Inhibition of Ligand-mediated HER2 Activation in Androgen-independent Prostate Cancer

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INTRODUCTION

Prostate cancer progression to androgen independence may be driven by several mechanisms. One such mechanism implicates overexpression and activation of HER2 receptor tyrosine kinase (1). In breast cancer, genomic amplification and overexpression of HER2 has been observed and is predictive of poor prognosis (2). The anti-HER2 antibody, Herceptin, which recognizes the extracellular domain of HER2 is used effectively as a therapeutic agent in breast cancer. Reports of HER2 expression in prostate cancers have been contradictory, and a potential role for HER2 amplification and overexpression in prostate cancer needs further clarification (3, 4). An alternate mechanism of HER2 activation is driven by ligand-mediated receptor activation. HER2, in complex with other members of the receptor family, EGFR, HER3, and HER4 is activated by ligand binding of the heterodimer complex (5, 6). Ligand activation of these receptor complexes drives cell proliferation and has been implicated in a variety of cancers (7–10). Anti-HER2 recombinant humanized monoclonal antibody (rhuMAb) 2C4. Furthermore, inhibition of tumor growth was observed in xenografts derived from 22Rv1 cells when treated with rhuMAb 2C4 in a dose-dependent manner. These studies provide a framework, both in vitro and in vivo, to examine the molecular mechanisms of ligand-driven HER2 activation in androgen-independent tumorigenesis.

MATERIALS AND METHODS

22Rv1 cells, deposited by Sramkoski et al. (11), were obtained from American Type Culture Collection (Manassas, VA). 22Rv1 cells were maintained in Ham’s F-12:high-glucose DMEM (50:50) supplemented with 10% heat-inactivated FBS and 2 mM l-glutamine. For FACS analysis, cells were detached with 5 mM EDTA in 150 mM NaCl, washed once with medium, and resuspended in 1% FBS/PBS. Cell suspensions were counted, adjusted to 1 × 10^6/ml and rotated, at 4°C for 1 h with 10 μg/ml 4D5 (anti-HER2), or 2F9 (anti-HER3), or 6C5 (anti-EGFR) monoclonal antibodies (14, Genentech, South San Francisco, CA) or assay buffer (1% FBS/PBS). Cells were washed, the cell pellets resuspended in assay buffer containing 15 μg/ml FITC-conjugated F(ab')2 fragment of goat antimouse IgG (Cappel/ICN, Aurora, OH) and incubated for 1 h at 4°C. After this incubation period, the cell suspensions were washed and resuspended in assay buffer for analysis using a FACSscan (Becton Dickinson, Bedford, MA). Cells expressing the specific receptors are represented as geometric mean fluorescence.

For immunoprecipitation experiments, 22Rv1 cells were serum starved for 4 h and incubated with 100 nM rhuMAb 2C4 (Ref. 14; Genentech, South San Francisco, CA) for 30 min at room temperature. Cells were stimulated with heregulin β1 (Genentech, South San Francisco, CA) at 1 nM for 15 min at room temperature. SKBR3 (American Type Culture Collection) and 22Rv1 cells. Cells were lysed with Tris-buffered saline and 1% Ipegal-630 and 1 mM of nitrocellulose membrane. HER2, EGFR, HER3, and phosphotyrosine were detected by immunoblotting with the following antibodies, MS-730 (NeoMarkers), SC-03 (Santa Cruz Biotechnologies, Santa Cruz, CA), and MS-310 (NeoMarkers), SC-8050 (Santa Cruz Biotechnologies) and 4G10 05-321 (Upstate, Waltham, MA), respectively. The antibodies were diluted in Tris-buffered saline/0.1% Tween 20 with 5% nonfat dry milk. Blots were detected by chemiluminescence, ECL Plus (Amersham, Piscataway, NJ).

Proliferation assays were performed with cells that were seeded into 96-well black microtiter plates at a density of 8000 cells/well in medium containing 10% FBS and were allowed to adhere overnight. The following day, the medium was removed and replaced with medium containing 0.1% FBS. After a 24-h period of serum starvation, the cells were treated with different concentrations of rhuMAb 2C4 for 1 h at room temperature, followed by the addition of either 1 nM heregulin or 1 nM TGF-α (Sigma). Cells were incubated for 4 days at 37°C. At the end of the incubation period, cell proliferation was measured using CellTiter-Glo Luminescent Viability assay (Promega, Madison, WI), and viable cells were reflected in relative luminescence units.

For xenograft studies, 1,000,000 22Rv1 cells were injected into 8-week-old female BALB/c nude mice (Charles River Labs, Wilmington, DE) in a 1:1 ratio of Matrigel (Collaborative Research, Bedford, MA) as described previously (11). When tumors reached ~100 mm^3, mice were randomly assigned to groups (n = 10) and were given i.p. injections of 100, 30, 10 mg/kg of rhuMAb 2C4, 100 mg/kg of isotype-matched control antibody, or vehicle control in a 100-μl volume. Injections and tumor measurements were carried out twice...
RESULTS

Because HER2 forms heterodimers with other EGFR/HER receptor family members, the AI cell line 22Rv1 was evaluated for expression of EGFR, HER2, HER3, and HER4. Lysates from 22Rv1 cells were run by SDS-PAGE and were Western blotted onto nitrocellulose. The blots were probed with specific EGFR/HER receptor antibody, and EGFR, HER2, and HER3 were detected (Fig. 1A). In contrast, HER4 was not detected (data not shown), as anticipated, based on previous reports (15). HER2 levels were low in 22Rv1 cells compared with the level of control cell line SKBR3 (Fig. 1A). A characteristic doublet of highly expressed HER2 was detected in SKBR3 cells as shown previously (16). The expression of EGFR/HER receptors was confirmed using FACS analysis, which corroborated the Western blot data (Fig. 1B).

22Rv1 cells were stimulated with heregulin or TGF-α, and cell proliferation was measured in response to ligand-mediated activation. Stimulation of serum-starved 22Rv1 cells with 1 nM heregulin showed increased cell proliferation compared with control (Fig. 2), whereas TGF-α did not mediate a similar proliferative response (data not shown). rhuMAb 2C4 inhibited cell proliferation, driven by 1 nM heregulin, in a dose-dependent manner (Fig. 2). In contrast, increasing concentrations of rhuMAb 2C4 alone did not affect cell proliferation. These observations suggest that rhuMAb 2C4 inhibits 22Rv1 cell proliferation driven by ligand-mediated HER2/HER3 activation. To directly examine the effects of rhuMAb 2C4 on HER receptor phosphorylation, 22Rv1 cells were treated with or without heregulin in the presence or absence of rhuMAb 2C4. Lysates were harvested as described in “Materials and Methods.” 22Rv1 cell lysate was immunoprecipitated with anti-HER2 antibody. Immunoprecipitates (IP) were run by SDS-PAGE, transferred and probed with antibodies to phosphotyrosine (P-Tyr), HER2, or HER3.

Fig. 1. A, EGFR/HER receptor expression in 22Rv1 cells observed by Western blot analysis. Lysate from 22Rv1 cells was subjected to SDS-PAGE and transferred to nitrocellulose. Blots were probed with antibodies that recognized the specified EGFR/HER receptors. Comparative Western blot of HER2 expression in 22Rv1 and SKBR3 cells. B, FACS analysis of EGFR/HER receptor expression in 22Rv1 cells. Specific antibodies that recognized EGFR, HER2, and HER3 were used to label 22Rv1 cells as described in “Materials and Methods,” and were counted by FACS.

Fig. 2. rhuMAb 2C4 inhibition of 22Rv1 cell proliferation stimulated by heregulin. Serum-starved 22Rv1 cells were treated with/without 1 nM heregulin (HRG) in the presence of increasing concentrations of rhuMAb 2C4, as described before. Dose-dependent inhibition by rhuMAb 2C4 of heregulin-stimulated cell proliferation is reflected as a decrease in relative luminescence.

Fig. 3. Inhibition of heregulin-mediated activation of HER2. 22Rv1 cells were treated with or without heregulin (HRG) in the presence or absence of rhuMAb 2C4. Lysates were harvested as described in “Materials and Methods.” 22Rv1 cell lysate was immunoprecipitated with anti-HER2 antibody, Immunoprecipitates (IP) were run by SDS-PAGE, transferred and probed with antibodies to phosphotyrosine (P-Tyr), HER2, or HER3.

Fig. 1. A, EGFR/HER receptor expression in 22Rv1 cells observed by Western blot analysis. Lysate from 22Rv1 cells was subjected to SDS-PAGE and transferred to nitrocellulose. Blots were probed with antibodies that recognized the specified EGFR/HER receptors. Comparative Western blot of HER2 expression in 22Rv1 and SKBR3 cells. B, FACS analysis of EGFR/HER receptor expression in 22Rv1 cells. Specific antibodies that recognized EGFR, HER2, and HER3 were used to label 22Rv1 cells as described in “Materials and Methods,” and were counted by FACS.

One million 22Rv1 cells, in a 1:1 ratio with Matrigel, were inoculated s.c. into BALB/c nude mice, and tumor growth was monitored. The histopathology of tumors revealed the growth of poorly differentiated prostatic adenocarcinoma. Immunohistochemical analysis...
showed low levels of HER2 expression (2+ HER2 by DAKO staining) in 22Rv1 tumor sections (data not shown). Once tumors were established, 100 mg/kg rhuMAb 2C4 or vehicle was administered to 10 mice/group, i.p., twice weekly for the duration of study. Inhibition of tumor growth was observed in rhuMAb 2C4-treated mice compared with the vehicle-treated control group (Fig. 4A).

To examine the dose response of rhuMAb 2C4 in these AI prostate tumors, 10, 30, and 100 mg/kg rhuMAb 2C4 were injected i.p., twice weekly into 10 mice/group, as described in the previous study. Iso-type-matched control antibody was also injected into 10 mice as a control group. As before, the highest dose group of rhuMAb 2C4 (100 mg/kg) demonstrated inhibition of tumor growth compared with the control group, and a dose response was observed in comparing the different treatments with 30 and 10 mg/kg rhuMAb 2C4 (Fig. 4B). These data show that rhuMAb 2C4 inhibited tumor growth in a dose-dependent manner. Collectively, our studies demonstrated that rhuMAb 2C4 inhibits ligand-driven receptor phosphorylation and cell proliferation of 22Rv1 tumor cells. These in vitro observations are extended in our in vivo analyses by rhuMAb 2C4 inhibition of tumor growth in this AI prostate tumor model.

**DISCUSSION**

The role of HER2 in AI prostate tumors has been controversial because conflicting reports of HER2 receptor overexpression have been reported. Unlike human breast cancers in which gene amplification and up-regulation of HER2 receptor is well documented, there is no clear evidence to suggest a similar correlation in AI prostate cancer. Craft et al. (1) demonstrate in vivo that overexpression of HER2 is correlated with AI prostate tumorigenesis, and they elucidate a possible mechanism for AI prostate cancer.

Our analysis explores a uniquely different mechanism through ligand-mediated HER2 activation, cellular proliferation, and tumorigenesis. The detection of low HER2 levels suggests that overexpression of HER2 does not drive 22Rv1 cell proliferation. Other investigators have reported similar expression patterns of HER2, HER3, HG4, and EGFR in the CWR22 and CWR22R parental tumors (15, 17), and our data for the derivative 22Rv1 are consistent with these observations. Our results show that heregulin mediates the activation of the HER2/HER3 receptor complex in these AI 22Rv1 cells. Moreover, we show that rhuMAb 2C4 blocks receptor phosphorylation, most likely through inhibition of heregulin-mediated HER2/HER3 receptor dimerization. We also demonstrate in vitro that heregulin-driven cell proliferation of 22Rv1 is inhibited by rhuMAb 2C4. HER3 harbors an inactive kinase domain, and ligand-mediated kinase activation is driven by HER2 in these heterodimer complexes. Tyrosine phosphorylation of HER2 immunoprecipitates, detected by the 4G10 antibody, recognizes phosphorylation of HER2 and HER3 in this complex. Our results show that rhuMAb 2C4 effectively blocks phosphorylation of the heterodimer complex.

This inhibition extends to our in vitro cell proliferation and in vivo analyses. We show inhibition of tumor growth with rhuMAb 2C4 treatment of 22Rv1 mouse xenografts compared with the control group. It is formally possible, although unlikely, that 2C4 rhuMAb inhibition of tumor growth may be attributable to general immune mechanism(s) still intact in the nude mouse. Investigation of anti-HER2 treatment has been carried out in the parental CWR22 and CWR22R xenograft tumor models (18–20). However, in vitro analysis of CWR tumors has been hampered by an inability to maintain and characterize these transplant model cells in culture. Extensive characterization by cytogenetic analysis shows the 22Rv1 cell line represents one hyperdiploid stem line from CWR22 tumor. Injection of 22Rv1 cells into a xenograft model shows formation of a poorly differentiated adenocarcinoma with irregular glandular appearance and elevated prostate-specific antigen (11). Our histopathology analyses of 22Rv1 tumor xenografts are in agreement with previous observations. The tumors express low levels of HER2 (data not shown), consistent with our in vitro observations of low HER2 expression by Western blot and FACS analysis. In conclusion, the 22Rv1 cell line offers the distinct advantage of studying ligand-activated HER2 activation and the prospect of anti-HER2 for therapeutic use in AI prostate tumors.

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