Toxicology in Vitro 27 (2013) 220–231

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Insights into the mechanism of cell death induced by saporin delivered into cancer cells by an antibody fusion protein targeting the transferrin receptor 1

Tracy R. Daniels-Wells [a,*], Gustavo Helguera [a,1], José A. Rodríguez [a,b], Lai Sum Leoh [a], Michael A. Erb [a], Graciel Diamante [a], David Casero [c,d], Matteo Pellegrini [c,d], Otoniel Martínez-Maza [e,f,g,h], Manuel L. Penichet [a,b,e,f]

[a] Division of Surgical Oncology, Department of Surgery, David Geffen School of Medicine, University of California, Los Angeles, CA, USA
[b] The Molecular Biology Institute, University of California, Los Angeles, CA, USA
[c] Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, CA, USA
[d] Institute for Genomics and Proteomics, David Geffen School of Medicine, University of California, Los Angeles, CA, USA
[e] Department of Microbiology, Immunology, and Molecular Genetics, David Geffen School of Medicine, University of California, Los Angeles, CA, USA
[f] Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA, USA
[g] Department of Obstetrics and Gynecology, David Geffen School of Medicine, University of California, Los Angeles, CA, USA
[h] Department of Epidemiology, Fielding School of Public Health, University of California, Los Angeles, CA, USA

ARTICLE INFO

Article history:
Received 24 August 2012
Accepted 8 October 2012
Available online 17 October 2012

Keywords:
Saporin
Immunotoxin
Transferrin receptor 1
Oxidative stress
Ribosome-inactivating protein

ABSTRACT

We previously developed an antibody-avidin fusion protein (ch128.1Av) that targets the human transferrin receptor 1 (TfR1) and exhibits direct cytotoxicity against malignant B cells in an iron-dependent manner. ch128.1Av is also a delivery system and its conjugation with biotinylated saporin (b-SO6), a plant ribosome-inactivating toxin, results in a dramatic iron-independent cytotoxicity, both in malignant cells that are sensitive or resistant to ch128.1Av alone, in which the toxin effectively inhibits protein synthesis and triggers caspase activation. We have now found that the ch128.1Av/b-SO6 complex induces a transcriptional response consistent with oxidative stress and DNA damage, a response that is not observed with ch128.1Av alone. Furthermore, we show that the antioxidant *N*-acetylcysteine partially blocks saporin-induced apoptosis suggesting that oxidative stress contributes to DNA damage and ultimately saporin-induced cell death. Interestingly, the toxin was detected in nuclear extracts by immunoblotting, suggesting the possibility that saporin might induce direct DNA damage. However, confocal microscopy did not show a clear and consistent pattern of intranuclear localization. Finally, using the long-term culture-initiating cell assay we found that ch128.1Av/b-SO6 is not toxic to normal human hematopoietic stem cells suggesting that this critical cell population would be preserved in therapeutic interventions using this immunotoxin.

1. Introduction

Saporin is a ribosome-inactivating protein (RIP) isolated from the plant *Saponaria officinalis* that strongly blocks protein synthesis (Lombardi et al., 2010). It is a Type I RIP in that it consists of a single catalytic polypeptide chain and lacks a cell-binding chain. It has similar catalytic activity to that of ricin, a Type II RIP that consists of both the catalytic and cell-binding domains (de Virgilio et al.,

Abbreviations: b-SO6, biotinylated saporin-6; BCA, bicinchoninic acid; BFU-e, burst forming unit-erythroid; BHLHB2, basic-helix-loop-helix transcription factor B2; BMMC, bone marrow mononuclear cells; CDC14B, dual specificity protein tyrosine phosphatase family; CFU-e, colony forming unit-erythroid; CFU-GM, colony forming unit-granulocyte/macorphage; CHX, cycloheximide; ch128.1Av, mouse/human chimeric antibody avidin fusion protein targeting CD71; DNS, dansyl hapten (5-dimethylamino naphthalene-1-sulfonyl chloride); FYTTD1, forty-two-three domain containing 1; GADD45B, growth arrest DNA damage-inducible gene 45β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIST2H4, Core histone 2H4; HRP, horseradish peroxidase; kDa, kilodalton; KLF6, Kruppel-like transcription factor 6; LFC, log base 2-fold change; LTC-IC, long-term culture-initiating cell; MM, multiple myeloma; mw, molecular weight; NAC, *N*-acetylcysteine; NFKBIE, NF-κB, inhibitor epsilon (IκBε); NHL, non-Hodgkin's lymhoma; NIH, National Institutes of Health; QPCR, quantitative polymerase chain reaction; RGS1, regulator of G-protin signaling; RIP, ribosomal-inactivating protein; ROS, reactive oxygen species; SO6, saporin-6; TBP, TATA-box binding protein; Tf, transferrin; TfR1, transferrin receptor 1 (also known as CD71); THUMD2, THUMP domain containing 2; TSC22D3, glucocorticoid-induced leucine zipper (TFGβ-stimulated clone 22 domain); TXNIP, thioredoxin interacting protein.

* Corresponding author. Address: Division of Surgical Oncology, Department of Surgery, UCLA, 10833 Le Conte Avenue, CHS 54-140, Box 951782, Los Angeles, CA 90095-1782, USA. Tel.: +1 310 825 0457; fax: +1 310 825 7575.
E-mail address: tdaniels@mednet.ucla.edu (T.R. Daniels-Wells).
[1] Present address: School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina.

0887-2333/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2012.10.006

2010). RIPs are *N*-glycosidases that depurinate specific adenine residues of the 23S/25S/28S ribosomal subunits leading to the irreversible block in protein synthesis. Saporin has also been reported to have DNase-like activity (Gasperi-Campani et al., 2005; Roncuzzi and Gasperi-Campani, 1996), although this is controversial (Lombardi et al., 2010). It has also been reported that the glycosidase activity of saporin is not required for its cytotoxicity (Cimini et al., 2011; Sikriwal et al., 2008). There are several isoforms of saporin that have been identified and named based on the tissue of origin and chromatographic peak in ion-exchange chromatography (Lombardi et al., 2010). Saporin-6 (SO6), one of the most active forms of the toxin, is produced in the seeds of the plant and represents the major peak (peak 6) in chromatography analysis of seed extracts (Lombardi et al., 2010). This peak contains up to 4 different isoforms of the toxin that has either an aspartic or glutamic acid residue in position 48 and either a lysine or arginine residue at position 91. Due to its high cytotoxicity, high stability and resistance to denaturation (Santanche et al., 1997), and inability to readily enter cells, saporin is a promising therapeutic agent for delivery into cancer cells.

An antibody-avidin fusion protein (ch128.1Av) was previously developed as a delivery system for a broad range of biotinylated therapeutic agents, such as SO6, into cancer cells (Daniels et al., 2007; Ng et al., 2002, 2006). This fusion protein contains avidin genetically fused to the C_H3 domains of the human IgG3 heavy chains. The antibody is specific for the human transferrin receptor 1 (TfR1, also known as CD71) and does not compete with the endogenous ligand transferrin (Tf) for receptor binding (Ng et al., 2006; Rodríguez et al., 2007). The TfR1 is a Type II transmembrane homodimeric protein involved in iron uptake and regulation of cell growth (Daniels et al., 2006b). It is widely expressed at low levels on many cell types, but shows increased expression on rapidly dividing cells including malignant cells due to their increased need for iron (Daniels et al., 2006b). Because of its central role in cancer pathology, its accessibility on the cell surface, and its ability to internalize through receptor-mediated endocytosis, the TfR1 has been used for the targeted delivery of numerous different therapeutic agents into cancer cells (Daniels et al., 2012, 2006a). The TfR1 can be targeted in two ways, either through the use of conjugates containing Tf, or through the use of antibodies like ch128.1Av. In addition to its delivery potential, ch128.1Av is cytotoxic to certain human malignant B cells, including multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL) cells (Ng et al., 2002, 2006; Ortiz-Sánchez et al., 2009), an activity that is higher compared to that of its parental antibody (ch128.1) without avidin (Daniels et al., 2011; Ng et al., 2006). This activity is due to an alteration in the TfR1 cycling pathway, increased TfR degradation, and the induction of lethal iron starvation in sensitive cells (Daniels et al., 2007; Ng et al., 2006; Rodríguez et al., 2011). However, both ch128.1Av and its parental antibody demonstrated *in vivo* anti-cancer activity in two xenograft mouse models of disseminated human MM (Daniels et al., 2011). Taken together, ch128.1Av is a versatile approach for the treatment of B-cell malignancies in that it can be directly cytotoxic through the disruption of iron metabolism or it can be used as a universal delivery system for different therapeutic agents.

Previously we have shown that ch128.1Av delivers the active b-SO6 toxin into human malignant B cells resulting in protein synthesis inhibition, caspase activation (especially caspase-2 and caspase-3), and the induction of apoptosis in both cells that are sensitive to the fusion protein alone and those that are resistant (Daniels et al., 2007). The cytotoxicity of b-SO6 conjugated to ch128.1Av in cells that are sensitive to the direct effects of ch128.1Av occurs much faster than that of the ch128.1Av alone. Additionally, the cytotoxicity of the conjugate could not be blocked by the addition of excess iron (Daniels et al., 2007), indicating that in contrast to ch128.1Av alone, iron starvation does not play a role in this cell death. These data suggest that the death induced by the conjugate is exclusively mediated by the toxin and not the direct cytotoxic effects of the fusion protein. A previous report on the gene expression analysis of ch128.1Av alone showed a transcriptional response consistent with iron deprivation mediated in part by p53 (Rodríguez et al., 2011). We now show that the ch128.1Av/b-SO6 immunotoxin induces a different transcriptional response, which is consistent with the induction of oxidative stress and DNA damage. The induction of lethal oxidative stress was confirmed through the analysis of cell death in the presence of an antioxidant. In addition, we have conducted studies that suggest nuclear localization of the toxin. Finally, we found that ch128.1Av/b-SO6 does not show toxicity to normal human hematopoietic stem cells or non-committed (early) progenitor cells.

2. Materials and methods

2.1. Cell lines

IM-9 (a human EBV-transformed B-lymphoblastoid cell line) and U266 (a human MM cell line) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Both malignant B-cell lines were grown in RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologicals Inc., Lawrenceville, GA) and grown in 5% CO_2 and 37 °C.

2.2. Recombinant antibody-avidin fusion protein production and immunotoxin formation

The antibody-avidin fusion protein ch128.1Av (formerly known as anti-human TfR IgG3-Av) has been previously described (Ng et al., 2002, 2006). It consists of a mouse/human chimeric IgG3 antibody genetically fused to avidin via its C_H3 domains. The IgG3 contains the variable regions of the murine antibody 128.1. A similar non-targeting isotype control fusion protein specific for the hapten dansyl (DNS): 5-dimethylamino naphthalene-1-sulfonyl chloride (IgG3-Av) has been previously reported (Ng et al., 2006). Both fusion proteins, expressed in murine myeloma cells, were purified from cell culture supernatants using affinity chromatography. Proteins were dialyzed into buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.8) and protein concentrations were determined by the bicinchoninic acid (BCA) Protein Assay (Thermo Fisher Scientific, Walnut, CA). Mono-biotinylated saporin (b-SO6, mw ~30 kDa) was purchased from Advanced Targeting Systems (San Diego, CA) as a custom conjugate of one biotin per toxin molecule. ch128.1Av or IgG3-Av was conjugated to b-SO6 in a 1:1 M ratio on ice for 30 min before the addition to cell culture medium as previously described (Daniels et al., 2007).

2.3. Microarray hybridization and data quality control

IM-9 and U266 cells were incubated for 1, 3, 9, or 24 h with 10 nM ch128.1Av alone or conjugated to b-SO6. Control samples consisted of cells incubated with an equal volume of buffer alone for the same time points. Total mRNA was collected from all samples using the RNeasy Kit (Qiagen, Valencia, CA). RNA was quantified and the integrity evaluated using a Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). RNA was hybridized onto HumanRef-8 v2 Expression BeadChips (Illumina, Inc., San Diego, CA) and global gene expression profiles for these samples were collected using the BeadArray software package (Illumina) in the UCLA DNA Microarray Core Facility. Quality control, preprocessing, data normalization, and statistical analysis of differential expression was performed as described previously (Rodríguez et al., 2011). All changes were deemed significant ($p < 0.05$) based on a