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Research paper

Antibody internalization studied using a novel IgG binding toxin fusion

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Abstract

Targeted therapy encompasses a wide variety of different strategies, which can be divided into direct or indirect approaches. Direct approaches target tumor-associated antigens by monoclonal antibodies (mAbs) binding to the relevant antigens or by small-molecule drugs that interfere with these proteins. Indirect approaches rely on tumor-associated antigens expressed on the cell surface with antibody–drug conjugates or antibody-based fusion proteins containing different kinds of effector molecules. To deliver a lethal cargo into tumor cells, the targeting antibodies should efficiently internalize into the cells. Similarly, to qualify as targets for such drugs newly-discovered cell-surface molecules should facilitate the internalization of antibodies that bind to them. Internalization can be studied be several biochemical and microscopy approaches. An undisputed proof of internalization can be provided by the ability of an antibody to specifically deliver a drug into the target cells and kill it.

We present a novel IgG binding toxin fusion, ZZ-PE38, in which the Fc-binding ZZ domain, derived from Streptococcal protein A, is linked to a truncated *Pseudomonas* exotoxin A, the preparation of complexes between ZZ-PE38 and IgGs that bind tumor cells and the specific cytotoxicity of such immunocomplexes is reported. Our results suggest that ZZ-PE38 could prove to be an invaluable tool for the evaluation of the suitability potential of antibodies and their cognate cell-surface antigens to be targeted by immunotherapeutics based on armed antibodies that require internalization.

Keywords: Targeted therapy; Fusion protein; Internalization; ZZ domain; PE38; Immunotoxin

Abbreviations: CDR, complementarity determining region; DMF, Dimethyl Formamide; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; FPLC, fast protein liquid chromatography; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; PCR, polymerase chain reaction; PE, *Pseudomonas* exotoxin; PE38, a truncated *Pseudomonas* exotoxin lacking domain I and part of domain Ib.; RT, room temperature (25 °C); scFv, single chain antibody fragment; TBS, Tris-buffered saline; V_H, heavy chain variable region; V_L, light chain variable region; VNTR, the variable-number-of-tandem-repeat region of the MUC1 protein.

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1. Introduction

Targeted therapy encompasses a wide variety of different strategies, which can be divided into direct or indirect approaches. Direct approaches target tumor-associated or-specific proteins to alter their signaling either by monoclonal antibodies (mAbs) binding to the relevant antigens or by small-molecule drugs that interfere with these proteins (molecular targeting). Indirect approaches rely on tumor-associated proteins expressed on the cell surface that serve as a target for fusion proteins containing different kinds of effector molecules (Schrama et al., 2006). To deliver a lethal cargo into tumor cells, the targeting antibodies should efficiently internalize into the cells. Similarly, to qualify as targets for such drugs, newly-discovered cell-surface molecules should facilitate the internalization of antibodies that bind to them. Internalization can be studied be several biochemical and microscopy approaches (Casalini et al., 1993; Blake, 2001; Lang et al., 2006).

To be an effective drug delivery vehicle, an antibody should not only internalize into the target cells, but should follow an intracellular route ending with the delivery of the drug to the correct intracellular address. Hence, an undisputed proof of the therapeutic potential of such an antibody can be provided by showing that it can, indeed, deliver a cytotoxic drug into such cells, resulting in their death. Such agents are known as immunoconjugates and immunotoxins. (FitzGerald et al., 1988, 2004; Pietersz and McKenzie, 1992; Schrama et al., 2006) To construct reagents with selectivity for certain tumor cells, immunotoxins were initially generated where mAbs or Fab' fragments were chemically linked to potent protein toxins derived from plants or bacteria like ricin, abrin, saporin, *Pseudomonas* exotoxin (PE), and diphtheria toxin (DT), which combined the selectivity of the carrier moiety with the potency of the toxin moiety. (Pastan and Kreitman, 2002). Immunotoxins kill cancer cells via binding to a surface antigen, internalization and delivery of the toxin moiety to the cell cytosol. In the cytosol, toxins catalytically inhibit a critical cell function and cause cell death. The antibody portion of the immunotoxins targets antigens that are expressed preferentially on the surface of cancer cells. This "first generation" of immunotoxins showed impressive results *in vitro* but in most cases disappointing anti-tumor effects in animals or humans. The "second generation" of recombinant immunotoxins are antibody-toxin chimeric molecules that are mostly, fully recombinant and consist of a targeting moiety, usually in the form of a single chain antibody, genetically linked to a truncated version of either DT or PE. (Reiter and Pastan, 1998).

Over the years, a large number of antibodies that bind tumor-associated antigens were isolated. Early on, the need for rapid identification of the potential of such an antibody was recognized, since internalization is a pre-requisite for most drug delivery approaches. (Casalini et al., 1993) A proof of internalization can be provided by linking the antibody to a cytotoxic cargo (such as a drug or a toxin) and testing the antibody's ability to deliver its cargo into a target cell. Basically, the first generation of immunotoxins, the antibody–toxin chemical conjugates, could provide such a tool. However, chemical conjugation may not work well with some antibodies, and surely can not be used with polyclonal serum. The generation of a recombinant immunotoxin from each candidate antibody is technically feasible, but extremely laborious. A few "general purpose" agents that could potentially link any IgG to a toxin were reported over the past two decades, most by fusing the IgG Fc-binding protein-A or fragments thereof to various toxins. However, none of these agents proved to be effective in target cell-killing. (Kim and Weaver, 1988; O'Hare et al., 1990; Madshus et al., 1991; Tonevitskii et al., 1991).

During the past few years we have been evaluating the therapeutic potential of the anti-Muc1 antibody H23 (Mazor et al., 2005). This study was carried out with the purpose of evaluating the potential of the H23 to deliver a cytotoxic payload to breast cancer cells, with the Fc-binding toxin fusion protein ZZ-PE38 serving as a tool for studying the potential of H23 to deliver a lethal cargo to target cells. The monoclonal anti-ErbB2 antibody FRP5 was chosen for the study as sort of a "positive control" since the potential of the scFv it was derived from to serve as the targeting moiety of recombinant immunotoxins is well established (Harwerth et al., 1992; Schmidt et al., 1996). Both antibodies that are murine IgG1 antibodies were converted to chimeric IgG1 antibodies to facilitate efficient binding to the ZZ domain and a potential for future use for human therapy. For that purpose we constructed a two-plasmid system to clone antibody variable domain for expression as IgG antibodies with the Fc of human IgG1 in transfected mammalian cells.

The antibody Fc-binding ZZ domain, a tandem repeated, mutated domain B derived from the antibody-binding protein-A of *Staphylococcus aureus* had been applied in a multitude of biotechnological applications (Nilsson et al., 1987, 1996). With the aim of evaluating the drug delivery potential of antibodies that bind tumor-associated antigens, we prepared a novel fusion protein, ZZ-PE38 where the ZZ domain is linked to a truncated *Pseudomonas* exotoxin A. We also describe a two-plasmid system for cloning antibody variable domain for expression as chimeric of human IgG1 antibodies. We further describe the preparation of complexes between