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Expression and purification of two anti-CD19 single chain Fv fragments for targeting of liposomes to CD19-expressing cells

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Abstract

Antibody-targeted liposomal anticancer drugs combine the specificity of antibodies with large payloads of entrapped drugs. We previously showed that liposomal doxorubicin (DXR) targeted via anti-CD19 monoclonal antibodies (mAb) or their Fab' fragments against the B-cell antigen CD19 led to improved therapeutic effects in murine B-cell lymphoma models relative to non-targeted liposomal DXR. We now are examining the use of anti-CD19 single chain fragments of the antibody variable region (scFv) as a targeting moiety, to test the hypothesis that scFv have advantages over full-sized mAb or Fab' fragments. We expressed two different anti-CD19 scFv constructs, HD37-C and HD37-CCH in *E. coli*, and purified the scFvs using two different methods. The HD37-CCH construct was selected for coupling studies due to its relative stability and activity in comparison to HD37-C. When coupled to liposomes, the HD37-CCH scFv showed increased binding in vitro to CD19-positive Raji cells, compared to non-targeted liposomes. Cytotoxicity data showed that HD37-CCH scFv-targeted liposomes loaded with DXR were more cytotoxic than non-targeted liposomal DXR. Our results suggest that anti-CD19 scFv constructs should be explored further for their potential in treating B-lymphoid leukemias and lymphomas.

Keywords: Anti-CD19; scFv; Liposome; Doxorubicin; B-lymphoid cell; Immunoliposome

1. Introduction

The use of antibodies and antibody fragments as cancer therapeutics have flourished in recent years and led to the clinical approval of several monoclonal antibodies, including Rituxan® (rituximab) and Herceptin® (trastuzumab) [1,2]. Currently, more than 150 mAbs are in clinical trials worldwide [3]. Immuno-conjugates, where mAbs are covalently linked to a few molecules of drugs, toxins or radioisotopes, have also been successfully commercialized, e.g., Zevalin® (ibritumomab tiuxetan), Bexxar (^{131}I-tositumomab), and Mylotarg® (gemtuzumab ozogamicin) [4–6]. Additive or synergistic effects have been demonstrated in patients when combinations of signaling antibodies such as rituximab and conventional chemotherapeutic drugs are administered [7,8].

Although mAbs can be highly selective for their targets, their properties are not ideal for continued administration in patients since immune reactions can occur, particularly to antibodies containing foreign (e.g., mouse) regions [9]. Antibody fragments, such as Fab' fragments (~55 kDa) and single chain fragments of the variable region (~35 kDa) lack the Fc antibody fragment, and thus the dominant immunogenic determinants. Fab' and scFv fragments can be selected by phage display and are more easily engineered than mAbs to control properties such as affinity or internalization capabilities [10]. They can be produced by a variety of methods including bacterial and algal fermentation, which should decrease their production costs [11]. Many laboratories are currently investigating the use of these small fragments for the treatment of cancers [12–17].

Liposomes are phospholipid bilayer spheres that can encapsulate drugs in their aqueous interior. The grafting of polyethylene glycol (PEG) to the surface of liposomes (termed Stealth® liposomes) serves two purposes: the polymer terminus is a convenient location for the coupling of targeting ligands such as mAbs and derived fragments, and these liposomes have circulation half-lives of several hours, which allows sufficient

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time for the liposomes to access and bind to the target cells [13,15,18,19]. Several liposomal drugs are currently on the market, including Caelyx/Doxil® (a Stealth® liposomal formulation of the anticancer drug doxorubicin (DXR), which is approved for AIDS-related Kaposi's sarcoma, refractory ovarian and metastatic breast cancers) [20]. Currently no ligand-targeted liposomes are in the clinic, but extensive preclinical research activity is taking place in this area. The concept is similar to that of antibody-drug conjugates, with the advantage that targeted liposomes can deliver a far greater payload of drug per antibody, i.e., several hundred drug molecules per antibody, relative to less than 10 drug molecules per antibody for the antibody–drug conjugates. This can result in increased therapeutic effect for fewer antibodies, leading to reduced antibody-associated side effects and reduced expense [20–23].

We have had a long-standing interest in disease targets that are readily accessible from the vasculature, including B and T cell haematological malignancies and the vasculature of solid tumors. Non-Hodgkin's lymphoma, the most common B-cell malignancy, is the 5th most common cause of new cases of cancer in North America and accounts for approximately 4 to 5% of new cases of cancer every year [24,25]. B-cell malignancies generally respond well to initial chemotherapy, especially the combination of Rituxan® plus CHOP (cyclophosamide, doxorubicin, vincristine and prednisone) [8,26,27]. Unfortunately, a substantial percentage of patients who respond to initial therapy will relapse. This is thought to result from the incomplete eradication of residual malignant B-lymphoid cells and/or their progenitors, and justifies the search for therapies that can eradicate these cells. The CD19, CD20 and CD22 antigens are expressed on a high percentage of malignant B-lymphoid cells in lymphoma patients [25,28,29]. Since these antigens are found primarily on mature B cells and not on their precursor cells, they are good targets for directing therapeutics against malignant B-lymphoid cells.

We had previously shown that anti-CD19-targeted liposomal DXR led to improved therapeutic effects in murine models of human B lymphoma (Namalwa) relative to free DXR or non-targeted liposomal DXR [16,18,30,31]. We are now exploring the potential advantages of using scFv fragments, in relation to mAb and Fab', for targeting liposomes to B-cell malignancies. In the current study, two constructs of anti-CD19 scFv were tested separately for ease of production from *E. coli* fermentation and for ease of purification, storage and stability. The most suitable candidate was selected and conjugated to liposomes. Cell binding and cytotoxicity of the anti-CD19 scFv immunoliposomes were compared to non-targeted liposomes.

2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) and methoxypoly(ethylene glycol) (MW 2000), covalently linked to distearoylphosphatidylethanolamine ($mPEG_{2000}$-DSPE), were generous gifts from ALZA Corporation, Inc. (Mountain View, CA). Cholesterol (Chol) and 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphoethanolamine (NBD-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Maleimide-derivatized PEG_{3400}-DSPE (Mal-PEG_{3400}-DSPE) was custom synthesized by Nektar Therapeutics (Huntsville, AL). Chol-[1,2-^{3}H-(*N*)] hexadecyl ether ([^{3}H]CHE, 1.48-2.22 TBq/mmol) and ^{125}I-NaI, 185 MBq were purchased from Perkin Elmer Life Sciences (Woodbridge, ON). Bio-Rad Protein Assay Reagent was purchased from Bio-Rad Laboratories (Mississauga, ON). β-mercaptoethanol (β-ME), 2-iminothiolane (Traut's Reagent), L-arginine (L-Arg), Protein L Agarose columns, goat anti-mouse fluorescein isothiocyanate (GAM-FITC) conjugate and RPMI 1640 media were obtained from Sigma Chemical Co. (St. Louis, MO). 1,4-dithiothreitol (DTT) was purchased from Fisher Scientific (Nepean, ON). Nuclepore polycarbonate membranes (pore sizes, 0.4, 0.2, 0.1, and 0.08 μm) were purchased from Northern Lipids (Vancouver, BC). Sephadex G-50, Sephadex G-25, Sepharose CL-4B and Aqueous Counting Scintillant (ACS) were purchased from Amersham Biosciences (Baie d'Urfe, PQ). Nickel-nitrilotriacetic acid (Ni-NTA) column and murine anti-polyhistidine (anti-His) mAb were purchased from Qiagen (Qiagen, Hilden, Germany). Penicillin-streptomycin-L-glutamine (P/S/G), and fetal bovine serum were obtained from Invitrogen (Burlington, ON). Bacto Tryptone, Bacto Yeast Extract and Bacto Agar were from BD (Sparks, MD). All other chemicals were of the highest grade possible.

2.2. Cell lines and antibodies

The human Burkitt's lymphoma cell lines (Namalwa and Raji) and the human T-lymphoma cell line (Molt 4) were purchased from the American Type Culture Collection. The cells were cultured in suspension in RPMI 1640 media supplemented with 10% (V/V) fetal bovine serum, and 1% (V/V) P/S/G in a humidified 37 °C incubator with a 5% CO_2 atmosphere. Cell surface expression of CD19 was determined using single-color flow cytometry. Briefly, 1×10^6 cells were stained with a CD19 mAb, followed by a secondary antibody, GAM-FITC. Cell-associated fluorescence was analyzed on a Becton Dickinson FACScan using Lysis II software (Becton Dickinson, San Jose, CA).

The murine anti-CD19 mAbs, FMC63 mAb (IgG_{2a}) and HD37 mAb (IgG_1), were produced from the FMC63 [32] and HD37 hybridomas [33], obtained from Dr. H. Zola (Children's Health Research Institute, Adelaide, Australia) and Dr. B. Doerken (Charité, University Medicine, Berlin, Germany) via Dr. E. Vitetta (University of Texas Southwestern Medical Center, Dallas, TX), respectively. Two scFv constructs were used. The scFvs from the HD37 mAb were courtesy of Dr. S. Kipriyanov from Affimed Therapeutics AG, Heidelberg, Germany [34]. HD37-c-*myc*-Cys-His5 scFv (HD37-CCH) contains the c-*myc* and His5 tags for identification and purification, and a cysteine residue for coupling to liposomes. HD37-Cys (HD37-C) scFv, which contains a terminal cysteinyl residue for coupling to liposomes, was prepared by removing the tags from a HD37-c-*myc*-His6-Cys (HD37-CHC) construct as described below.

The PCR method was used to amplify the HD37-C scFv from HD37-c-*myc*-His6-Cys (HD37-CHC) in the pSKK vector [35]. Gene specific primers (forward primer, P1, 5' CTG CTG GCA GCT CAG CCG GCC ATG GCG CAG 3'; and Backward primer, P2, 5' TTA GCA CAG GCC TCT AGA TTA GCA GGA TCC AGC ATC AGC CCG TTT GAT TTC CAG CTT GGT GCC 3') were designed with suitable restriction enzyme sites (*Nco*I in P1 and *Xba*I in P2) in both primers, respectively, and a Cys codon was added before the stop codon in the P2 primer. PCR products were analysed by 1% agarose/TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) gel electrophoresis and amplified products were purified using a QIAquick gel extraction kit (Qiagen, Germany). Both the PCR product and the pSKK vector were digested with *Nco*I+*Xba*I endonucleases, gel purified and ligated overnight at 14 °C. The ligation mixture was transformed into *E. coli* Top10F electrocompetent cells (Invitrogen, Burlington, ON) by electroporation. Recombinant clones were screened by restriction digestion fragment analysis of the plasmid DNA, digested with *Nco*I and *Xba*I endonucleases. Digested products were analyzed by 1% agarose/TAE gel electrophoresis and the correct size insert was confirmed by restriction digestion fragment mapping.

2.3. Expression of scFv clones

For HD37-C, a recombinant plasmid carrying the scFv gene was used to transform *E. coli* RV308 by a heat shock method [36]. *E. coli* RV308 transformants were propagated in 5 mL of 2YT (1.6% tryptone, 1% yeast extract and 0.5% NaCl, pH 7.5), containing 100 μg/mL of ampicillin, and were