RESEARCH COMMUNICATION

Promising Fusion Protein Design to Target the U87 MG Glioma Cell Line

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Abstract

Gliomas, with a poor clinical course, account for 30% to 40% of all intracranial tumors. Immunotherapy with monoclonal antibodies has emerged as a promising area of investigation and recently it has been shown that antibodies utilize complementarity-determining regions (CDRs) of their variable domains to bind to antigens with high affinity and specificity. Here, we designed an antibody mimetic fused with diphtheria toxin to target the U87 MG glioma cell line. VHCDR1 and VLCDR3, together with 5 amino acid residues on both side of the CDRs, through a cognate framework region (VHFR2) yielded a mimetic of BT32/A6 (United States Patent number: 5639863). We fused the mimetic with the first 388 amino acid residues of diphtheria toxin and E. coli strain BL21 (ED3) was used to express the soluble immunotoxin DT-MG. The immunotoxin DT-MG alone did not kill Raji up to the maximal concentration tested (10-6M) in vitro. By contrast, concentrations ≥10-9M, of the fused DT-MG killed more than 95% of U-87 MG cells. It is suggested that the mimetic maintained the synergic interactions and high-affinity associated with the parent antibody. This construct holds promise for targeting specific cancer epitopes and may be useful when incorporated into diagnostic and therapeutic regimens.

Keywords: Glioma therapy - momoclonal antibodies - toxins - fusion proteins

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Introduction

Gliomas account for 30% to 40% of all intracranial tumors. About half of all gliomas in adults are glioblastomas. The incidence of primary brain tumors in the USA is estimated to be 10 per 100 000 persons per year, in a male-female ratio of 6:4. Gliomas are typical tumors of middle age, with peak incidence between the ages of 40 and 65 years (Zülch, 1986).

Thanks to recent advancement in the application of monoclonal antibodies(mAbs) to oncology, available clinically useful mAbs typically use a combination of mechanisms in directing cytotoxic effects to a tumor cell. However, those intact antibodies may experience difficulty in approaching their targets through neoplastic "binding site barriers" owing to their greater molecular weight (Fujimori et al., 1991). Comprising both heavy chain (VH) and light chain (VL) domains, single-chain variable fragments (scFvs) often posses the specific, monovalent, antigen-binding affinity of the parent antibodies, which show improved pharmacokinetics for tissue penetration (Holliger and Hudson, 2005). Unfortunately, they are poorly soluble and prone to aggregation. Single domains, which present in camels and cartilaginous fish, ignore the synergismof VH and VL domains. Researchers have found that such antibodies utilize complementarity-determining regions (CDRs) of their variable domains to bind antigens with high affinity and specificity (Qiu et al., 2007; Zhen et al., 2009).

Previous studies used key residues of CDRs for interaction of the antibody with its antigen, while of the ignoring binding residues located within their framework regions (Qiu et al., 2007) (FRs). Therefore, we wanted to fuse two CDRs,VHCDR1 and VLCDR3, together with 5 amino acid residues on both side of the CDRs, through a cognate framework region (VHFR2) that possesses mimetic properties. This was then genetically fused with the first 388 amino acid residues of diphtheria toxin to form a mimetic-toxin (immunotoxin) which retaines the antigen recognition of the parent antibodies and reacts only with targeted gliomas cells of U87.

Materials and Methods

Bacterial strains and plasmids

We used Escherichia coli (E. coli) strain JM109 to amplification of plasmids and cloning. Furthermore, E. coli strain BL21 was applied to expression of soluble immunotoxin. The plasmid pGEX-T containing the DT388 fragment was fused by our group.

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Preparation of mimetics and fusion protein

The DNA sequences of a human monoclonal antibody specific to the cell cycle independent gliomas surface antigen of BT32/A6 were published previously (United States Patent number: 5639863). The genetically engineered plasmid fusion, expression and purification were as described previously (Qiu et al., 2007; Zhen et al., 2009).

The amino acid sequence for the 5aa-VHCDR1-5aa-VHFR2-5aa-VLCDR3-5aa mimetic of the parent molecules were:5aa-VHCDR1-5aa-VHFR2-5aa-VLCDR3-5aa: GFTFSSYAMHWVRQAAPGKGLE Y VSGVYYCMQRIEFPFTFGGGT.

Cell culture and killing activity in vitro

The human glioma cell line U-87 MG (ATCC HTB-14) and Burkitt's lymphoma (Raji, ATCC CCL-86) were used in our experiments. Both cells were inoculated in Falcon 3046 six-well tissue culture plates(Becton Dickinson Co.) with 3ml RPMI 1640 (GIBCO BRL) at an initial cell density of 5×105 cells/ml. All media were supplemented with 10% fetal bovine serum, penicillin (200U/ml) and streptomycin (200μg/ml), L-glutamine (2mM), and amphotericin B (Fungizone; 1.5μg/ml). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 (Sanyo Electro. Biomed).

Different concentrations of BT-MG ranging from 10-9 M to 10-6 M were used in the experiments and physiological saline served as control. Live and dead cells were counted with 50nM acridinorange (AO)/500 nM Propidium Iodide (PI) double staining. Vital staining pictures were collected using an inverted fluorescent microscope (IX-71, Olympus) at 400× with U-MWU2, U-MVB2 and U-MNG2 filters. Each experiment was repeated at least five times.

Results

Preparing the toxin-mimetic fusion peptides DT-MG

Design of the mimetics was guided by four precepts (Qiu et al., 2007). First, as antigen recognition by intact Fab is synergistically produced by all six CDRs residing in both the VH and VL domains, it should contain at least two antigen-binding sites: one from the VH and the other from the VL domain. Such synergistic recognition cannot be accomplished if the CDR loops all originate from one variable domain (Ewert et al., 2003; Holliger and Hudson, 2005). Second, as the CDR3 loop is the central, most accessible antigenbinding segment in an intact Fab, it should be regarded as an essential component of the mimetic (Aburatani et al., 2002; Ewert et al., 2003; Holliger and Hudson, 2005).

Third, the CDR3 loop should be complemented by either the CDR1 or CDR2 loop of the other variable domain, as these are normally the closest to CDR3 in the parental antibody. Fourth, the C-terminus of the selected CDR1 or CDR2 loop and the N-terminus of the selected CDR3 loop should be joined with a framework region selected from either the VH or the VL to approximate the linkage of CDR in the parent molecule. Furthermore, we added the 5 amino acids on both sides of CDRs. The

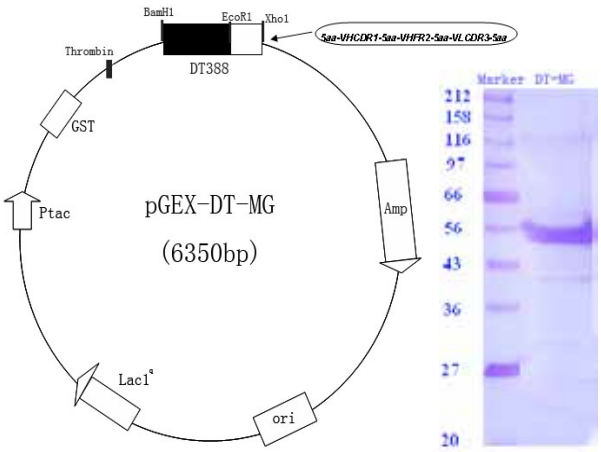

Figure 1. a) The DT-MG Plasmid Containing the DT388 gene and 5aa-VHCDR1-5aa-VHFR2-5aa-VLCDR3-5aa of BT32/A6. b) SDS-PAGE of DT-MG fusion protein

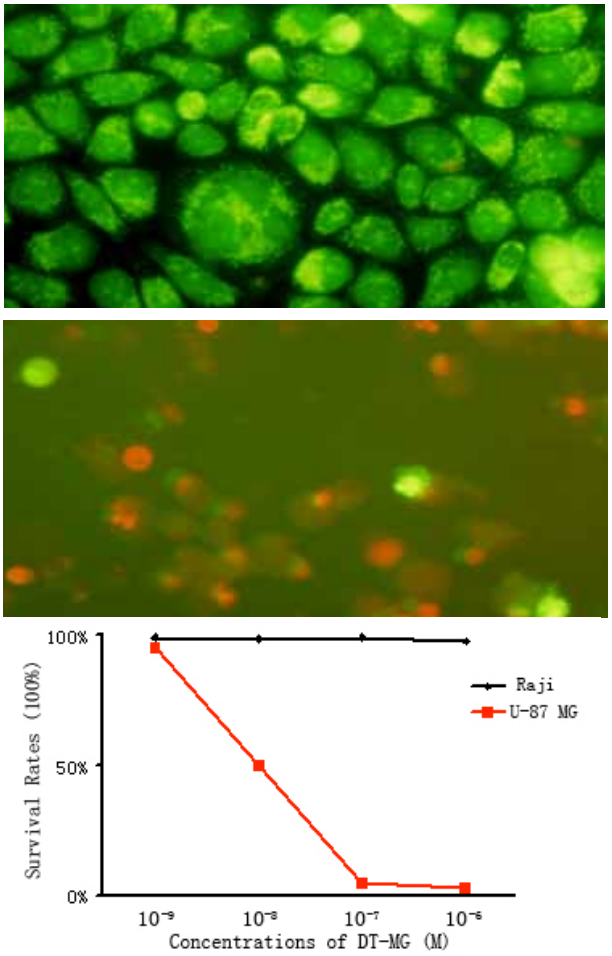

Figure 2. a) Normal U 87 MG Cells; b) 10-9M of BT-MG Killed 95% of U-87 MG Cells; c) Killing Ability of the DT-MG Fusion Peptides

genes of 5aa-VHCDR1-5aa-VHFR2-5aa-VLCDR3-5aa of BT32/A6 were constructed to follow position DT388 by double-stranded oligonucleotide mutagenesis (QuickChange Kit, Stratagene) using the plasmid containing the DT388 gene to form DT-MG (Figure 1). The fusion peptide was expressed and purified, and the silver staining indicated there was a very bulky band about

52KD (Figure 1b).

In vitro killing activity of BT-MG fusion peptides

Physiological saline could not affect the survival rate of either U-87 MG or Raji. The U-87 MG cell was chosen since it is reactive to the parent antibody found in the patient. These U-87 MG cells were incubated with various concentrations of DT-MG. Raji served as a control. We found that DT-MG could not kill these cells up to the maximal concentration tested (10-6M). In contrast, at concentrations ≥10-7M, BT-MG killed 95% of U-87 MG cells (Figure 2).

Discussion

Over 100 years ago, Paul Ehrlich hypothesized that 'magic bullets' could be developed to selectively target some diseases with very little side effects. This vision became manifest with the development of hybridoma technology (Kohler and Milstein, 1975) which provide mAbs capable of highly specific associations with their targeted antigens. However, the limited ability of native antibodies and antibody fragments to penetrate solid cancers has stimulated the search for smaller alternatives. Most methods have focused on developing antibody fragments derived from the three antigen-binding entities that reside in a variable domain of either the heavy chain (VH) or light chain (VL) of the antigen-binding fragment (Fab) of immunoglobulins. Although it is acceptable that antigen recognition by whole antibodies requires multiple noncovalent forces involving all six CDR loops residing in the VH and VL domains (Ewert et al., 2003; Holliger and Hudson, 2005), the contributions of synergic interactions of VH and VL domains are not well understood (Binz et al., 2005). Derivatives of CDR sequences retain antigen-recognition abilities, but antibody mimetics with modified CDR structure are inactive in vivo, possibly owing to inappropriate CDR modification or the absence of spacers between the CDR derivatives,which could mimic the four framework regions that separate the hypervariable regions of the variable domains.

Qiu et al (2007) firstly demonstrated that a variety of specific antibodies can be tailored to construct mimetics comprising two CDRs, each selected from the VHCDR1 and VLCDR3 domain of a Fab, oriented by means of a framework region. While, Zhen et al (2009) found that the 5 amino acid residues on both side of the CDRs played important parts in retain the structure and antigen recognition ability of CDRs. Therefore, we designed such a mimetic which retains immunological characteristics of its high-affinity parent antibody.

We have determined this fusion mimetic-toxin kills positive glioma U-87MG cells but not control Raji cells. This killing activity supports our view that this 5aa-VHCDR1-5aa-VHFR2-5aa-VLCDR3-5aa mimetic retaines the synergic interactions and high-affinity of its parent antibody. Such mimetics may hold promise for markedly improving cancer diagnostics and therapeutics, especially as the small size of the mimetic may confer other useful properties, such as enhanced intracellular delivery.

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