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Development of a sensitive screening method for selecting monoclonal antibodies to be internalized by cells

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ABSTRACT

Antibody–drug conjugates (ADCs), drugs developed by conjugation of an anticancer agent to a monoclonal antibody (mAb), have lately attracted attention in cancer therapy because ADCs can directly bind cancer cells and kill them. Although mAbs for ADCs must be internalized by the target cells, few methods are available for screening mAbs for their ability to be internalized by cells. We have developed a recombinant protein, termed DT3C, which consists of diphtheria toxin (DT) lacking the receptor-binding domain but containing the C1, C2, and C3 domains of Streptococcus protein G (3C). When a mAb–DT3C conjugate, which functions *in vitro* like an ADC, reduces the viability of cancer cells, the mAb being tested must have been internalized by the target cells. DT3C can thus be a tool to identify efficiently and easily mAbs that can be internalized by cells, thereby enhancing the development of promising ADCs.

1. Introduction

Chemotherapy with anticancer agents often causes various side effects, such as pancytopenia, diarrhea, and skin ulcer, since anticancer drugs affect not only cancer cells but also normal cells in almost the same way. There is an urgent need for development of drugs that can selectively target and kill malignant cells without exposing the patient to broad toxic effects. Antibody-conjugated drugs fulfill this selectivity requirement.

We have developed a mutant adenovirus vector that can selectively bind the Fc portion of antibodies to improve the efficacy of gene therapy in the treatment of cancer [1–4]. In our laboratory, we have 603 monoclonal antibodies (mAb) that bind a cancer antigen and could deliver the adenovirus vector to cancer cells. We have already identified the antigens to which 422 out of the 603 antibodies bind, and the antigens detected are derived from no more than 62 molecules and include potential therapeutic targets in cancer treatment such as CD20, CD44, CD47, CD81, CD155, CD276, CEACAMs, EGFR, EpCAM, EphA2, IGF1R, IL13Ra2, L1CAM, CSPG4, and PSMA [5–9].

Antibody–drug conjugates (ADCs), drugs developed by conjugation of an anticancer agent to a mAb, have lately attracted considerable attention in cancer therapy because of the ability of ADCs to directly bind cancer cells and effectively induce cell death [5,10]. ADCs are called immunotoxins because they are internalized by cancer cells through an antigen–antibody reaction, and then the anticancer agent takes effect to induce cell death. Although the internalization of mAb by cells is essential for effective cancer treatment by ADCs, as far as we know, Mab–ZAP is the only tool that has been reported to be able to measure the efficiency of mAb internalization by cells. Mab–ZAP consists of an anti-mouse antibody and a ribosome inactivating protein, saporin. When mAb–Mab–ZAP conjugates are internalized by cells, the cells undergo apoptosis due to the toxicity of saporin and, as a result, we can recognize whether or not the tested mAbs are internalized by cells. However, one cannot exclude the possibility that Mab–ZAP itself decreases, to some extent, the efficiency of mAb internalization since Mab–ZAP is a macromolecule that includes an anti-mouse antibody [11].

Here, we propose a new tool to evaluate more precisely the efficiency of mAbs' internalization by cells. This tool, termed DT3C, is a recombinant protein that is composed of diphtheria toxin (DT) and the C1, C2, and C3 domains of Streptococcus protein G (3C). We can now easily measure the efficiency of mAb internalization by cells

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by monitoring to what extent mAb–DT3C conjugates induce cell death.

2. Materials and methods

2.1. Cell lines used and culture conditions

We used a human lung cancer cell line (PC-9), two human prostate cancer cell lines (PC-3 and LNCaP), and a human melanoma cell line (A375). PC-9 cells were purchased from IBL (Gunma, Japan), and the other cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 supplemented with 10% Super Low IgG-FBS (v/v, Hyclone, Thermo Scientific, Rockford, IL, USA), 1 mM sodium pyruvate (Life Technologies, CA, USA), and 1% streptomycin–penicillin–glutamine solution (v/v, Life Technologies) at 37 °C in a 5% CO_2 incubator.

2.2. Antibodies used in this study

We used two commercial antibodies, anti-TROP2 antibody (77220, R & D Systems Inc., Minneapolis, MN) and a control antibody (P3, eBioScience, Affymetrix, Japan). A total of 47 antibodies were developed in our laboratory and used in the present study: anti-EpCAM (AY12, AY24, AY40, MY15, MY24, MY25 and 1B7), anti-CD276 (me4A2), anti-CD73 (AY30), anti-CD71 (6E1, HU10, 3C2,Yuk9, 510B and 2D1), anti-EGFR (AY13 and me1B3), anti-CD59 (Yuk1, OV9A2, MY5 and Ch9F4), anti-TROP2 (Pr1E11 and Pr8H10), anti-CD155 (AY19, AY34, and 2H7), anti-PSMA (I2, I17, I21, I26 and H28), anti-CEACAMs (L31, L34 and L38), anti-Na/K ATPase β1 (OV2C9 and 6F8), anti-Na/K ATPase β3 (4A8, OV3E10 and OV5D2), anti-CD105 (SHM51, SHM71 and SHM82), anti-IL13Ra2 (SHM28, SHM31 and SHM38), and anti-CSPG4 (SHM44 and NS110).

2.3. Expression and purification of DT3C

A bacterial expression vector for the N-terminally 6xHis-tagged DT3C protein, termed pQE30-DT3C, was constructed in several steps. A BamHI–BspEI fragment coding for the catalytic and translocation domains of DT [12,13] was generated by PCR using the genomic DNA from Corynebacterium diphtheria (PW8; kindly provided by Nobuhiro Fujii, Sapporo Medical University) as template and a pair of primers (5′-CGGGATCCGGCGCTGATGATGTTGTTGATTCTTC-3′; 5′-GGGGTACCCCTATTATCCGGATTTATGCCCCGGAGAATACGCGGGA-3′). In parallel, a BspEI–KpnI fragment encoding the 3C [14,15] was generated by PCR using the genomic DNA from Group G Streptococcus (also provided by Nobuhiro Fujii) as template and a pair of primers (5′-GGGTCCGGAGGATCCACTAGTATAGATGAAATTTTAGCTGCATTACCTAAG-3′; 5′-GGGGTACCTTATCCGGAAGATCTTTCAGTTACCGTAAAGGTCTTAGTCGC-3′). The two fragments were inserted between the BamHI and KpnI sites of pQE-30 (QIAGEN, Hilden, Germany), resulting in pQE30-DT3C (Fig. 1A). *Escherichia coli* strain M15 [pREP4] (QIAGEN) was transformed with pQE30-DT3C, grown in liquid culture, and incubated with isopropyl-β-D-thiogalactoside for induction of DT3C expression. The cells were lysed with sonication and the DT3C protein was purified using Ni–NTA Agarose (QIAGEN), according to the manufacturer's protocol.

2.4. Cytotoxicity assay

A mAb and DT3C or Mab–ZAP (Advanced Targeting Systems, San Diego, CA 92121 USA) were incubated at room temperature for 30 min to form mAb–DT3C or mAb–Mab–ZAP conjugates. Cancer cells were seeded and incubated in the presence of mAb–DT3C or mAb–Mab–ZAP conjugate for 72 h, and the number of viable cancer cells was then estimated using a WST-1 assay (Roche Diagnostics, Indianapolis, IN, USA.

3. Results

3.1. Production of DT3C

We constructed a bacterial expression vector for DT3C as illustrated in Fig. 1A. A DNA fragment encoding the catalytic and translocation domains of DT fused with the Streptococcal protein G-derived 3C domain, which is capable of binding to the Fc portion of IgG was placed behind the sequence for 6xHis tag in frame. We purified bacterially produced DT3C protein and analyzed the protein by SDS–PAGE. DT3C migrated at approximately 70 kDa (Fig. 1B). DT3C lacks the receptor-binding domain so that cells could not be killed in the absence of antibodies. DT3C was designed to form mAb–DT3C conjugates readily through the 3C domain, which would mimic ADCs.

3.2. DT3C indicates the efficiency of mAb internalization by cells

We assessed whether or not MY5 (an anti-CD59 antibody)-DT3C conjugate and 6E1 (an anti-CD71 antibody)-DT3C conjugate could induce cell death in PC-9 cells. Fig. 2 shows that each conjugate actually decreased the viability of PC-9 cells in a concentration-dependent manner, with the viability becoming less than 10% at 1 μg/mL of DT3C, while neither DT3C alone nor P3 (a control antibody)-DT3C conjugate was able to induce cell death even at 10 μg/mL of DT3C. These findings suggested that (1) DT3C does not interfere with the internalization of the mAbs tested, and (2) DT3C alone does not have any negative effect on cell viability. Consequently, we can efficiently detect antibodies that are actually internalized by cancer cells through administration of a mAb–DT3C conjugate to the cells.

3.3. Only nine of 47 mAbs were internalized by cancer cells

We then examined the internalization efficiency of 47 mAbs developed in our laboratory. As illustrated in Fig. 3, only nine of the 47 (19%) mAb–DT3C conjugates significantly reduced the cell viability by more than 90%, and the ten antibodies were identified in anti-CD59, CD71, IL13Ra2, or TROP2 antibodies tested. Interesting, not all of the anti-CD59, CD71, IL13Ra2, and TROP2 antibodies ($n = 16$) were internalized (Fig. 3). These results suggested that epitopes, but not antigens, recognized by mAbs, play a key role in internalization of antibodies.

3.4. DT3C is superior to Mab–ZAP in selecting antibodies internalized by cells

We finally examined if DT3C was a better tool than Mab–ZAP to identify antibodies that can be internalized by cells. Both 77220 (an anti-TROP2 antibody)-DT3C conjugate and 77220-Mab–ZAP conjugate reduced viability of PC-9 cells in a dose-dependent manner (Fig. 4). More than 30% of PC-9 cells, however, survived in the presence of 77220-Mab–ZAP conjugate at 10 μg/mL (=∼48 nM) of Mab–ZAP, while 77220-DT3C conjugate significantly lowered cell viability by ∼94% at 1 μg/mL (=∼14 nM) of DT3C. We thus concluded that DT3C is superior to Mab–ZAP in screening antibodies internalized by cells. Mab–ZAP alone as well as DT3C alone had no negative effects on cell viability.

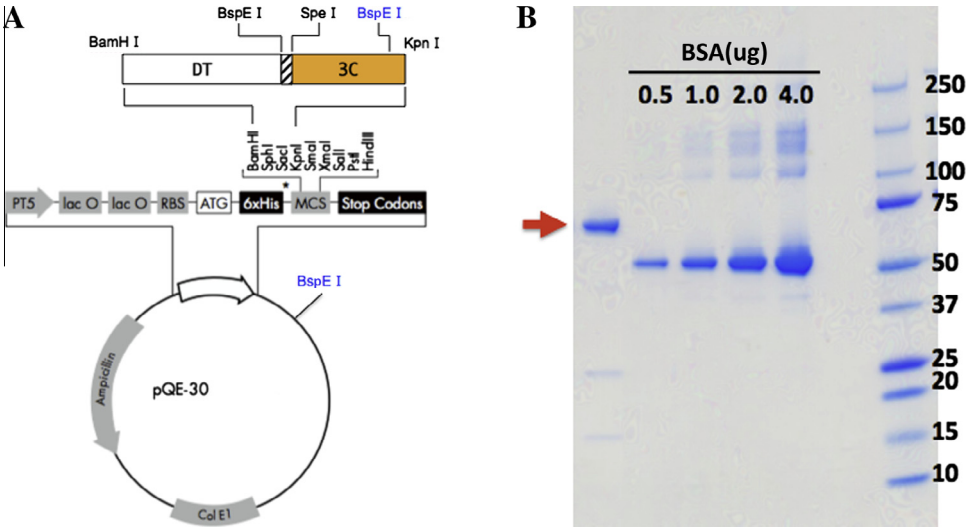

Fig. 1. Structure and molecular weight of DT3C. (A) Schema showing the structure of pQE30-DT3C. (B) The molecular weight of DT3C is indicated (arrow).

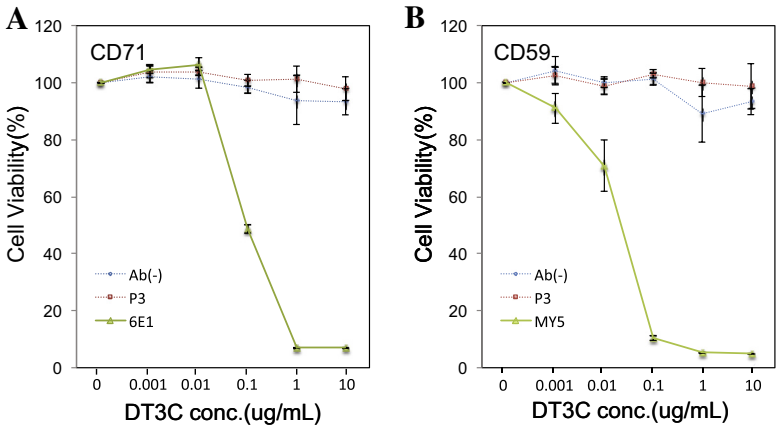

Fig. 2. Induction of cell death in PC-9 cells. 6E1 (an anti-CD71 antibody)-DT3C conjugate and MY5 (an anti-CD59 antibody)-DT3C conjugate markedly induced cell death in PC-9 cells. (A) PC-9 cells were seeded and incubated with DT3C alone, P3-DT3C conjugate, or 6E1-DT3C conjugate for 72 h. The number of viable cells was evaluated using the WST-1 assay. Values are means ± SD of triplicate cultures. The concentration of the antibodies used was 10 μg/mL. (B) PC-9 cells were seeded and incubated with DT3C alone, P3-DT3C conjugate, or MY5-DT3C conjugate for 72 h. The number of viable cells was evaluated using the WST-1 assay. Values are means ± SD of triplicate cultures. The concentration of the antibodies used was 10 μg/mL.

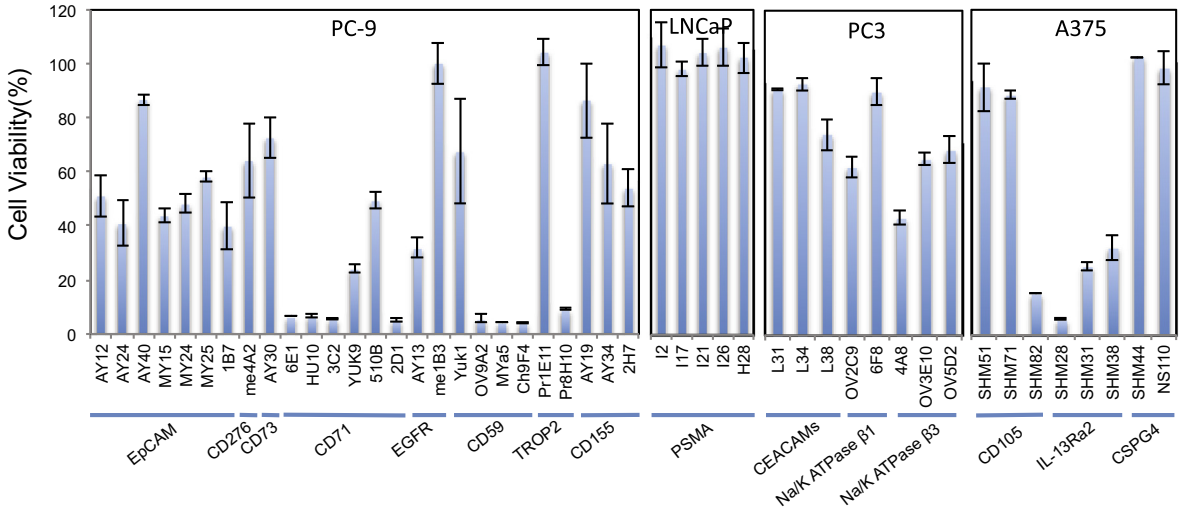

Fig. 3. Summary of cell viability reductions by 47 mAb–DT3C conjugates. The concentration of mAbs or DT3C used in this experiment was 1 μg/mL.

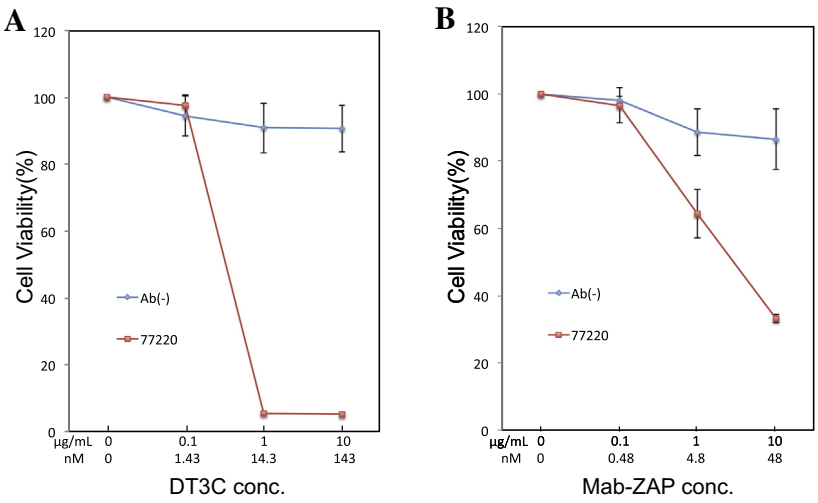

Fig. 4. Comparison of DT3C and MabZAP effectiveness. A mAb-DT3C conjugate induced cell death more effectively than a mAb–Mab–ZAP conjugate. (A) PC-9 cells were seeded and incubated with DT3C alone, or 77220-DT3C conjugate for 72 h. The number of viable cells was evaluated using the WST-1 assay. Values are means ± SD of triplicate cultures. The concentration of the antibody used was 10 μg/mL. (B) PC-9 cells were seeded and incubated with Mab–ZAP or 77220-Mab–ZAP conjugate for 72 h. The number of viable cells was evaluated using the WST-1 assay. Values are means ± SD of triplicate cultures. The concentration of the antibody used was 10 μg/mL.

4. Discussion

Here, we have developed a new screening method that is able to efficiently and easily select antibodies that can be internalized by cells; internalization is a critical process that must take place before ADCs can exert their effect in cancer cells. The DT3C we have described in this study has three major features. First, a mAb–DT3C conjugate that functions *in vitro* like a true ADC administered to patients is easily produced by a 30 min incubation at room temperature. Second, DT3C binds any IgG derived from various species, such as human, mouse, rabbit, goat, to form a mAb–DT3C conjugate. Third, mAb–DT3C conjugates markedly decrease cell viability only when the conjugates are internalized by target cells. These findings suggest that DT3C would be an efficient tool to select antibodies that can be internalized by cells, thereby facilitating the development of new ADCs.

It seems likely that DT3C is superior to Mab–ZAP in screening for antibodies internalized by cells (Fig. 4). A mAb–DT3C conjugate almost always consists of one mAb (~150 kDa) and two DT3Cs (~140 kDa), resulting in a macromolecule of approximate 290 kDa. This is because two DT3Cs bind the two Fc portions of a mAb like protein G. On the other hand, it appears to be hard to control the number of Mab–ZAPs that bind a mouse mAb (~150 kDa), because a Mab–ZAP (~210 kDa) is composed of an anti-mouse antibody and a ribosome inactivating protein, saporin. Although the molecular weight of a mAb–Mab–ZAP conjugate is at least 360 kDa, we surmise that a mAb–Mab–ZAP conjugate often consists of one mAb and several Mab–ZAPs to form a very large ($\geqslant$360 kDa) macromolecule, which could inhibit the mAb internalization by cells.

In conclusion, DT3C, which we have developed, appears to be a more efficient tool than Mab–ZAP to screen for antibodies that can be internalized by cells. DT3C can efficiently and easily select monoclonal antibodies with the ability to be internalized by cells, thereby enhancing the development of new ADCs.

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