The HER2-Binding Affibody Molecule \((Z_{\text{HER2:342}})_2\)
Increases Radiosensitivity in SKBR-3 Cells

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Abstract

We have previously shown that the HER2-specific affibody molecule \((Z_{\text{HER2:342}})_2\) inhibits proliferation of SKBR-3 cells. Here, we continue to investigate its biological effects \(\textit{in vitro}\) by studying receptor dimerization and clonogenic survival following irradiation. We found that \((Z_{\text{HER2:342}})_2\) sensitizes the HER2-overexpressing cell line SKBR-3 to ionizing radiation. The survival after exposure to \((Z_{\text{HER2:342}})_2\) and 8 Gy \((S_{\text{8 Gy}} \, 0.006)\) was decreased by a factor four compared to the untreated \((S_{\text{8 Gy}} \, 0.023)\). The low HER2-expressing cell line MCF-7 was more radiosensitive than SKBR-3 but did not respond to \((Z_{\text{HER2:342}})_2\). Treatment by \((Z_{\text{HER2:342}})_2\) strongly increased the levels of dimerized and phosphorylated HER2 even after 5 minutes of stimulation. The monomeric \(Z_{\text{HER2:342}}\) does not seem to be able to induce receptor phosphorylation and dimerization or sensitize cells to irradiation.

Introduction

The tyrosine kinase receptor HER2 (ErbB2/neu) is one of four members of the epidermal growth factor receptor (EGFR) family. Abnormal expression and signaling of the receptor is associated with development and progression of several forms of cancer, and is also associated with enhanced invasiveness and resistance to chemotherapy and radiation \([1,2]\). This makes it an important cell-surface structure for development of targeting agents, both for therapy and imaging, or as a prognostic biomarker for e.g. trastuzumab therapy \([3]\).

While the other members of the EGFR family (EGFR, HER3 and HER4, also denoted as ErbB1-4) can be bound by many different growth factors (e.g. EGF and neuregulins), HER2 does not have any natural ligand. Nevertheless, HER2 is known as the most potent receptor and the preferred dimerization partner \([4,5]\). Activation of the receptors occurs through hetero- or homodimerization with another member of the EGFR family, resulting in trans-phosphorylation of tyrosine residues in the intracellular part of the receptor. These phosphorylation sites serve as initiation points for various signaling pathways leading to cellular processes such as proliferation, migration and apoptosis. The effect on downstream signaling, and hence the biological outcome, depends on the composition of the receptor pair and the identity of the ligand \([5]\).

Affibody molecules (Affibody®) are based on the 58 amino acid bundle of the Z domain of staphylococcal protein A. They are usually generated by phage display-based selection from libraries where 13 surface-exposed amino acids have been randomized. High affinity binders to a variety of proteins, e.g. insulin, EGFR and Amyloid-\(\beta\), have been identified \([6]\) and an imaging study on breast cancer patients showed promising results \([7]\). In a previous study, we have shown that \((Z_{\text{HER2:342}})_2\) inhibits proliferation of SKBR-3 \([8]\).

In this study we investigated the effect of the HER2-binding affibody molecule, \((Z_{\text{HER2:342}})_2\), in combination with external \(\gamma\)-radiation and found that \((Z_{\text{HER2:342}})_2\) sensitizes SKBR-3 cells to radiation. We have also studied \((Z_{\text{HER2:342}})_2\)'s ability to induce receptor phosphorylation and dimerization.

Materials and Methods

Cell Lines

The human breast cancer cell lines SKBR-3 and MCF-7 were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). SKBR-3 cells express approximately \(2-6 \times 10^6\) HER2 and \(10^5\) EGFR receptors per cell \([9,10]\). MCF-7 cells express low levels of both EGFR and HER2, about \(10^4\) receptors per cell, and high levels of HER3 \([11]\).

SKBR-3 and MCF-7 cells were cultivated in RPMI1640 culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamin, 100 IU/mL penicillin and 100 \(\mu\)g/mL streptomycin (Biochrom KG, Germany). For MCF-7, Non-Essential Amino Acids (1\(\times\)) and Sodium Pyruvat (1 mM) were also added to the culture medium.

Reagents

The HER2-specific antibody was from Santa Cruz Biotechnology (Santa Cruz, USA), and the EGFR specific antibody from Cell Signaling Technology (Boston, USA). The antibody directed to \(\beta\)-actin was from Sigma-Aldrich (Saint Louis, USA). Anti-mouse and
HER2-Binding (Z342)2 Increases Radiosensitivity

Statistical Methods
For the survival assays, GraphPad Prism 4 (GraphPad Software, Inc., San Diego, USA) was used to perform an unpaired t-test. A P-value <0.05 was considered significant.

Results

Binding Assay
To verify that (ZHER2:342)2 binds to SKBR-3 cells, real-time binding of 125I-labelled affibody was measured using LigandTracer. Cell binding at two different concentrations and retentions were measured. As can be seen in Figure 1A, (ZHER2:342)2 shows an increasing binding trace with increasing concentration. Using these data, in combination with data acquired from off-rate at the higher concentration (Figure 1B), kinetic evaluation of (ZHER2:342)2 was estimated with TraceDrawer. The Kp was calculated to be 6 pM, using a 1:1-binding model. Due to limitations in the assay, concentrations that rendered ligand depletion were used. However, during kinetic evaluation the software could adjust for ligand depletion and the obtained curve fit conform well with measured data.

Receptor Dimerization and Phosphorylation
To determine if binding of (ZHER2:342)2 could influence the receptor activation state, we investigated the phosphorylation and dimerization of HER2. Since HER2 can form heterodimers with other members of the EGFR family, we also studied EGFR and HER3. Expression of HER4 is too low in the current cell lines to be of interest [11]. Analysis of dimerization was done in SKBR-3 and MCF-7 cells by crosslinking proteins with BS3 [Bis[SulfosuccinimidyI] suberate] before cell lysis. The monomeric and dimeric receptors were then separated by SDS-PAGE and detected by western blot. As can be seen in Figure 2A, lane 2–5, treatment by (ZHER2:342)2 (0.2 μM) increases phosphorylation of HER2. As shown in Figure 3B, no significant difference in phosphorylation of HER2 was detected with (ZHER2:342)2 (0.2 μM) versus control (0.2 μM) for SKBR-3 cells. In contrast, treatment with the monomeric affibody molecule, Z342, did not result in any receptor dimerization (lane 8). Except for a tendency for increased dimeric EGFR upon EGF stimulation, no large effects on EGFR dimerization could be detected (Figure 2B). HER3 was only detected in monomeric form in SKBR-3 cells (data not shown). In MCF-7 neither EGFR nor HER2 could be detected and HER3 was only detected in monomeric form (data not shown). Phosphorylation of the receptors was measured by ELISA. Unstimulated cells had low baseline of phosphorylated EGFR, HER2 and HER3. The only exception was as expected high levels of p-HER2 in SKBR-3. In accordance with a previous result by western blot [8], HER2 was phosphorylated by (ZHER2:342)2 in SKBR-3 cells (Figure 3A). This was also seen in MCF-7 cells, although decreased over time (Figure 3D). HER2 was not phosphorylated by EGF or NRG1-B1 in SKBR-3. In MCF-7, NRG1-B1 activated HER2 but not to the same extent as (ZHER2:342)2. As shown in Figure 3B, no significant difference in the levels of phosphorylated EGFR could be seen for SKBR-3 when incubated with (ZHER2:342)2. EGFR was stimulated only by EGF (10 nM for 5 min). For MCF-7 cells, the levels of p-EGFR were too low to draw any conclusions (data not shown). Treatment with (ZHER2:342)2 phosphorylated HER3 in SKBR-3 but not in MCF-7 cells (Figure 3C and E). Stimulation with 10 nM of NRG1-B1 for 5 minutes was used as a positive control for phosphorylation of HER3 (Figure 3C and E).

Survival Assays
To investigate if (ZHER2:342)2 could sensitize breast cancer cells to ionizing radiation we analyzed clonogenic survival on SKBR-3 and MCF-7 cells. As shown in Figure 4A, treatment of SKBR-3...
cells with 10 nM (ZHER2:342)2 decreased survival 4-fold after irradiation with 8 Gy compared to the irradiated control (S 8Gy 0.006 and 0.023 respectively, P < 0.01). After 4 Gy of radiation, the two groups did not significantly differ. For MCF-7 cells, treatment with (ZHER2:342)2 did not alter the survival, neither at 4 nor 8 Gy (Figure 4B). Survival for the non-irradiated control, plating efficiency (PE), was low for the SKBR-3 cell line; PE (ZHER2:342)2 0.05 and PE (control) 0.09, compared to PE (ZHER2:342)2 0.37 and PE (control) 0.38 for MCF-7 cells.

To verify the results, a different survival model was also used. Growth of SKBR-3 cells was followed after irradiation. This time, treatment with monomeric ZHER2:342 was also included. Figure 4C shows the number of cells after 28 days of cultivation after irradiation by 0, 2 and 6 Gy. In addition a clear effect of (ZHER2:342)2 was seen with this assay. At both doses the survival of SKBR-3 cells with 10 nM (ZHER2:342)2 decreased survival 4-fold after irradiation with 8 Gy compared to the irradiated control (S 8Gy 0.006 and 0.023 respectively, P < 0.01). After 4 Gy of radiation, the two groups did not significantly differ. For MCF-7 cells, treatment with (ZHER2:342)2 did not alter the survival, neither at 4 nor 8 Gy (Figure 4B). Survival for the non-irradiated control, plating efficiency (PE), was low for the SKBR-3 cell line; PE (ZHER2:342)2 0.05 and PE (control) 0.09, compared to PE (ZHER2:342)2 0.37 and PE (control) 0.38 for MCF-7 cells.

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Figure 1. The binding trace of 125I-(ZHER2:342)2 to SKBR-3 cells. The interaction was monitored in real-time at room temperature using LigandTracer Grey. A) In the first experiment two concentrations of 125I-(ZHER2:342)2 were added after each other. First, cells were incubated with 58 pM (ZHER2:342)2 and thereafter, when equilibrium had been reached, more substance was added to a total concentration of 174 pM. B) In the second experiment, the off-rate after equilibrium of 174 nM exposure was followed for 24 hours. Data evaluation and estimation of the kinetic parameter Kd were performed using the software TraceDrawer, using a one-to-one binding model with depletion correction. CPS (Counts per second).

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Figure 2. Receptor dimerization. SKBR-3 cells were treated with 10 nM (ZHER2:342)2 for 5, 15, 60 and 120 minutes. ZHER2:342 was used at 100 nM for 1 h. Untreated cells (−), and cells treated with EGF and nrg1-β1 for 15 minutes were used as controls. The SKBR-3 cells were cross-linked by 5 nM BS3 for 30 minutes before cell lysis. Total cell lysates were then subjected to SDS-PAGE and western blot with antibodies specific for HER2 (A) and EGFR (B), (ZHER2:342)2 treatment resulted in dimerization of HER2 even after 5 minutes of incubation. Numbers indicate minutes of stimulation.

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Figure 3. Phosphorylated levels of EGFR, HER2 and HER3, as measured by ELISA. The relative levels for the treated samples were determined by comparing those with the untreated control (∼) which was arbitrarily set as 1. Numbers indicate minutes of stimulation by (ZHER2:342)2–EGF and NRG1-β1 was used for 5 minutes. A–C) SKBR-3 cell line. D–E) MCF-7 cell line. Mean values and standard deviation from at least two independent measurements are presented.

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Figure 4. Survival after exposure to ionizing radiation. A–B) Clonogenic survival. Cells were treated with 10 nM (ZHER2:342)2 for 2 hours prior to irradiation and allowed to recover for 16 hours before reseeding. Mean values and standard deviations are calculated on at least 7 replicates. The linear quadratic model was used for curve fitting and an unpaired t-test to test for significance. A) SKBR-3. Inset; after irradiation with 8 Gy the cells treated with (ZHER2:342)2 had a significantly lower (P<0.01) survival than the control group. B) MCF-7 cells. Inset; after irradiation with 8 Gy the cells treated with (ZHER2:342)2 did not differ from the control group. C) Growth extrapolation method. SKBR-3 cells were treated with 16.6 nM (ZHER2:342)2, ZHER2:342 or left untreated for two hours, irradiated and then reseeded. The figure shows the number of cells normalized to the starting value in each group after 28 days of cultivation in normal cell culture medium.

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HER2-Binding (Z342)2 Increases Radiosensitivity

(ZHER2:342)2-treated cells was significantly lower. Extrapolation of the curve fits based on the data points where exponential growth has been reached, results in an estimate of the surviving fraction of cells that are responsible for the regrowth [14]. The surviving fraction calculated by this method was similar to the result from the clonogenic survival, rendering a significant decrease in survival compared to the control (S6Gy, 0.002 for (ZHER2:342)2, compared to S6Gy, 0.02 for the control (P<0.01)). Notably, the monomeric ZHER2:342 did not sensitize for radiation, on the contrary there seemed to be a protective effect at the lower dose.

It should be noted that MCF-7 cells are more radiosensitive than SKBR-3 cells, mean survival after 8 Gy was 0.6% compared to 2.3%. It has been suggested that increased HER2 expression is correlated with radioresistance [15,16].

Discussion

The HER2-binding affibody molecule (ZHER2:342)2 clearly binds to SKBR-3 cells (Figure 1). The KD was calculated to be 6 pM as many as 2–6 homodimers when overexpressed [18,19] and SKBR-3 cells have HER2 could be detected even in unstimulated SKBR-3 cells. By western blot, while ELISA, which is a more sensitive method, could detect phosphorylated receptor. The same difference in phosphorylation could be detected, the phosphorylation must have been induced by (Z HER2:342)2 can simultaneously bind two HER2 receptors and downstream signaling [8,13], it is possible that the dimeric phosphorylated HER2 has a dimerization arm that is always open [27], so the effect of the dimeric (ZHER2:342)2 did not sensitize for radiation, for example, cetuximab [13,22]. The mechanism for this action is not yet fully understood. However, it has been shown that the repair process for radiation-induced DNA damage can be effected by the EGFR-receptors, both by the receptor itself and also through its downstream signaling effectors phosphatidylinositol 3 kinase (PI3K)/Akt and mitogen-activated protein (MAP) kinases/Erk [23,24]. We have previously shown that the affibody molecules, trastuzumab and cetuximab can effect the level of phosphorylated Akt and Erk [8,13,25]. Thus, it is possible that the radiosensitizing effects of the HER2-binding agents are conferred through these signaling pathways. Since the effects of the monomeric ZHER2:342, and the dimeric (ZHER2:342)2 differ so much in terms of radiosensitizing, receptor dimerization and phosphorylation, and as we have previously shown, with regards to proliferation and downstream signaling [8,13], it is possible that the dimeric (ZHER2:342)2 can simultaneously bind two HER2 receptors and thereby induce homodimerization. Ligand-induced dimerization is common for the tyrosine kinase receptors, but for the EGFR family dimerization is considered as entirely receptor mediated. As a result of binding of a ligand, the receptor undergoes conformational changes that open up a dimerization arm and thus enable the receptor to dimerize [26]. HER2, on the other hand, has a dimerization arm that is always open [27], so (ZHER2:342)2 could in principle span the dimer interface and induce homo-dimerization by pulling two receptors together. (ZHER2:342)2 binds to the junction of domain III and IV on HER2, but not to the same site on domain IV as Trastuzumab [28].

To conclude, we have shown that the HER2-binding affibody molecule (ZHER2:342)2 significantly decreases survival after γ-irradiation. This radiosensitizing effect makes (ZHER2:342)2 interesting for therapy purposes.

Author Contributions

Conceived and designed the experiments: LE, JL, LG. Performed the experiments: LE, LG. Analyzed the data: LE, JL, LG. Contributed reagents/materials/analysis tools: JL, LG. Wrote the paper: LE.

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