the endocytic pathway as methylamine, which increases pH of the endosomal vesicles, inhibited the down-regulation of IR (data not shown). We have previously shown that IGF-I given over the same time period did not down-regulate IGF1R (11) and it also did not decrease IR over the same time period (see Fig. 3). Because EM164 is an IGF1R antagonist but still causes receptor down-regulation, it does not appear that receptor tyrosine phosphorylation is required for these effects.

It is possible that the antibodies down-regulated hybrid receptor and the apparent decrease in total IR levels were a result of diminished hybrid levels. To determine if the antibodies down-regulated hybrid IGF1R/IR or holol-IR, MCF-7 cell lysates were examined for IR by immunoblot after immunoprecipitation with either anti-IGF1R (Fig. 1B, top, lanes 4-6) or anti-IR antibodies (Fig. 1B, top, lanes 7-9). Twenty-four-hour treatment with scFv-Fc caused a down-regulation of the IR within the IGF1R/IR hybrid complex (lane 6 compared with lane 4) and also the IR/IR holoreceptor (lane 9 compared with lane 7). In lanes 10 to 12, total cell lysates were immunoblotted for IR and a 24-hour treatment with scFv-Fc caused a down-regulation of IR. As a control, in lanes 1 to 3 lysates were immunoprecipitated with IGF1R and immunoblotted with IGF1R and as we have previously reported (11), 24-hour treatment with scFv-Fc down-regulated IGF1R. Similar results were seen with EM164 (Fig. 1B, bottom), which also decreased IR within the IR/IR holoreceptor following a 24-hour treatment compared with a 15-minute treatment (lane 6 compared with lane 3) and compared with untreated or insulin-treated cells. Thus, antibodies directed only against IGF1R down-regulate IR in MCF-7 cells.

These results were specific for IGF1R and IR, as neither β1 integrin or epidermal growth factor receptor (EGFR) levels were affected by EM164 and scFv-Fc (data not shown). Moreover, other antibodies (rituximab and trastuzumab) did not affect IR or IGF1R (data not shown). Thus, only antibodies directed against IGF1R can down-regulate IR and IGF1R.

**Short-term treatment with EM164 did not inhibit insulin signaling in MCF-7 cells.** In MCF-7 cells, IRS-1 is the major adaptor protein phosphorylated by IGF-I and insulin (29). IGF-I treatment resulted in detection of a 185 kilodaltons phosphorylated band in cells (Fig. 2A, lane 2) and in cells treated with IGF-II (lane 3) or insulin (lane 4) but not in untreated cells (lane 1). Pretreatment of cells with 120 nmol/L EM164 for 15 minutes before stimulation with IGF-1 inhibited IGF-1-induced phosphorylation of IRS-1 (lane 6 compared with lane 2) and also inhibited phosphorylation of IRS-1 mediated by IGF-II (lane 7 compared with lane 3), but did not inhibit phosphorylation of IRS-1 by insulin (lane 8 compared with lane 4). Fifteen-minute treatment with EM164 also did not inhibit insulin-stimulated phosphorylation of IR (Fig. 2C, top). Because EM164 does
not directly bind to IR, insulin-stimulated phosphorylation of IRS-1 is not inhibited by EM164 following a short-term treatment.

Twenty-four-hour pretreatment with antibodies against IGF1R inhibited insulin signaling and insulin-stimulated proliferation of MCF-7 cells. We hypothesized that if the antibodies down-regulated the holo-IR, then they should more completely inhibit insulin signaling after a 24-hour exposure. MCF-7 cells were pretreated with EM164 for 24 hours (Fig. 2B, lanes 5-8), or not (lanes 1-4), before exposure to IGF-I, IGF-II, or insulin. Figure 2B (lanes 1-4) shows that IGF-I, IGF-II, and insulin exposure resulted in phosphorylation of IRS-1 in control cells not pretreated with EM164. When cells were exposed to EM164 for 24 hours followed by treatment with IGF-I (lane 6), IGF-II (lane 7), or insulin (lane 8), phosphorylated IRS-1 was not detected. In contrast, cells treated with insulin for 24 hours still retained the ability to be further stimulated by treatment with additional insulin (Fig. 2B, lane 10 compared with lane 9).

To directly assay the effect of EM164 on IR activation, cellular proteins were immunoprecipitated with an antibody against IR followed by immunoblotting with an antiphosphotyrosine antibody. Short-term treatment with EM164 did not inhibit phosphorylation of IR (Fig. 2C, top, lane 8 compared with lane 4), indicating that EM164 does not directly block insulin binding to IR. However, a 24-hour treatment with EM164 inhibited IR phosphorylation by insulin (lane 12 compared with lane 4). Similarly, a 24-hour treatment with scFv-Fc also decreased phosphorylation of IR (Fig. 2C, lane 16 compared with lane 4). As a control, the membrane in Fig. 2C (top) was stripped and reprobed for IR (bottom) and shows the levels of IR in the immunoprecipitates. These data show that following a 24-hour treatment with scFv-Fc...
and EM164, down-regulation of IR levels resulted in inhibition of IR activation by insulin.

Furthermore, as shown in Fig. 2D, treatment of cells with EM164 for 24 hours also blocked insulin-stimulated phosphorylation of Akt and phosphorylation of p44/p42 ERK1/2 (lane 6) compared with the phosphorylation in cells that were not pretreated with EM164 (lane 3). These data show that down-regulation of IR by EM164 partially blocked the ability of insulin to initiate downstream signaling.

To test the effect of EM164 on insulin-stimulated proliferation, MCF-7 cells were treated with 10 nmol/L insulin in the absence or presence of EM164. Insulin stimulated the growth of MCF-7 cells as shown in Fig. 2E (filled column) and EM164 did not significantly inhibit insulin-stimulated proliferation of MCF-7 cells when added simultaneously (left). We tested if a 24-hour pretreatment of cells with EM164 before exposure to additional insulin would have any effect on the growth of cells. Cells were pretreated with either insulin as control or EM164 for 24 hours in Fig. 2E (right). After this pretreatment, cells were incubated with or without additional insulin. Pretreatment with insulin followed by treatment with subsequent insulin resulted in increased proliferation compared with the control as shown in Fig. 2E. In contrast, pretreatment with EM164 inhibited proliferation in response to insulin.

Figure 2. Continued. D, 24-hour treatment with EM164 inhibited downstream signaling pathways activated by insulin. Cells without exposure to EM164 (lanes 1-3) or following exposure to EM164 for 24 hours (lanes 4-6) were untreated (lanes 1 and 4), treated with IGF-I (lanes 2 and 5), or 10 nmol/L insulin for 5 minutes (lanes 3 and 6). Cellular proteins were immunoblotted for phospho-Akt, total Akt, phospho-p44/p42 ERK1/2, and total p44/p42 ERK1/2. All experiments shown in (A-D) were repeated thrice with similar results and a representative experiment is shown. E, pretreatment of cells with EM164 inhibited growth stimulation by insulin. Left, MCF-7 cells in serum-free conditions were treated with insulin, EM164, or insulin and EM164 together for 5 days. Proliferation was then measured by the uptake of the MTT reagent followed by measurement of absorbance at 570 nm. Growth is shown as the absorbance at 570 nm. Columns, mean of triplicate samples; bars, SE. Right, MCF-7 cells in serum-free conditions were pretreated with either insulin (columns labeled 24h pre-Tx: Insulin) or EM164 (columns labeled 24h Tx: EM164) for 24 hours. Medium was removed and cells were treated without (SFM) or with 10 nmol/L insulin for 4 days. Proliferation was then measured by the uptake of the MTT reagent followed by measurement of absorbance at 570 nm. Growth is shown as the absorbance at 570 nm. Columns, mean of triplicate readings; bars, SE. Experiments were repeated thrice with similar results.
Thus, cells pretreated with insulin still have ample receptor on the cell surface, allowing for further proliferation in response to insulin. In contrast, when cells pretreated with EM164 were exposed to insulin, the cells had decreased cell surface IR and did not proliferate in response to insulin.

scFv-Fc and EM164 did not down-regulate IR in Hs578T breast cancer cells with very low levels of IGF1R expression. Because the antibodies against IGF1R have no immunoreactivity with IR (12), we next examined if expression of IGF1R was required for IR down-regulation. Hs578T expressed low levels of IGF1R when compared with MCF-7 (Fig. 3A). Hs578T and MCF-7 cells had equivalent amounts of IR expression (Fig. 3A, top). In Hs578T cells, 24-hour treatment with scFv-Fc did not result in decreased levels of IR (Fig. 3A), whereas a similar treatment decreased IR levels in MCF-7 cells. In both cells, a 15-minute treatment with scFv-Fc did not down-regulate IR. In Hs578T cells, scFv-Fc was able to down-regulate the very low levels of IGF1R in these cells (Fig. 3A, middle, lane 12 compared with lane 10). Figure 3A (bottom) shows the total levels of ERK1/2 as a loading control. Similar results were seen with EM164 (Fig. 3B).

scFv-Fc- and EM164-mediated down-regulation of IR required coexpression of both IGF1R and IR on the same cell. R− cells are fibroblasts derived from mice with a genetic deletion of IGF1R (28) and do not express IGF1R (Fig. 3C, top). In these cells, neither scFv-Fc nor EM164 affected IR levels (Fig. 3D, top, lanes 9 and 10) after 24-hour treatment.

Our data from Hs578T and R− cells suggested that both IGF1R and IR need to be expressed on the same cell to see IR down-regulation by anti-IGF1R antibodies. To test this hypothesis, we transfected the cDNA for human IGF1R into R− cells to generate R−/IGF1R cells. Two different clones were characterized and the IGF1R stably expressed in these cells was functional as measured by the ability of IGF-I to activate downstream signaling pathways. To test the effect of EM164 and scFv-Fc on IR levels, R−/IGF1R #11, and R−/IGF1R #8 were exposed to IGF-I, insulin, EM164, scFv-Fc, or no additional treatment for 15 minutes or 24 hours and immunoblotted for IGF1R (Fig. 3C) or IR (Fig. 3D). In both clones expressing IGF1R, 24-hour treatment with either EM164 (lane 9) or scFv-Fc (lane 10) caused down-regulation of IGF1R as shown in Fig. 3C. Furthermore, in R−/IGF1R #11 cells, both EM164 (Fig. 3D, lane 9) and scFv-Fc (lane 10) also caused down-regulation of IR after a 24-hour treatment compared with untreated cells (lane 6) or cells treated with the antibodies for only 15 minutes (lanes 4 and 5) as shown in Fig. 3D. Neither IGF-I nor insulin affected IGF1R or IR levels (Fig. 3C and D). Figure 3E shows the levels of total ERK1/2 to indicate equal loading of samples in Fig. 3C and D.
Thus, expression of IGF1R in R−/C0 cells leads to the down-regulation of IR by both EM164 and scFv-Fc. These results support the observation that expression of both receptors on the surface of the same cell is required for down-regulation of IR by anti-IGF1R antibodies.

scFv-Fc down-regulated IGF1R and IR in lipid rafts. Several reports have described the presence of both IGF1R and IR in plasma membrane lipid rafts in adipocytes and fibroblasts (32–35). Therefore, we determined if IGF1R and IR are found in lipid rafts in MCF-7 cells. Both IGF1R and IR were present in the Triton-soluble cytosolic and membrane fraction (Fig. 4A and B, lanes 1-3) and scFv-Fc down-regulated both receptors. In addition, IGF1R and IR were also found in the Triton-insoluble/octylglucoside-soluble fraction in lanes 4 to 6, which represents the lipid raft proteins. About 20% to 25% of the total IGF1R and IR found in MCF-7 cells was in lipid rafts. Furthermore, 24-hour treatment with scFv-Fc down-regulated both IR and IGF1R in the lipid raft (lane 6) in Fig. 4A and B, respectively. Figure 4D shows the quantitation of the levels of IGF1R and IR in the Triton-insoluble/octylglucoside-soluble fraction in Fig. 4A and B with the relative levels of either expressed as percent of the respective receptor levels in untreated cells. G protein α inhibitory subunit (Gαi2) was used as a loading control and marker for lipid rafts (36) and was present only in lanes 4 to 6, which represent the raft fraction (Fig. 4C).

Disruption of rafts partially rescued down-regulation of IR by antibodies directed against IGF1R. Because both IGF1R and IR were also found in rafts, we hypothesized that IGF1R antibodies down-regulated IR due to receptor colocalization in rafts. In this model, initiation of endocytosis or transcytosis of IGF1R in the rafts also down-regulated IR in this membrane compartment. Therefore, we disrupted lipid rafts with either filipin III or methyl-β-cyclodextrin and determined the effect on down-regulation of IR levels by the antibodies. In Fig. 5A, both EM164 (lane 4) and scFv-Fc (lane 5) down-regulated IR after a 24-hour exposure when rafts were intact. When cells were treated with 2 μg/mL filipin III (Fig. 5B) or 2 mmol/L methyl-β-cyclodextrin (Fig. 5C) for 24 hours, down-regulation of IR by EM164 (lane 4) and scFv-Fc (lane 5) was partially rescued. Immunoblotting for ERK1/2 was used as loading control and total ERK1/2 levels were equal in all samples (data not shown). Figure 5D represents densitometric quantitation of the

**Figure 3** Continued. C, D, and E, scFv-Fc and EM164 down-regulated IR in R− cells expressing IGF1R. R−, R−/IGF1R #11, and R−/IGF1R #8 cells were untreated (lanes 1 and 6), treated with 5 nmol/L IGF-I (lanes 2 and 7), 10 nmol/L insulin (lanes 3 and 8), 120 nmol/L EM164 (lanes 4 and 9), or 250 nmol/L scFv-Fc (lanes 5 and 10) for 15 minutes (lanes 1-5) or 24 hours (lanes 6-10). Cellular lysates were immunoblotted for IGF1Rβ (C), IRβ (D), and total ERK1/2 to show equal loading of all samples (E). Experiments were repeated four times and a representative experiment is shown.
regulation in xenograft tumors. Figure 6A shows the levels of IR in the tumors before treatment (before PBS and before scFv-Fc lanes). Twenty-four hours after treatment, tumors taken from animals treated with scFv-Fc had significant down-regulation of IR (bottom, after scFv-Fc lanes). No change in IR was observed in PBS-treated animals (top, after PBS lanes). Densitometric analysis showed that after treatment with scFv-Fc, IR levels in xenograft tumor extracts were ∼38% (n = 3) of that in tumor extracts before scFv-Fc treatment. In contrast, in mice that received control PBS injections, the IR levels were ∼99% of that before treatment.

**EM164 inhibited xenograft growth of MCF-7 cells and down-regulated IGF1R and IR in vivo.** Maloney et al. (12) have previously shown that EM164 inhibits IGF-1-mediated proliferation of MCF-7 cells in vitro and the xenograft growth of BxPC-3 human pancreatic cancer cells. Figure 6B shows that EM164 inhibited xenograft growth of MCF-7 cells compared with control antibody. Animals treated with EM164 showed significant decrease in tumor volume (P = 0.0033 at day 39, t test; n = 5). Tumor volumes were significantly lower in EM164-treated animals over the period of treatment. After 4 weeks of treatment, mice were sacrificed, tumors were harvested, and tumor extracts from mice treated with EM164 or control antibody were assayed for levels of IGF1R and IR. As shown in Fig. 6C, EM164 caused down-regulation of both IGF1R and IR, whereas the control antibody did not affect levels of either. Figure 6C shows the quantitation of IR and IGF1R levels in the xenograft tumors. In EM164-treated tumors, IR levels were decreased by 32% and IGF1R levels by 75% compared with levels in control antibody-treated tumors.

Furthermore, the inhibition of xenograft tumor growth by EM164 (Fig. 6B) and decrease in receptor levels (Fig. 6C and D) was also paralleled by the inhibition of IGF-1-mediated activation of IGF1R and phosphatidylinositol-3 kinase pathway in MCF-7 xenograft tumors in vivo by EM164 (Fig. 6D). Treatment with IGF-1 for 30 minutes before sacrifice led to phosphorylation of IRS-1 and Akt in MCF-7 xenograft tumors (Fig. 6D, lane 5) compared with mice injected with PBS (lane 4). Treatment with EM164 24 hours before treatment with IGF-I caused inhibition of IRS-1 phosphorylation (lane 6). Similar inhibition of phosphorylation of Akt was seen in the xenograft tumor following a 24-hour treatment with EM164. Total levels of Akt were unchanged by treatment with EM164.

**Discussion**

Perhaps the best example of a well-validated target for cancer therapy is estrogen receptor (ER) α in breast cancer. By disrupting receptor function with selective ER modulators or by lowering serum estradiol levels via ovarian suppression or aromatase inhibition, essentially all stages of breast cancer are effectively treated. Although the idea that ovarian-derived factors stimulated breast cancer growth preceded the molecular identification of EREs, we now understand that only tumors that express this target benefit from its inhibition, although other ERS have been identified. Thus, a firm understanding of the effector of estrogen action has provided substantial clinical benefit for breast cancer patients.

There are potentially many other growth regulatory pathways that affect cancer cell biology. It is evident that IGF system regulates various aspects of the malignant phenotype. All of the IGF ligands (insulin, IGF-I, and IGF-II) have been implicated in cancer cell biology. Thus, strategies to inhibit IGF1R have emerged as potential...
cancer treatments. However, in addition to IGF1R, preclinical data support an important role for IR regulating IGF action, either as a hybrid or holoreceptor (37). An optimal anti-IGF therapy in cancer would block all of the receptors responsible for IGF signaling, including IR. Certainly, IGF1R selectivity is desirable in host tissues but this may not be the case in cancer cells.

Antibodies that target IGF1R have been developed for clinical use. We have previously shown that scFv-Fc and EM164 inhibit IGF-I-mediated effects and down-regulate IGF1R efficiently (12), suggesting that down-regulation of IGF1R is an important mechanism of action for antibodies against IGF1R. Therefore, we sought to determine the effects of scFv-Fc and EM164 on IR and insulin signaling. In this study, we show that both these antibodies also down-regulate IR, inhibit signaling via IR, and inhibit insulin-stimulated proliferation of breast cancer cells in vitro. This was surprising because neither antibody binds IR. It is possible that the decrease in IR levels reflects the down-regulation of IR, which is present as part of the hybrid IGF1R/IR via direct binding of the antibodies to the hybrid receptor. However, we show that both scFv-Fc and EM164 also down-regulate holo-IR in MCF-7 cells.

These unexpected results raise the question, how did scFv-Fc and EM164 decrease levels of holo-IR?

Our data show that scFv-Fc and EM164 down-regulate IR by endocytosis of IR present in lipid rafts along with IGF1R. Indeed, lipid rafts have gained prominence as specialized platforms that can bring together components of a signaling cascade in a spatial manner in the plasma membrane (38). Many reports have shown that IGF1R and IR can be found in lipid rafts (32–34). In this study, we show that IGF1R was found in lipid rafts in MCF-7 cells (Fig. 5). Furthermore, because MCF-7 cells lack caveolin-1 (39), these cells should only have noncaveolin flat lipid rafts and not the caveolin-containing invaginated lipid rafts or caveolae. It is possible that the binding of antibodies to IGF1R may result in the sequestration of some of the antibody-IGF1R complexes in lipid rafts from where they undergo endocytosis or transcytosis. In addition to the classic clathrin-dependent endocytic pathways (40), endocytosis can also occur via clathrin-independent caveolar pathways (41, 42). Thus, although internalization of receptors via caveolae has been documented (41, 43–45), endocytosis of cell surface receptors from noncaveolin flat lipid rafts has not yet been

Figure 5. Disruption of lipid rafts reversed down-regulation of IR by antibodies. A to C, MCF-7 cells were untreated (lane 1), or treated with either 5 nmol/L IGF-I (lane 2), 10 nmol/L insulin (lane 3), 120 nmol/L EM164 (lane 4), or 250 nmol/L scFv-Fc (lane 5) for 24 hours in the absence (A) or presence of either 2 μg/mL filipin III (B) or 2 mmol/L methyl-β-cyclodextrin (C) for 24 hours. Cellular proteins were separated by SDS-PAGE and immunoblotted for IRβ (left) or IGF1Rβ (right). Equal loading of samples was ensured by immunoblotting for total levels of ERK1/2 (data not shown). D, densitometric quantitation of the levels of IRβ (left) and IGF1Rβ (right) in (A–C). Columns, percentage of the level of the respective receptor in untreated cells in (A). Experiments disrupting lipid rafts were repeated four times with similar results and a representative experiment is shown.
reported. However, it has recently been shown that SV40 can enter cells via an endocytic pathway that involves neither clathrin nor caveolae (46), suggesting that receptors can be internalized from flat lipid rafts. We have shown for the first time that tyrosine kinase receptors can also be internalized from noncaveolin lipid rafts.

We were able to show that scFv-Fc decreases the amount of IGF1R in lipid rafts (Fig. 4). Furthermore, in MCF-7 cells, IR was also significantly downregulated after treatment with scFv-Fc (Fig. 6A). This effect was observed in vivo as well, as EM164 did not significantly decrease IGF1R levels in the tumors of mice treated with PBS (Fig. 6B). In addition, EM164 significantly inhibited the growth of MCF-7 xenograft tumors in vivo (Fig. 6B). EM164 also significantly decreased the levels of IGF1R in xenograft tumors (Fig. 6C). Moreover, EM164 inhibited IGF-I-mediated activation of IGF1R in MCF-7 xenograft tumors (Fig. 6D).

Figure 6. Antibodies down-regulated IR in vivo and inhibited xenograft growth. A, scFv-Fc down-regulated IR in vivo. Female athymic mice bearing MCF-7 xenograft tumors on each side were studied. Tumors from the left side were resected from each mouse before treatment. The next day, three mice were injected i.p. with 500 µg scFv-Fc and three with PBS. Twenty-four hours after treatment, tumors were collected. Tumor samples were frozen in liquid nitrogen and homogenized in TNE5V buffer using a pulverizer as described in Materials and Methods. Tumor extracts (100 µg) were immunoblotted for total IR levels. Lanes 1 to 3 (top and bottom), total IR levels in the tumor extracts in the six mice before treatment. IR levels in the mice that were injected with scFv-Fc is shown in lanes labeled “after scFv-Fc” (lanes 4 to 6, bottom). Lanes 4 to 6 (top) show IR levels in tumor extracts from mice after treatment with PBS. Experiments were repeated thrice and a representative one is shown.

B, EM164 inhibited xenograft growth of MCF-7 cells in athymic mice. Sixty-day-release E2 pellets were implanted in 4- to 5-week-old female mice. The next day, 5 x 10⁶ MCF-7 cells were injected into the second mammary fat pad on the left side of each mouse. Twelve days after cells were injected, when the tumors were in the exponential phase of growth, five mice were injected with 800 µg EM164 and five were injected with 800 µg of an isotype-matched control antibody. Treatments were continued for 4 weeks. Tumor growth is shown as the tumor volume over time. Points, average volume (n = 5 for each group); bars, SE. Statistical significance of inhibition of tumor growth in EM164-treated animals compared with control antibody-treated animals was assessed using a t test. **, P < 0.005; *, P < 0.03, significant inhibition of tumor growth. Tumor growth experiments were repeated thrice and a representative experiment is shown. C, EM164 down-regulated IR in xenograft tumors. At the end of the experiment in (B), mice were sacrificed and tumors were harvested. Tumor extracts (100 µg) from three mice treated with the control antibody (lanes 2-4) and three mice treated with EM164 (lanes 5-7) were subjected to SDS-PAGE and immunoblotted for IGF1R and IR. MCF-7 cell lysate (40 µg) was run as a control (lane 1). Bottom graph, quantitation of the average levels of IR in the tumors expressed as percentage of the level in control antibody (C-Ab)–treated tumors. D, EM164 inhibited IGF-I–mediated activation of IGF1R in MCF-7 xenograft tumors. Female athymic mice bearing MCF-7 xenograft tumors were treated i.p. either with 800 µg EM164 (lane 6) or PBS (lanes 4 and 5). Twenty-four hours later, mice were injected i.p. with 200 µg IGF-I in a volume of 200 µL (lanes 5 and 6) or PBS (lane 4). Thirty minutes later, mice were sacrificed and tumors were harvested. The tumors were snap frozen, homogenized, and 100 µg extracts were immunoblotted for phosphotyrosine (top), phospho-Akt (middle), and total Akt (bottom).
found in lipid rafts. Thus, the ability of antibodies against IGF1R to down-regulate holo-IR may be due to the localization of IR in the same membrane compartment as some of the antibody-IGF1R complexes undergoing endocytosis. This might represent only a small fraction of the total IR and explains why the antibodies down-regulate IGF1R more efficiently than IR as IGF1R antibodies can down-regulate IGF1R in and out of the lipid rafts. This mechanism is also supported by our finding that reagents that disrupt lipid rafts inhibit down-regulation of IR by EM164 and scFv-Fc. Both filipin III and methyl-β-cyclodextrin reversed down-regulation of IR by EM164 and scFv-Fc. In contrast, these agents did not have a significant effect on down-regulation of IGF1R by EM164 and scFv-Fc as these antibodies should bind IGF1R in any cell surface membrane compartment. Other growth factor receptors have been shown to be internalized by multiple pathways. Thus, transforming growth factor-β receptors can be internalized via the clathrin-dependent endocytic and lipid raft caveolar pathways and reside in both lipid raft and nonraft membrane domains (47). Similarly, IFN-γ receptors have been reported to be internalized by both clathrin-dependent and caveolar endocytic pathways (48).

We have also shown that expression of both IGF1R and IR is required for scFv-Fc or EM164 to down-regulate IR and thus inhibit insulin signaling in breast cancer cells. This characteristic of these antibodies may be highly advantageous as insulin target organs, such as liver, which lack IGF1R, may be unaffected by antibodies directed against IGF1R. Furthermore, in HepG2 cells, which are a hepatoma cell line widely used to study insulin effects, scFv-Fc and EM164 did not down-regulate IR (data not shown). This further supports the notion that in liver cells anti-IGF1R antibodies should not disrupt insulin function.

Unlike HER2, which is amplified in cancers, IGF1R and IR are ubiquitously expressed and the effects of systemic therapy targeting IGF1R are unknown. However, the existence of human populations with low levels of IGF-I (49) provides hope that inhibition of IGF effects may be tolerated without morbidity. Furthermore, humans with IGF1R mutations have been described (50), suggesting that disruption of IGF1R can be tolerated. It is true that maximum benefit would be derived from targeting all receptors responsible for the action of IGF-I and IGF-II, and that would include IR-A, then unbalancing glucose homeostasis would not be a desirable effect of anti-IGF1R therapy especially if it has to be given over a long period of time. In that respect, we have shown that antibodies against IGF1R may be efficient at inhibiting mitogenic effects mediated by insulin and IGF-II via the IR only in tumor cells without affecting IR in target organs such as the liver. Currently, an antibody against IGF1R is in clinical trial for multiple myeloma (http://www.mayoclinic.org/multiple-myeloma/clinicaltrials.html or http://www.moffitt.usf.edu/about_moffitt/publications/clinical_trials_update/nbcmonths/2004s4.pdf) and others will soon be in clinical trials for solid tumors. In conclusion, it is a certainty that anti-IGF1R reagents will be tested in human cancer. Understanding the key targets, mechanism of action, and toxicities associated with the different types of reagents will be required to most appropriately identify patients who could benefit from such a therapy. Identifying the role of IR in solid tumors needs to be more completely defined.

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Down-regulation of Insulin Receptor by Antibodies against the Type I Insulin-Like Growth Factor Receptor: Implications for Anti–Insulin-Like Growth Factor Therapy in Breast Cancer


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