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Research paper

Unique therapeutic properties and preparation methodology of multivalent rituximab-lipid nanoparticles

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ABSTRACT

Therapeutic monoclonal antibodies hold great promise in the treatment of cancer and other diseases, but their unclear mechanism of action makes it difficult to identify features that will increase their efficacy. One such feature may be antibody valence, since enhanced therapeutic efficacies have been observed using multivalent, as opposed to bivalent, antibodies. For example, multivalent antibody-lipid nanoparticles (Ab-LNPs) containing rituximab (Rtx) or trastuzumab show significantly increased therapeutic activity compared to equivalent doses of the bivalent antibodies. To more fully understand this phenomenon, we created a methodology reliant on biotin-neutravidin interactions for preparing specific valences of Ab-LNPs that shows improvements in reproducibility, preparation time and overall yield of coupled Ab (up to 80%). We subsequently prepared a series of valences of Rtx-LNPs to examine binding characteristics to $CD20^+$ lymphoma cells, distribution of Rtx-LNPs on the cell surface, modulation of CD20 expression, cytotoxicity of the constructs and ability of the different valences to directly induce apoptosis. As the valence of Rtx-LNP was increased, the amount of Rtx bound to cells increased up to ~10-fold higher compared to bivalent Rtx. Although more Rtx was bound to cells, there were also surprising increases in the levels of unbound CD20. This suggested the formation of Rtx-enriched microdomains that were confirmed using confocal fluorescence laser-scanning microscopy. Multivalent Rtx-LNPs were significantly more cytotoxic than Rtx; for equivalent doses of drug, Rtx-LNPs elicited apoptosis in two lymphoma cell lines in a valence-dependent manner up to levels that were 14-fold higher than bivalent Rtx. It is suggested that CD20-enriched microdomains may play a role in the mechanism of action of Rtx. This new preparation methodology can be used in future studies evaluating the mechanism of action of multivalent Ab-LNPs prepared with Rtx or other therapeutic Abs.

1. Introduction

Therapeutic monoclonal antibodies (Abs) are a class of drugs that have demonstrated great success in the treatment of cancer and other diseases due to their ability to induce direct and indirect therapeutic effects on target cells [1–4]. The indirect mechanisms of action of these drugs include complement-dependent cytotoxicity (CDC) and Ab-dependent cell-mediated cytotoxicity (ADCC), which are both mediated by the Fc region of the IgG molecule [5–7]. The direct mechanism, on the other hand, varies from Ab to Ab since it results from the physical interaction of the Fab regions with the cell-surface target [8]. For example, one of the direct mechanisms of action of rituximab (Rtx), an anti-CD20 monoclonal Ab used in the treatment of B-cell malignancies, is the induction of apoptosis in target cells [5,9–11]. The activity of a given therapeutic Ab is therefore viewed as a result of the unique combination of indirect and direct effects that occur after the Ab binds to its target *in vivo* [5]. Because of this complexity, in general, the precise mechanism of action of most therapeutic Abs is unclear, and it is therefore often difficult to define features that will increase or decrease the efficacy of a given therapeutic Ab.

One feature of therapeutic Ab design that is gaining increasing attention is the valence of the Ab, which is the number of target-binding sites the Ab contains. Most therapeutic Abs are IgG molecules and are therefore bivalent Abs since they contain two identical Fab regions. Multivalent Ab constructs possess valences of three

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or higher, and consist of two or more Ab molecules and/or Fab fragments bound together in a stable configuration. In numerous cases, the *in vitro* and *in vivo* therapeutic activity of multivalent Ab constructs has been reported as superior to equivalent doses of traditional bivalent Ab. This is the case, for example, with numerous multivalent anti-CD20 Ab designs such as tetravalent Rtx dimers [12], Rtx-dextran polymers (valence ~10) and nanoparticles prepared by coupling Rtx to Dynabeads [13], trivalent and tetravalent Rtx constructs obtained through protein engineering [14], Rtx coupled to gold nanoparticles [15], anti-CD20 multivalent branched copolymer-Fab conjugates [16], hexavalent anti-CD20 created using the Dock-and-Lock method [17], protein-polymer nanoworms [18] and multivalent Ab-lipid nanoparticles (Ab-LNPs; described below) [19,20]. Such effects have also been noted using multivalent Abs against other targets such as ErbB2, DR5 and EGFR [14,21,22]. The increased efficacies are related to the fact that multivalent Abs hypercrosslink their target on the cell surface and as a result exhibit different properties compared to their bivalent counterparts such as increased functional affinity (avidity) toward the cell-surface target, decreased dissociation rates, enhanced biodistribution, and longer *in vivo* half-life [23–25]. This provides the basis for mechanisms of enhancements (or inhibitions) of therapeutic activity that are fundamentally different in the multivalent case compared to the bivalent one [26].

Ab-LNPs make use of liposomes as nanoscale scaffolds for creating multivalent therapeutic Abs. Liposomes are traditionally employed in the context of drug delivery where a toxic drug is encapsulated within the internal aqueous compartment which increases the efficacy of the drug by altering its pharmacokinetics and by enhancing delivery to the site of disease [7,27,28]. To achieve further increases in the delivery to the site of disease, many investigators have used target-specific Abs that can be tethered to the liposome, creating immunoliposomes [29–31]. We have developed constructs consisting of Rtx or trastuzumab (Trz) attached to liposomes devoid of encapsulated drug and we have shown that these formulations exhibit significantly higher efficacy *in vitro* and *in vivo* than Ab or bare liposomes alone [19,20]. Importantly, when Rtx or Trz are conjugated to nanoparticles, the specificity of the antibody remains intact [15] and there is no nonspecific binding of Rtx to cells lacking the target receptor CD20 [20]. These studies demonstrated that the direct and indirect modes of action of multivalent Rtx-LNPs are improved when compared to Rtx [19,20], however it was unclear how the multivalent constructs were able to induce these effects at a molecular level, and to what extent the valence played a role in these improvements. To begin to address these issues, it was necessary to prepare specific valences of Rtx-LNPs. Here we describe a reproducible methodology for preparing Ab-LNPs with defined valence. The method draws on desirable features from earlier methods such as the post-insertion technique [32,33], while eliminating thiol-maleimide chemistry and implementing improved analytical assays for the characterization of intermediates. Using this methodology to produce Rtx-LNPs of varying valence, we have uncovered unique biological properties of these constructs in terms of their binding characteristics to $CD20^+$ lymphoma cells, distribution on the cell surface, cytotoxicity, and ability to directly induce apoptosis. This information sheds light on the unique mechanism of action of these multivalent Ab constructs and lends support to their further development.

2. Materials and methods

2.1. Materials

The following lipids were obtained from Avanti Polar Lipids (Alabaster AL, USA): 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG-biotin). [^{3}H]cholesteryl hexadecyl ether ([^{3}H]CHE) and Pico-Fluor 15 scintillation fluid were from Perkin-Elmer Life Sciences (Woodbridge ON, Canada). Neutravidin (Neut) and immobilized iminodiacetic acid gel were obtained from Pierce/Thermo Fisher Scientific (Rockford IL, USA). Rituximab (Rtx) and trastuzumab (Trz) were obtained from the BC Cancer Agency pharmacy (Vancouver BC, Canada). *N*-Hydroxysuccinimide ester-dPEG$_4$-biotin (NHS-PEG-biotin) was purchased from TimTec (Newark DE, USA). Disposable PD-10 columns (which contain Sephadex G-25 medium) were obtained from GE Healthcare Life Sciences (Piscataway NJ, USA). 4-Hydroxyazobenzene-2-carboxylic acid (HABA)/avidin reagent was purchased from Sigma-Aldrich (St. Louis MO, USA) as a lyophilized powder. ATTO-TAG 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA), AlamarBlue, Annexin-V binding buffer, recombinant human Annexin-V labeled with fluorescein isothiocyanate (Annexin-V-FITC), Alexa Fluor 488 (A488)-labeled cholera toxin subunit B, and Alexa Fluor 568 (A568) monoclonal antibody labeling kit were from Invitrogen (Burlington ON, Canada). Hanks' balanced salt solution (modified, without phenol red) was purchased from Stemcell Technologies (Vancouver BC, Canada). FITC-labeled anti-Rtx Ab (FITC-anti-Rtx), A647-labeled monoclonal anti-CD20 Ab (A647-mCD20), and A647-labeled mouse IgG2a negative control Ab (A647-neg) were from AbD Serotec (Kidlington, UK). Streptavidin-coated polystyrene microspheres with a diameter of 100 nm were purchased from Bangs Laboratories (Fishers IN, USA). A goat anti-human-Fcγ-fragment secondary Ab was acquired from Jackson immunoResearch (West Grove PA, USA). Glass cover slips (8 mm) and paraformaldehyde were purchased from Electron Microscopy Sciences. Phycoerythrin-conjugated anti-human-IgG Ab (PE-anti-IgG) and polyclonal anti-CD20 Ab were purchased from Santa Cruz Biotechnology (Santa Cruz CA, USA). FluorSave reagent was obtained from Calbiochem (Billerica MA, USA). Unless noted otherwise, all other reagents were obtained from Sigma-Aldrich and were of the highest quality available.

2.2. Cell lines

Ramos cells were a gift from Dr. Robert Kay (Terry Fox Laboratory, Vancouver BC, Canada), and Z138 cells were generously provided by Dr. Zeev Estrov (University of Texas) and previously characterized [34]. Ramos cells have a higher expression of CD20 on their cellular surface, approximately 330,000 molecules/cell [35]; while CD20 is expressed in lower amounts in the case of Z138 cells, 60,000 molecules/cell [36,37]. Both cell lines were maintained in RPMI 1640 medium from Stemcell Technologies (Vancouver BC, Canada) supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine from Invitrogen, as well as penicillin-streptomycin from Stemcell Technologies. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of Neut-micelles

Suspensions of DSPE-PEG and DSPE-PEG-biotin were prepared by gently dissolving the lipids in HBS (25 mM HEPES, 150 mM NaCl, pH 7.4) then they were mixed in various mole ratios corresponding to different Neut-LNP formulations. While vortexing, suspensions were slowly added to a threefold mole excess of Neut over DSPE-PEG-biotin, then were incubated for 30 min at room temperature with stirring.