Human Monoclonal Antibodies Targeting Nonoverlapping Epitopes on Insulin-like Growth Factor II as a Novel Type of Candidate Cancer Therapeutics

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Abstract

Soluble ligands are important targets for therapy of cancers and other diseases. Therapeutic monoclonal antibodies (mAb) against such ligands block their interactions with corresponding receptors but do not enhance their removal from the circulation and can increase their half-lives because of the long half-lives of the antibodies. We have hypothesized that mAbs targeting two or more nonoverlapping epitopes on the same ligand could form oligomeric antibody–ligand complexes that can bind to cells expressing Fc gamma receptors (FcγRs) with high avidity leading to their fast and irreversible removal from the circulation. Insulin-like growth factor II (IGF-II) is an example of such ligands and an important target for human cancer therapy. We identified two mAbs, m610.27 and m630.3, which bound to nonoverlapping epitopes on IGF-II with nanomolar affinity, and generated a bispecific antibody, m660. m660 inhibited the interaction of human IGF-II (hIGF-II) with the human breast cancer cell line MCF-7, hIGF-II–mediated IGF receptor type I and insulin receptor phosphorylation, and cell growth. In the presence of hIGF-II, large complexes of m660 were formed that bound to FcγRII-expressing BJAB cells much more efficiently than the monospecific antibody–hIGF-II complexes and were presumably phagocytosed by phorbol 12-myristate 13-acetate–stimulated macrophage-like U937 cells. A mixture of m610.27 and m630.3 exhibited similar properties. To our knowledge, these mAbs are the first reported to target nonoverlapping epitopes on a cancer-related ligand and could represent a novel class of candidate therapeutics against cancers. This approach could also be used to irreversibly eliminate other disease-related soluble ligands.

Introduction

Insulin-like growth factors (IGF), IGF-I and IGF-II, are circulating small soluble ligands (1, 2). They bind to IGF receptor type I (IGF-IR) and activate multiple intracellular signaling pathways resulting in cell proliferation, survival, differentiation, and transformation. IGF-II also binds to insulin receptor (IR), primarily A isoform (IR-A), with high affinity, as well as to hybrid receptors containing IGF-IR and IR chains. Elevated expression of these receptors and/or ligands has been detected in some cancer tissues such as human breast carcinomas and linked to their pathogenesis. Therefore, the IGF-mediated pathways are attractive targets for cancer therapy. Small-molecule tyrosine kinase inhibitors and monoclonal antibodies (mAb) against IGF-IR have shown benefits in human clinical trials (1). However, resistance to the IGF-IR–directed agents has developed (3). A possible mechanism is the involvement of IR when the IGF-IR–mediated pathways are blocked. Thus, targeting both IR and IGF-IR may be necessary to completely inhibit the signal transduction mediated by IGF-II. Although IR is also functionally important for glucose homeostasis, targeting IGF-II may be an ideal strategy, which could leave the insulin–IR interactions unaffected. Recently, several mAbs specific to IGF-II or cross-reactive against IGF-I and IGF-II have been identified that potently inhibit the growth and migration of human cancer cells in vitro and in vivo (4–8).

Unlike the antibodies against receptors, many of which inhibit tumor growth by modulating ligand–receptor interactions and by receptor downregulation, ligand-specific antibodies could act as carrier proteins and help ligands evade some clearance mechanisms such as renal filtration and proteolytic digestion because of the long half-lives and high stability of antibodies (9, 10). The dissociation of immune complexes would allow for slow release of ligands that continue to function. This possibility has been evidenced by previous studies on cytokines showing that murine mAbs prolonged the serum half-life and bioactivity of human interleukins in mice.
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although they completely neutralized the interleukins in vitro (10–12). Therefore, strategies to eliminate ligands efficiently and irreversibly are desirable to improve the inhibitory activities of antibodies.

An antigen with 2 or more nonoverlapping antibody-binding sites is likely to cross-link antibodies, resulting in large immune complexes that can be cleared as a result of activation of conventional immune clearance mechanisms such as complement deposition and Fc gamma receptor (FcγR)-mediated internalization and degradation (13). In this study, we describe the generation and characterization of 2 human mAbs, m610.27 and m630.3, targeting nonoverlapping epitopes on IGF-II, and a bispecific antibody, m660, based on them. m660 not only inhibited IGF-II–mediated receptor phosphorylation and cancer cell growth but also formed large soluble immune complexes with IGF-II, which were presumably phagocytosed by phorbol 12-myristate 13-acetate (PMA)-stimulated macrophage-like U937 cells, suggesting the same possibility in vivo.

Materials and Methods

Cells, plasmids, soluble ligands, antibodies, and phagocytosis inhibitors

The BJAB cells and U937 cells (clone P) were a gift from Anu Puri (National Cancer Institute, Frederick, MD, USA). The 293 free style cells, human insulin, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD64 (FcγRI), CD32 (FcγRII), and CD16 (FcγRIII) antibodies, and streptavidin–PE conjugate were purchased from Invitrogen. Mature human IGF-I (hIGF-I) and IGF-II (hIGF-II), mouse IGF-II (mIGF-II), and IGF-binding proteins (IGFBP) were purchased from R&D Systems. A precursor form of hIGF-II (long hIGF-II) was produced in our laboratory. Horseradish peroxidase (HRP)-conjugated mouse anti-FLAG tag antibody, HRP-conjugated goat anti-human IgG (Fc-specific) antibody, HRP-conjugated rabbit anti-c-Myc tag antibody, FITC-conjugated goat F(ab′)2 anti-human IgG (Fc-specific) antibody, and cytochalasin D were products of Sigma-Aldrich.

Panning and screening of a human engineered antibody domain (eAd) library

To select antibodies noncompeting with a previously reported mAb m610, biotinylated hIGF-II was used to pan a large (size, 2.5 × 1010) phage-displayed human eAd library (17) according to the previously described protocol (18). Clones that specifically bound to hIGF-II were identified from the 4th round of panning using soluble expression-based monoclonal ELISA (semELISA) as described previously (19).

Construction, panning, and screening of randomly mutagenized and light chain–shuffled libraries

To affinity mature m630, a phage-displayed library of m630 mutants (size, 109) was constructed. To introduce point mutations, we conducted random DNA mutagenesis with the Gene-Morph PCR Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. m630 gene fragments with mutations were PCR amplified by using m630-encoding plasmid as a template and primers m36F1 (5′-TGGTTCCTACCTACCGGCGCCGCGGTGCCCGCCTCGAAGGGTGCAGCAGCTTGCCCTCCGCTCCGCTCAGGAGGTGGCCTGGCAGCTGTGAGTGG-3′; sense) and bnIgG20L3, 5′-CTTCTACAGATGCCACTTGGCTGAGAGAAAGACAC-3′ (antisense); m36R1, 5′-CACTCTGGTACGCGGACCTGACTCAGGAGGGAGGTGGTTTCTCT-3′ (sense); and H4, 5′-AGAGCCACCTCCGGCCGACCCGCGGCGCTCAGGTGGCTGTTTCTCTCTACACTCAGGAGGGAGGTGGTTTCTCT-3′ (antisense). The PCR products were gel-purified, digested with SfiI, and gel-purified again. The purified fragments were then cloned into pComb3X linearized by SfiI. A phage library was prepared by electroporation of Escherichia coli (E. coli) strain TGI electroporation-competent cells (Stratagene) with desalted and concentrated ligation, as described previously (17). The phage library was panned against hIGF-II coated on 96-well plates and screened for higher affinity binders using monoclonal phage ELISA (mTE-LISA) as described previously (21).

Cloning of fusion proteins

The following primers were used: m36F, 5′-TGGTTCCGCTACCCTCGGCCACGCGGTGGCTG-3′ (sense); m36R1, 5′-GTCATTGTTTGGCCCGCTCGAAGGGTGCAGCAGCTTGCCCTCCGCTCCGCTCAGGAGGTGGCCTGGCAGCTGTGAGTGG-3′ (antisense); m36.4L4, 5′-GGAGTGG-3′ (antisense); CLF, 5′-CTTCTACAGATGCCACTTGGCTGAGAGAAAGACAC-3′ (sense); and HISR (5′-CACTCTGGTACGCGGACCTGACTCAGGAGGGAGGTGGTTTCTCTCTACACTCAGGAGGGAGGTGGTTTCTCT-3′; antisense). The PCR products were gel-purified, digested with SfiI, and gel-purified again. The purified fragments were then cloned into pComb3X linearized by SfiI. The new library was panned against hIGF-II coated on 96-well plates and screened for higher affinity binders using monoclonal phage ELISA (mTE-LISA) as described previously (21).

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heavy and light chains using protocols and reagents similar to those we used previously (5). Briefly, the heavy chain variable region was first cloned into pDR12 via XhoI and SacI sites. The light chain sequence was then cloned into pDR12 via HindIII and EcoRI sites. To clone m630.3Fc, m630.3 gene fragment was PCR-amplified with primers m36F and m36R1, digested with SfiI and Apal, and cloned into pSecTagB-Fc. To generate the bispecific antibody m660, the heavy chain leader peptide gene fragment (Hleader) and the single-chain variable fragment (scFv) of m610.27 were PCR-amplified with primer pairs bnIgG20H1/bnIgG20H3 and m610.27H2/m610.27H4, respectively. Hleader was joined to scFv m610.27 by overlapping PCR conducted in a volume of 50 μL by using both templates (in the same molarities) for 7 cycles in the absence of primers and 15 additional cycles in the presence of primers (500 pmol/L of bnIgG20H1 and m610.27H4). The product was digested with XhoI and SacI, and cloned into pDR12. To fuse m630.3 to the N terminus of the human IgG1 light chain constant region, the light chain leader peptide (Lleader), m630.3, and the human IgG1 kappa light chain constant region (CK) were amplified by PCR with primer pairs bnIgG20L1/bnIgG20L3, m36.4L2/m36.4L4, and CLF/bnIgG20L4, respectively. Lleader was linked to m630.3 and CK by overlapping PCR with primers bnIgG20L1 and bnIgG20L4 as described earlier. The Lleader–m630.3–CK fragment was then digested with EcoRI and HindIII, and cloned into the pDR12 construct containing scFv m610.27.

Protein expression and purification
Fabs and eAds were expressed and purified from E. coli HB2151 periplasm, and all fusion proteins were produced in 293 free style cells, as described previously (22).

Size exclusion chromatography
Size exclusion chromatography was done as described previously (23) except a Superdex200 10/300 GL column (GE Healthcare) was used in this study.

ELISA
ELISA was done as described previously (22). Bound Fabs and eAds were detected by HRP-conjugated mouse anti-FLAG tag antibody. The antibody–Fc fusion proteins binding to IGF-II directly coated on 96-well plates were detected by HRP-conjugated goat anti-human IgG (Fc-specific) antibody. In the competition ELISA with IGF-II captured by IgGs, bound m630.3Fc was detected by HRP-conjugated rabbit anti-c-Myc tag antibody. In the competition ELISA with IGF-II captured by IGFBPs, bound antibodies were detected by HRP-conjugated goat anti-human IgG (Fc-specific) antibody. The half-maximal binding (EC50) was calculated by fitting the data to the Langmuir adsorption isotherm.

Flow cytometry (FACS)
To measure the interactions of IGF-II with MCF-7, BJAB and U937 cells, biotinylated IGF-II was mixed with or without antibodies and added to approximately 10⁸ cells in 200 μL PBS containing 0.1% BSA (PBSA). After 1-hour incubation on ice, the cells were washed twice and resuspended in 200 μL PBSA, and 1 μL streptavidin–PE conjugate was added. Following 30-minute incubation on ice, the cells were washed twice and then subjected to fluorescence-activated cell sorting (FACS). For detection of the expression of FcγRII and FcγRI, 10¹ BJAB and PMA-stimulated U937 cells in 200 μL PBSA were mixed at different ratios (v/v) with FITC-conjugated mouse anti-human CD32 (FcγRII) and CD64 (FcγRI) antibodies, respectively, and incubated for 30 minute on ice. The cells were washed twice with 200 μL PBSA and then used for FACS analysis. Antibody binding to BJAB and U937 cells in the absence or presence of IGF-II was detected by FITC-conjugated goat F(ab’2) anti-human IgG (Fc-specific) antibody at a 1:200 dilution (v/v).

Phosphorylation assay
Inhibition of IGF-II–mediated receptor phosphorylation by the antibodies was measured as described previously (5).

Cell growth assay
Cell growth assay was done as described previously (5) except that live cells were determined by using the CellTiter-Glo® Luminescent Cell Viability Assay System (Promega) according to the manufacturer’s instructions.

Results
Selection and affinity maturation of IGF-II–specific mAbs
We identified previously a human mAb to IGF-II, m610, which potently inhibited IGF-IR and IR signaling pathways and cancer cell growth in vitro (5) and in a mouse model with implanted human bone (6). To further increase its affinity, a Fab library with shuffled light chains was generated and panned against hIGF-II. One of the mutants selected, m610.27, had a light chain derived from the same germline (IGKV1-39*01) but with 8 mutations compared with the original; its heavy chain remained the same as m610 that was closest to the family IGHV1-46*01 (Supplementary Fig. S1A). m610.27 in Fab format bound to hIGF-II with an EC50 of 5 nmol/L, 4-fold lower than that (20 nmol/L) of m610 (data not shown). We also identified a novel eAd, m630, by panning and screening one of our large phage-displayed eAd libraries against hIGF-II. m630 did not compete with m610 in binding to hIGF-II and mIGF-II in ELISA-based assays (data not shown). m630 was affinity matured by panning and screening of a phage-displayed library generated by random mutagenesis with error-prone PCR. Four m630 mutants, designated m630.1, m630.3, m630.4, and m630.9, were selected (Supplementary Fig. S1B). The highest affinity binder, m630.3, bound to hIGF-II with an EC50 (2 nmol/L) 25-fold lower than that (50 nmol/L) of m630 (data not shown).
Generation and characterization of monospecific and bispecific IgG1-like mAbs

Fab m610.27 was converted to an IgG1 (Supplementary Fig. S2A). A fusion protein, m630.3Fc, was made by attaching m630.3 to the N terminus of a human IgG1 Fc via a hinge (Supplementary Fig. S2B). An IgG1-like bispecific antibody, m660, was generated by fusing scFv m610.27 and m630.3 to the N termini of the heavy and light chain constant regions of a human IgG1, respectively, via a linker composed of 3 repeats of the G4S motif (Supplementary Fig. S2C). IgG1 m610.27, m630.3Fc, and m660 were well expressed and purified from 293 free style cell culture supernatants with yields of 5.5, 12, and 2.0 mg/L, respectively. They migrated on a SDS-PAGE under reducing and nonreducing conditions with apparent molecular weights (aMWs) comparable with their calculated molecular weights (cMWs) or slightly higher because of glycosylation (Supplementary Fig. S2D).

Binding activity and specificity of the antibodies were analyzed by ELISA. m630.3Fc bound to hIGF-II directly coated on 96-well plates with an EC50 (0.6 nmol/L) about 6-fold lower than that (3.5 nmol/L) of IgG1 m610.27 (Fig. 1A). It was cross-reactive against hIGF-I although very weakly while no significant binding of IgG1 m610.27 to hIGF-I was observed at a concentration up to several μmol/L. Neither m630.3Fc nor IgG1 m610.27 interacted measurably with human insulin. Both antibodies bound strongly to mIGF-II with an EC50 of 0.7 nmol/L for m630.3Fc and 8 nmol/L for IgG1 m610.27 (Fig. 1B). As expected for noncompeting antibodies, m630.3Fc bound to the hIGF-II (Fig. 1C) and mIGF-II (Fig. 1D) captured by IgG1 m610.27. In contrast, it did not bind to the IGF-II captured by IgG1 m708.5 (24), which is a human mAb cross-reactive to hIGF-I and hIGF-II, suggesting a competitive binding of m630.3Fc with IgG1 m708.5. m660 bound to hIGF-II with an EC50 comparable to that of m630.3Fc and 3-fold lower than that of IgG1 m610.27 (Fig. 1E). To find out whether the lack of additional avidity effects of m660 could be because of the possible inaccessibility of one of the antibody epitopes, we used long...
hIGF-II, which provides larger surface areas for coating. m660 exhibited stronger binding to long hIGF-II than both monospecific antibodies (Fig. 1F). We further tested the possible interaction of the antibodies with IGF-II/IGFBP complexes, which represent >90% of the forms of IGF-II in humans. m630.3Fc showed no detectable binding to the hIGF-II captured by IGFBP2 (Fig. 1G) or IGFBP3 (Fig. 1H), which were coated on 96-well plates. In contrast, IgG1 m610.27 strongly bound to the captured hIGF-II with EC\textsubscript{50} comparable to that of the antibody with hIGF-II directly coated on 96-well plates (Fig. 1A), suggesting that the antibody epitope does not overlap with the IGFBP2- and IGFBP3-binding sites. m660 displayed a decreased binding activity against the captured hIGF-II compared with IgG1 m610.27.

Inhibition of hIGF-II binding, receptor phosphorylation, and cancer cell growth

Signaling mediated by IGF-IR and IR is initiated by binding of their ligands. To test whether our antibodies could efficiently block the binding, we incubated MCF-7 cells, which are known to express high levels of IGF-IR, with 1 nmol/L hIGF-II in the absence or presence of the antibodies at different concentrations. At 2 nmol/L antibody concentration, m630.3Fc did not show any significant inhibition in a flow cytometry–based assay (Fig. 2). IgG1 m610.27, m660, and IgG1 m610.27 mixed with the same molar concentration of m630.3Fc completely inhibited the binding. m660 at 1 nmol/L still gave 100% inhibition whereas reduced inhibitory activity was observed with IgG1 m610.27 alone or in combination with m630.3Fc at the same concentration.

Upon ligand binding, IGF-IR undergoes phosphorylation on tyrosine residues of the 2 β-subunits. To find out whether the antibodies could inhibit the transmembrane signaling mediated by IGF-IR, we measured the receptor phosphorylation in MCF-7 cells. Although the serum concentration of free IGF-II in healthy individuals is approximately 2.3 nmol/L according to a previous study (25) and the half-maximal effective dose (EC\textsubscript{50}) of IGF-II to mediate receptor phosphorylation could be even lower, we used 5 nmol/L or higher IGF-II concentrations in this and our previous studies (5, 24) because the levels of free IGF-II in some patients with cancer could be higher than those in healthy individuals. MCF-7 cells kept in serum-free DMEM displayed nondetectable phosphorylation of IGF-IR (Fig. 3A). After incubation with 5 nmol/L hIGF-II for 30 minutes, the phosphorylation of IGF-IR was readily detected. In the presence of 5 nmol/L m660, however, the phosphorylation was completely inhibited. IgG1 m610.27
and a mixture of IgG1 m610.27 with m630.3Fc at 5 nmol/L also gave strong but incomplete inhibition. m630.3Fc showed no obvious inhibitory activity at the same concentration. When 1 nmol/L antibody concentration was tested, no significant inhibition was seen in all cases. In addition to IGF-IR, IGF-II also binds to and activates IR. As shown in Fig. 3A, m660, IgG1 m610.7, or IgG1 m610.27 plus m630.3Fc at 5 nmol/L completely blocked the effects of 5 nmol/L hIGF-II on IR phosphorylation. Marked inhibition was also observed when the antibody concentration was decreased to 1 nmol/L. m630.3Fc seems to slightly inhibit IR phosphorylation but its effect was not concentration-dependent.

To find out whether the antibodies could inhibit cell proliferation, we used MCF-7 cells in a cell growth assay. m630.3Fc at a concentration of 200 nmol/L did not measurably affect the growth of the cells incubated with 10 nmol/L hIGF-II (Fig. 3B). By comparison, m660 (P < 0.001), IgG1 m610.27 alone (P = 0.017) or mixed with m630.3Fc at the same concentration (P = 0.002) exhibited potent inhibitory activity with IC50s of 1.6 to 8 nmol/L. However, none of the 3 groups was significantly better than the others (P > 0.05).

Formation of large soluble m660–IGF-II complexes and enhanced interaction with FcγRII-expressing cells

Multivalent antibody–antigen interactions can induce formation of large immune complexes that either precipitate or remain soluble, depending on the degrees to which antigens are cross-linked by antibodies. To find out whether large m660–IGF-II complexes could be formed, m660 was mixed with hIGF-II at different molar ratios and analyzed by size exclusion chromatography. m660 alone was monomeric with aMW of approximately 180 kDa (Fig. 4). With hIGF-II at the same molar concentration, about one-half of m660 eluted as a dimer with aMW of approximately 380 kDa; a small percentage of the antibody was trimerized and eluted at a position corresponding to an aMW of 580 kDa. When the m660:hIGF-II ratio was changed to 1:2, the antibody monomer quantity decreased significantly and larger amounts of trimer were observed. With a further increase of the amount of hIGF-II (ratio 1:4 or 1:8), almost all the antibody was in a trimeric state. To test whether the presence of IGFBP would affect the formation of the complexes, we used a mixture of m660, hIGF-II, and IGFBP3 at a 1:4:2 ratio. IGFBP3 was
first mixed with hIGF-II. Following 30-minute incubation on ice, m660 was then added. The result showed that about 30% of the antibody was in a dimeric state while the rest eluted as a monomer (Fig. 4). The elution peak corresponding to the antibody dimer showed a shift compared to those in the absence of IGFBP3, suggesting the engagement of IGFBP3 in the complexes. In all cases, no precipitation was observed. Similar complexation was observed when m660 was replaced with IgG1 m610.27 plus m630.3Fc at an equal molar concentration (data not shown). However, when hIGF-II was mixed with either IgG1 m610.27 or m630.3Fc, they eluted at positions similar to those for the antibodies alone, suggesting no cross-linking between the monospecific antibodies and hIGF-II (data not shown).

Large antibody–antigen complexes are able to trigger immune clearance mechanisms with efficient recognition of multiple antibody Fc domains by effector cells expressing FcγRs because of avidity effects. To determine the effects of m660 oligomerization through cross-linking of IGF-II, we used as a model BJAB cells, a human B cell line. According to a previous study (26), B cells express only FcγRII, which weakly (equilibrium dissociation constant of approximately 1 μmol/L) binds to naturally occurring antibodies with monovalent Fc. Therefore, the differences in binding between m660–IGF-II and monospecific antibody–IGF-II complexes could be better observed by using this cell line. Our results from flow cytometry analysis confirmed the expression of FcγRII using the FITC-conjugated mouse anti-human FcγRII and FcγRIII antibodies (Fig. 5A). We also found that BJAB cells did not significantly interact with hIGF-II at a concentration up to 1 μmol/L (Fig. 5B). Monospecific antibody IgG1 m610.27 or m630.3Fc showed weak interactions with BJAB cells and their binding was not or only slightly altered when hIGF-II was added at a 1:2 molar ratio (antibody:BJAB cells) (Fig. 5C). As expected, the presence of hIGF-II at a 1:4 ratio dramatically enhanced the interactions of m660 or IgG1 m610.27 plus m630.3Fc at an equal molar concentration with the cell line (Fig. 5C). hIGF-II did not significantly affect the control antibodies IgG1 m102.4, a human mAb specific for henipaviruses (21), plus m36h1Fc, an antibody–Fc fusion protein against HIV-1 (22), in binding to BJAB cells.

**Presumably enhanced phagocytosis of m660–IGF-II complexes by macrophage-like U937 cells**

Macrophage-mediated phagocytosis is one of the mechanisms for clearance of pathogens, cellular debris, and large immune complexes in vivo. To see whether m660–IGF-II complexes could activate this mechanism, we used PMA-stimulated macrophage-like U937 cells, which express considerable levels of FcγRII (Fig. 6A). The cells strongly interacted with hIGF-II alone at various concentrations suggesting the expression of IGF-IR and/or IR on the cell surface (Fig. 6B). To detect the interactions of the antibodies with or without hIGF-II, we...
used an FITC-conjugated goat F(ab’)2 anti-human IgG (Fc-specific) antibody. In a flow cytometry-based assay, in which the antibodies were incubated with the cells on ice for 1 hour, the presence of 20 nmol/L hIGF-II slightly decreased or did not alter the binding of 10 nmol/L IgG1 m610.27 and m630.3Fc to the cells (Fig. 6C). In contrast to the enhanced interactions with BJAB cells, however, increased binding of m660 at 10 nmol/L to U937 cells was not observed in the presence of 40 nmol/L hIGF-II. In the other assay, we incubated the antibodies with the cells at 37°C for 2 hours where phagocytosis is supposed to be more efficient than at 4°C. Although hIGF-II did not affect the interactions of the monospecific antibodies, m660 binding strength was decreased by approximately 80%, indicating internalization of the receptor/IGF-II/antibody complexes. To further assess the possibility of phagocytosis, we applied 50 μmol/L cytochalasin D, a phagocytosis inhibitor, to both thermal conditions. As expected, binding of m660 was increased at 4°C and partially restored at 37°C whereas cytochalasin D impaired the monospecific antibody binding.

Discussion
Soluble ligands are important targets for therapy of many diseases. In this study, we used IGF-II as a model to test our hypothesis that mAbs targeting nonoverlapping epitopes on a single ligand molecule are capable of driving the formation of large soluble immune complexes, which, because of avidity effects of multivalent Fc, could activate the immune clearance mechanisms in vivo leading to efficient and irreversible removal of ligands from the circulation.
Our results from the size exclusion chromatography showed that at an antibody-hIGF-II ratio of 1:4 or higher, almost all m660 was in trimeric states (Fig. 4). The level of antibody–antigen complexation could be affected by a number of other factors including the orientations of epitopes and paratopes, and the relative affinity of binding moieties. In humans, the complexation could be further affected by the existence of a large amount of IGF-II in complex with IGFBPs and acid labile subunit (ALS; ref. 27). Our results showed that the m610.27 arm of m660 was able to interact with IGF-II/IGFBP complexes (Fig. 1G and 1H) and an addition of IGFBP3 resulted in a reduced level of antibody cross-linking (Fig. 4). However, whether m610.27 would bind to the IGF-II/IGFBP/ALS binary complexes remains unknown. We did not test the effects of ALS because of the difficulty of obtaining soluble functional ALS. The m630.3 arm of m660 bound to free IGF-II only, which will ensure the formation of large immune complexes albeit to a lesser extent because of the low concentration of free IGF-II in 
vivo. However, the removal of free IGF-II would lead to a shift in the equilibrium between free and complexed IGF-II, which finally may result in complete irreversible depletion of total IGF-II. A note of concern is the possibility for side effects because of the complexation. It is known that immune complex deposition is a prominent feature of several autoimmune diseases such as the systemic lupus erythematosus (28). However, the serum concentration of IGF-II is relatively low leading to a relatively low rate of their formation so such possibility seems unlikely.

The binding avidity of large m660–hIGF-II complexes was shown by using BJAB cells, a human B lymphoma cell line (Fig. 5). Although mammalian B cells lack phagocytic capabilities, previous studies showed that they could preferentially uptake antigen–antibody complexes through FcγRIII-mediated endocytosis (29). Moreover, there is supporting evidence that B cells evolve from an ancestral phagocytic cell type (30). These findings indicate a previously unknown function of B cells in the innate immunity of mammals. Other cell types expressing FcγRII or FcγRIII may also have similar activities especially when large immune complexes with multivalent Fc are presented. Figure 6 showed that after incubation of m660–hIGF-II complexes with PMA-stimulated macrophage-like U937 cells at 37°C for 2 hours, the antibody binding strength was decreased by approximately 80%.
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whereas no significant decrease in binding was observed in the control groups (monospecific antibodies mixed with hIGF-II). Moreover, phagocytosis inhibitor cytochalasin D enhanced binding of m660-hIGF-II but not the monospecific antibody–hIGF-II complexes to the cells. These results suggest that the Fc receptors on the cells incubated with m660–hIGF-II complexes could be down-regulated presumably by co-internalization with the complexes.

One of the advantages of antibodies capable of irreversibly removing soluble ligands (iAbs) compared with reversible blocking antibodies (rAbs), could be that the former may function efficiently even with relatively low nmol/L affinity. Although the interactions between a receptor and iAbs competing for a ligand in vivo are complex, one could assume that following iAb administration, the total concentration of soluble ligand (in this case, IGF-II) should be gradually decreased to levels much lower than those achievable with rAbs. In contrast, rAbs tend to increase ligand concentrations because of the long half-lives of the antibodies, which serve as a reservoir. As a result, high pmol/L affinity is generally required for rAbs to efficiently block receptor–ligand interactions whereas low nmol/L affinity may be sufficient for iAbs efficacy. Our results showed that in vitro, the IGF-IR and IR phosphorylation mediated by hIGF-II at 5 nmol/L, a concentration higher than its average physiological concentration in healthy individuals (2.3 nmol/L), was 100% inhibited by the bispecific iAb m660 at 5 nmol/L final concentration (Fig. 3A). Typically, therapeutic antibody concentrations in the circulation are much higher (on the order of 1,000 nmol/L); therefore, we could expect that our antibodies would be capable of preventing signal transduction in vivo.

Further studies in animal models and humans are needed to find out whether m660 or a mixture of m610.27 with m630.3 could have potential as candidate human IGF-related cancer therapeutics. The antibodies can be used alone or in combinations with IGF-IR-directed agents and other antitumor therapeutics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: W. Chen, Y. Feng, D.S. Dimitrov
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References


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Human Monoclonal Antibodies Targeting Nonoverlapping Epitopes on Insulin-like Growth Factor II as a Novel Type of Candidate Cancer Therapeutics

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