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Targeting HER2 + breast cancer cells: Lysosomal accumulation of anti-HER2 antibodies is influenced by antibody binding site and conjugation to polymeric nanoparticles

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Humanized monoclonal antibodies (mAb) against HER2 are being engineered to treat cancer. We utilized phage-display technology to generate a novel anti-HER2 mAb (named 73JIgG) that binds an epitope of HER2 distinct from that of trastuzumab. Although these mAbs bind to the same cell surface receptor, they have different cell distribution profiles. After 3 h of incubation, almost 10% of the total 73JIgG reaches the lysosome compared to less than 3% of trastuzumab. Interestingly, 73JIgG disassociates from HER2 whereas trastuzumab remains bound to the receptor. Importantly, HER2 distribution is not affected by the antibody binding epitope, thus negating this mechanism as the reason for the difference in intracellular trafficking of 73JIgG versus trastuzumab. Each of trastuzumab and 73JIgG was chemically-modified with either a small molecule or polymeric nanoparticle to better understand the influence of conjugation on cellular localization. Relative to antibody alone, antibody–nanoparticle conjugates resulted in a higher concentration of antibodies in the lysosome whereas antibody–small molecule conjugates did not affect cell trafficking to the lysosome. Given the importance of lysosomal targeting, these results demonstrate the importance of understanding the influence of the antibody-conjugate on cell trafficking for ultimate optimization of treatment selection.

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1. Introduction

The human epidermal growth factor receptor type 2 (HER2, also referred to as HER2/neu or ErbB2) is a member of the HER family of transmembrane receptor tyrosine kinases. Elevated levels of HER2 in cancer are associated with poor prognosis, and thus HER2 has become a major therapeutic target [1–3]. Trastuzumab (Herceptin®) is a humanized IgG used clinically to treat patients with HER2-dependent tumors [4]. Several mechanisms have been proposed to explain trastuzumab (Tras) activity and trafficking [5–7]. The conserved Fc region is important in recruiting immune cells, leading to antibody-dependent cell-mediated cytotoxicity (ADCC). Tras may prevent HER2 dimerization, or inhibit shedding of the extracellular domain (ECD) of HER2, both of which result in decreased signaling [8,9]. Tras may also decrease the cell surface levels of HER2 by increasing endosomal/lysosomal destruction of the receptor [10]. Nevertheless, conflicting reports suggest that the majority of trastuzumab remains bound to HER2 and does not influence the internalization/recycling profile [11].

While the cell profile and mechanism of action of trastuzumab remain unclear, even less is known about newer anti-HER2 antibodies [5–7]. Several additional anti-HER2 antibodies are in development (i.e. pertuzumab and MM-111), each binding an epitope on HER2 that is unique from the Tras binding site [12–16]. We have used phage display technology to generate antibody libraries against a desired target [17,18] and herein describe the cellular disposition of one of these antibodies (73JIgG) that was generated against the ECD of HER2. Despite their proposed therapeutic mechanisms of action, the majority of therapeutic antibodies shows insufficient efficacy as a monotherapy and must be used concomitantly with additional chemotherapeutics. An exciting approach to increase the efficacy of therapeutic antibodies is to conjugate the antibodies with either potent, cytotoxic small molecule drugs [19–21] or with large drug-laden delivery vehicles (e.g. polymeric nanoparticles, liposomes, micelles) [22–28]. The first approach is designed to improve overall efficacy while maintaining desirable

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pharmacokinetic (PK), therapeutic (e.g. ADCC, CDC), and targeting properties of the antibody [29]. The second approach is designed to utilize the antibody as a combined therapeutic and targeting ligand while delivering a high drug load preferentially to cancer cells [30].

Antibodies conjugated to small molecules (Ab–SM) bind to target antigens, facilitating endocytosis [31]. In fact, Tras has been derivatized with the cytotoxic agent maytansine (DM1) to produce the conjugate trastuzumab-DM1 (Tras-DM1), which is clinically approved for HER2-postive cancer [20,21]. The ability to internalize and traffic to the lysosome is important for Ab–SM conjugates to release the small molecule drug and for relevant cytotoxic pathways. For example, Tras-DM1 contains a non-reducible thioester bond between the antibody and drug, requiring internalization and proteolytic degradation of the conjugate in order for drug to be released [21,32]. Likewise, the Ab–SM brentuximab-vedotin (Adcetris®) has an enzymatically degradable linker between the parent antibody and drug, requiring this Ab–SM to reach the lysosome in order for the conjugated drug to be released and be efficacious [33,34].

Antibody–nanoparticle (Ab–NP) constructs are designed to bind specifically to the target cell via overexpressed antigens on the surface and then internalized through receptor-mediated endocytosis [30]. In this approach, the antibody is used as a therapeutic and targeting ligand; however, conjugation to nanoparticles may alter the pharmacokinetic profile and cell endocytosis mechanism of the parent antibody [35–37]. We studied cell endocytosis of Ab–NPs using polymeric nanoparticles comprised of self-assembled, amphiphilic poly(lactide-co-2-methyl, 2-carboxy-trimethylene carbonate)-graft-poly(ethylene glycol), P(LA-co-TMCC)-g-PEG. Previous studies with trastuzumab-modified P(LA-co-TMCC)-g-PEG NPs have shown specific binding and toxicity to HER2+ vs. HER2− cells [22,38].

To gain greater insight into antibody targeting and cellular distribution for Abs modified with either small molecules vs. nanoparticles, we compared a novel antibody (called 73JIgG and its corresponding fragment, 73JFab) to that of clinically approved trastuzumab (TrasIgG, and its corresponding fragment, TrasFab) in terms of cell binding and lysosomal accumulation for the Ab alone, Ab–SM, and Ab–NP. While there has been a surge in popularity of antibody therapeutics and targeting ligands, there are few systematic studies that compare cellular distribution, even though cellular distribution impacts ultimate efficacy. Here we demonstrate that: 73JIgG (and 73JFab) bind an epitope on HER2 that is distinct from that of trastuzumab (and TrasFab); this binding is specific to HER2+ cells; and the cellular trafficking of 73JIgG differs from that of trastuzumab. We provide a quantitative analysis of the levels of trastuzumab and 73JIgG that remain bound to HER2, as well as the levels that reach the lysosome in two HER2+ cell lines. Interestingly, lysosomal accumulation is influenced by binding to nanoparticles. Developing a detailed understanding of cell trafficking for antibodies and emerging novel antibody drug or nanoparticle conjugates, will facilitate the selection of the most appropriate antibody system to achieve therapeutic efficacy.

2. Experimental

2.1. Materials

Herceptin® (trastuzumab) was purchased through Hoffmann-La Roche Limited (Mississauga, ON). The following antibodies were purchased: anti-LAMP2 [Rb] (Abcam 37024), anti-HER2 [Ms] (Thermo MS-301), anti-Rabbit 647 [Gt] (Invitrogen A2555A), anti-Mouse Alexa-546 [Gt] (Invitrogen A21043), and anti-Human IgG-H&L Fluorescein [Rb] (Sigma F4512). 5-((2-(and-3)-S-(acetylmercapto)succinoyl)amino) (SAMSA) fluorescein was purchased from Invitrogen (Eugene, OR). P(LA-co-TMCC)-g-PEG-furan was synthesized using previously reported protocols [39]. Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Pierce (Rockford, IL). All other solvents and reagents were purchased from Sigma-Aldrich and were used as received, unless otherwise noted.

2.2. Trastuzumab Fab

Trastuzumab Fab was produced from Trastuzumab IgG using a Fab Preparation Kit (Thermo Scientific Pierce, Rockford, IL) according to the manufacturer's guidelines.

2.3. Selection and characterization of anti-HER2 Fabs

Phage from Library F was cycled through rounds of binding selection with HER2 coated on 96-well Maxisorp Immunoplates (NUNC, Rochester, NY) as the capture target, as described [40]. After four rounds of selection, phage was produced from individual clones grown in a 96-well format and the culture supernatants were used in phage ELISAs to detect specific binding clones. Clones that bound to HER2 but not to bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO) were subjected to DNA sequence analysis.

2.4. Expression and purification of 73JFab protein

The Fab expression vector was derived from the phage display phagemid by inserting an amber stop codon upstream of the sequence encoding for cP3. Fab protein was produced by growing the transformed 55244 *Escherichia coli* cells as previously described [40]. The crude lysate was spun down, and the supernatant was applied to an rProtein affinity column (GE Healthcare); the column was washed with 100 column volumes of wash buffer (50 mM Tris–HCl, 150 mM NaCl, pH 8.0). Fab protein was eluted with nonpyrogenic elution buffer (50 mM NaH_2PO_4, 100 mM H_3PO_4, 140 mM NaCl, pH 2.8) and neutralized with nonpyrogenic neutralization buffer (1 M Tris–HCl, pH 8.0). Elutant was subsequently concentrated and buffer exchanged into PBS using the Amicon Ultra 30K Centrifugal Filters (Millipore, Carrigtwohill, CO) and protein concentrations were determined by a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) using human Fab (Jackson Immunoresearch, West Grove, PA) as a standard.

2.5. Conversion of 73JFab to 73JIgG1

The VH and VL sequences of the 73JFab clone were PCR-amplified and the resulting VL and VH cassettes were subcloned into EcoRI/BsiWI and EcoRI/NheI restriction sites in expression vector pFUSE2ss-CLIg-hk or pFUSEss-CHIg-hG1, respectively. Correct variable region sequences were verified by sequencing. Mammalian vector pFUSE2ss-CLIg-hk contains an expression cassette of the constant region of human κ light chain, and pFUSEss-CHIg-hG1 contains an expression cassette of the constant region of human γ1 heavy chain (Invitrogen, San Diego, CA).

2.6. IgG production in mammalian cells

73JIgG1 was produced using the FreeStyle™ 293 Expression System as per manufacturer's instructions (Invitrogen, Burlington, ON, Canada). Briefly, 250 mL of 293F cells in suspension were cultivated in 1 L shaker flasks (Fisher Scientific, Ottawa, ON, Canada) to a density of ~1–1.2 × 10^6 cells/mL. For transfection, 250 μg of the heavy chain DNA and 250 μg of the light chain DNA were combined with 250 μL of 293fectin and then added to the cells. Cells were fed ~24 h after transfection with 0.5% (w/v) Tryptone (Bio Basic, Markham, ON, Canada) and harvested by centrifugation 5 days post-transfection. Conditioned medium was diluted with 1/10th volume of 10× PBS and incubated with 1 mL of rProtein A-Sepharose (GE Healthcare, Sweden) for 1 h at RT while shaking. The gravity-flow column was loaded with the conditioned media and the beads were washed with 100 CV of nonpyrogenic wash buffer (1× PBS). IgG was eluted off the column with nonpyrogenic

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