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Research paper

Human kappa light chain targeted *Pseudomonas* exotoxin A — identifying human antibodies and Fab fragments with favorable characteristics for antibody–drug conjugate development

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ABSTRACT

Antibody–drug conjugates (ADC) represent promising agents for targeted cancer therapy. To allow rational selection of human antibodies with favorable characteristics for ADC development a screening tool was designed obviating the need of preparing individual covalently linked conjugates. Therefore, α-kappa-ETA′ was designed as a fusion protein consisting of a human kappa light chain binding antibody fragment and a truncated version of *Pseudomonas* exotoxin A. α-kappa-ETA′ specifically bound to human kappa light chains of human or human–mouse chimeric antibodies and Fab fragments. Antibody-redirected α-kappa-ETA′ specifically inhibited proliferation of antigen-expressing cell lines at low toxin and antibody concentrations. Selected antibodies that efficiently delivered α-kappa-ETA′ in the novel assay system were used to generate scFv-based covalently linked immunotoxins. These molecules efficiently triggered apoptosis of target cells, indicating that antibodies identified in our assay system can be converted to functional immunoconjugates. Finally, a panel of human epidermal growth factor receptor (EGFR) antibodies was screened — demonstrating favorable characteristics with antibody 2F8. These data suggest that antibodies with potential for *Pseudomonas* exotoxin A-based ADC development can be identified using the novel α-kappa-ETA′ conjugate.

1. Introduction

Antibody–drug conjugates (ADC) represent promising agents in tumor therapy, potentially overcoming some of the short-comings of “naked” antibodies or antibody derivatives that often are dependent on a functional host immune system (Carter, 2006; Schrama et al., 2006; Weiner, 2007; Weiner et al., 2009, 2010). ADC are bifunctional molecules that are composed of a targeting moiety represented by a monoclonal antibody or antibody fragment and a cytotoxic compound that is either chemically cross-linked or genetically fused (Pastan et al., 2006; Carter and Senter, 2008; Schirrmann et al., 2009). The antibody moiety is used to deliver cytotoxic compounds to distinct antigen-positive cells recognized by the respective antibody. In the past, a variety of substances have been tested for ADC design. These include radio-isotopes, chemotherapeutic agents as well as toxins derived from plants or bacteria (Wu and Senter, 2005). Often the toxic component only displays cytotoxicity when internalized. Therefore, for the development of ADC target antigens such as CD7, CD22, CD30 or CD33 on leukemias and lymphomas (Peipp et al., 2002; Pastan et al.,

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2006; Schwemmlein et al., 2006; Sutherland et al., 2006) and EGFR or HER2 on solid tumors were selected (Wels et al., 1992, 1995) due to a high internalization capacity after antibody binding or a high intrinsic turn over (Wu and Senter, 2005; Pastan et al., 2006; Carter and Senter, 2008).

To date only a small number of ADC have been clinically approved with gemtuzumab-ozogamycin representing a prototypic conjugate composed of calicheamycin chemically linked to a CD33 IgG4 antibody (Sievers et al., 2001; Linenberger, 2005). Recently, novel ADC demonstrated impressive clinical results. Trastuzumab-DM1 (derivative of maytansine 1) showed promising results in phase I and phase II clinical trials in patients with HER2-positive metastatic breast cancer (Lewis Phillips et al., 2008; Krop et al., 2010; Burris et al., 2011). Treatment with brentuximab vedotin, a chimeric CD30 antibody linked to the antimitotic agent monomethyl aurastatin E resulted in tumor regression for most patients with relapsed or refractory CD30-positive Hodgkin's lymphomas in a phase I study (Younes et al., 2010). BL22, a CD22-directed ADC, representing a group of single-chain immunotoxins with scFv fragments fused to a truncated version of *Pseudomonas aeruginosa* exotoxin A, showed high response rates in phase II clinical trials in patients with hairy cell leukemia, achieving up to 47% complete remissions (Kreitman et al., 2001, 2009).

Irrespective of the ADC format, as chemical conjugate or single-chain fusion protein, selection of the targeting antibody is critical. Depending on epitope specificity, antibody binding to the targeted surface receptor may severely compromise surface redistribution, internalization and subsequently the intracellular routing of the receptor and potentially routing of the ADC/receptor complex. For example, different CD20 antibodies have been demonstrated to either recruit CD20 into lipid rafts or not, resulting in altered indirect effector functions such as CDC (Teeling et al., 2004; Glennie et al., 2007). In addition, EGFR-directed antibodies have been demonstrated to significantly differ in their capacity to trigger receptor down modulation and internalization (Lammerts van Bueren et al., 2006; Bhattacharyya et al., 2010; Spangler et al., 2010). Furthermore, most toxic compounds only display potent cytotoxicity when delivered to the correct intracellular compartment (*e.g.* the endoplasmic reticulum (ER) for *P. aeruginosa* exotoxin A; Spooner et al., 2006). Therefore, different antibodies may significantly differ in their capacity to deliver selected cytotoxic compounds. As a consequence, selecting the most promising antibody at early developmental stages may reduce costs and obviates the need for testing many candidate conjugates in parallel in complex test systems such as animal models. Simple screening tools that allow identification of promising antibody candidates in an easy but highly predictive way may speed up the developing process. Cytotoxic compounds fused to an antibody-binding domain allowing formation of a stable non-covalent ADC may represent universal tools for fast screening of a large number of antibodies. Recently, fusion proteins of the IgG-binding motif from Streptococcal protein A or protein G and diphtheria toxin or *P. aeruginosa* exotoxin A have been reported (Mazor et al., 2007; Kuo et al., 2009). Although these molecules allowed formation of non-covalent ADC and screening of different murine and mouse/human chimeric antibodies for their potential to deliver a cytotoxic compound, this strategy displayed some limitations. As protein A and G display no species specificity and therefore also bind to bovine IgG present in high concentrations in most tissue culture media, establishing a high-throughput screening assay may be complicated, and may not allow screening by simple addition of antibody and antibody-binding toxin to the assay system (Kuo et al., 2009). Using antibody-binding domains with higher species specificity may overcome these shortcomings. Most therapeutic antibodies that enter clinical trials today are chimeric, humanized or fully human IgG antibodies containing human kappa light chains (Ruuls et al., 2008). Therefore, in the current report a novel *P. aeruginosa* exotoxin A based fusion protein which specifically binds human kappa light chains was characterized as a screening tool to identify internalizing antibodies with potential in ADC development.

2. Materials and methods

2.1. Cell lines

Raji, Ramos, Daudi, ARH-77, CEM, L363 and A431 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). JK-6L cells (Meister et al., 2007) were established in our laboratory. Cell lines were cultured in RPMI 1640-Glutamax-I medium (Invitrogen, Karlsruhe, Germany), containing 10% fetal calf serum, penicillin and streptomycin ($R10^+$).

2.2. Antibodies

See Table 1.

2.2.1. Construction of α-kappa-ETA′

A truncated version of *Pseudomonas* exotoxin A (ETA′) was synthesized according to published sequences (Entelechon GmbH; Regensburg, Germany). During that process, the codon usage was adjusted to *E. coli* and a mutation reported to enhance cytotoxicity (R490A) was introduced (Bang et al., 2005). The KDEL ER-retention motif was C-terminally introduced (Seetharam et al., 1991). In addition, restriction sites allowing cloning into vector pet27b (Novagen) and the insertion of antibody binding domains such as scFv-fragments or domain antibodies as SfiI-cassettes were added (Krebber et al., 1997; Peipp et al., 2002). The kappa light chain specific domain antibody was synthesized according to published sequences (Hermans et al., 2006) and codon usage was adjusted to *E. coli*. The correct sequence of the final construct, pet27b-α-kappa-ETA′, was verified by Sanger sequencing.

2.3. Expression and purification of immunotoxins

The ETA′ fusion proteins were expressed under osmotic stress conditions as described (Barth et al., 2000). Induced cultures were harvested 16–20 h after induction. The bacterial pellet from one liter culture was resuspended in 200 ml of extraction buffer (0.5 M sucrose, 0.1 M Tris, 1 mM EDTA, pH 8.0). The suspension was stirred for 3 h at 4 °C and cleared by centrifugation for 30 min at 20,000 *g*. The ETA′ fusion proteins were enriched by two step affinity chromatography using streptactin agarose matrix and Ni-NTA agarose (Qiagen,