Cancer Letters 365 (2015) 149–155

Contents lists available at ScienceDirect

Cancer Letters

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

Original Articles

Targeted killing of rhabdomyosarcoma cells by a MAP-based human cytolytic fusion protein

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ARTICLE INFO

Article history:
Received 30 October 2014
Received in revised form 2 April 2015
Accepted 4 April 2015

Keywords:
Rhabdomyosarcoma
Human cytolytic fusion protein
Fetal acetylcholine receptor
Microtubule-associated protein
Immunotherapy

ABSTRACT

The treatment of rhabdomyosarcoma (RMS) is challenging, and the prognosis remains especially poor for high-grade RMS with metastasis. The conventional treatment of RMS is based on multi-agent chemotherapy combined with resection and radiotherapy, which are often marked by low success rate. Alternative therapeutic options include the combination of standard treatments with immunotherapy. We generated a microtubule-associated protein (MAP)-based fully human cytolytic fusion protein (hCFP) targeting the fetal acetylcholine receptor, which is expressed on RMS cells. We were able to express and purify functional scFv35-MAP from *Escherichia coli* cells. Moreover, we found that scFv35-MAP is rapidly internalized by target cells after binding its receptor, and exhibits specific cytotoxicity toward FL-OH1 and RD cells *in vitro*. We also confirmed that scFv35-MAP induces apoptosis in FL-OH1 and RD cells. The *in vivo* potential of scFv35-MAP will need to be considered in further studies.

Introduction

Rhabdomyosarcoma (RMS) is the most common pediatric soft-tissue sarcoma, accounting for 50% of all neoplasias. It is one of the 10 most frequent childhood/adolescent tumors, with an incidence of 4–5 cases per million per year [1–3]. RMS may occur anywhere in the body but it is often found in the head and neck region or the genitourinary tract [4]. There are three main subtypes: the more aggressive alveolar subtype (aRMS), the embryonal subtype (eRMS) and the pleomorphic subtype (pRMS). Approximately 70% of all neoplasias are classed as eRMS, which frequently involves loss of heterozygosity mutations at 11p15.5 and 9p22. This disrupts the expression of tumor suppressor genes such as *PTCH1* and proto-oncogenes such as *HRAS* [2,5]. The aRMS subtype often involves a chromosome translocation that fuses the transcription factor genes *PAX3* or *PAX7* with the *FOXO1* locus (t(2;13)(q35;q14) or t(1;13)(q36;q14)). This creates a potent chimeric oncogene containing a PAX DNA-binding domain and a FOX transactivation domain, which induces cell proliferation and blocks apoptosis and differentiation during myogenesis [5–7]. The rare pRMS subtype appears almost exclusively in adults and is characterized by different genetic aberrations, such as the loss of chromosomes 2, 3, 14, 15, 16 and 19 [5,8].

The treatment of RMS has improved over the last two decades and the 5-year event-free survival rate has increased from 20% to 70%. Conventional treatment is multimodal, comprising multi-agent chemotherapy with resection and radiotherapy. Nevertheless, the outcome for patients with relapsed and metastatic aRMS is still poor with a 5-year event-free survival rate of 15% [2,9,10]. New therapeutic options are therefore urgently required, especially for high-risk patients.

One such option is immunotherapy targeting the fetal acetylcholine receptor (fAChR), which is a suitable target for three reasons. First, the fAChR has five subunits (α2, β, δ and γ) and the embryonic γ-subunit is replaced by the ε-subunit during development to form the adult acetylcholine receptor (AChR) [11–13], which means that the fAChR is not expressed in healthy children and adolescents, except in certain extraocular muscle and thymic myoid cells [14–18]. Second, the fAChR is overexpressed on the surface of RMS tumor cells [19,20]. Third, the fAChR is internalized when it is bound by monoclonal antibodies, and internalization is necessary for immunotherapeutic molecules such as immunotoxins to exert their effects [21].

Immunotherapy involves the delivery of therapeutic molecules using antibodies or their fragments to target particular cell types.

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http://dx.doi.org/10.1016/j.canlet.2015.04.004
0304-3835/

Such modalities include antibody drug conjugates (ADCs), where antibodies are chemically conjugated to a therapeutic effector molecule [22], and classical immunotoxins comprising an antibody and a toxin of bacterial, fungal or plant origin [23,24] presented as a fusion protein to increase stability [25]. Although many ADCs and immunotoxins have been based on full-length antibodies, their large size (~150 kDa) reduces their ability to penetrate solid tumors, which is a disadvantage when treating RMS [26]. Therefore, single-chain variable fragments (scFvs) have been used instead because they are much smaller (~30 kDa) [22,27].

Despite promising results in preclinical and clinical studies [24,28], classical immunotoxins containing non-human effector molecules are rapidly cleared since they induce the production of neutralizing antibodies and this reduces their efficacy [29]. They also cause side effects such as vascular leak syndrome and liver toxicity which limit the doses that can be used [30]. These drawbacks have led to the development of fusion proteins containing human effector molecules, such as the serine proteases granzyme B and granzyme M, the ribonuclease angiogenin and death-associated kinase 2 [31–34]. Microtubule-binding protein tau (MAP) is another human effector molecule, which has been tested as a fusion with the natural ligand epidermal growth factor (EGF) and with two different scFvs targeting the surface antigens EpCAM and CD30 [35–37]. MAP binds specifically to microtubules and thereby blocks mitosis and presumably vesicular transport, ultimately inducing apoptosis [38]. Unlike other human effector molecules, MAP is not expected to be inactivated by endogenous inhibitors.

Here, we created a fusion construct joining the fully human scFv35 to the human effector molecule MAP to generate a novel, recombinant human cytolytic fusion protein (hCFP) against RMS. The fAChR-specific scFv35 was previously combined with a truncated version of *Pseudomonas* exotoxin A (ETA') and bound specifically to fAChR$^+$ RMS cells (FL-OH-1 and RD) resulting in the induction of apoptosis [39]. The scFv35-MAP fusion was successfully expressed in *Escherichia coli* cells and the purified protein bound specifically to FL-OH-1 and RD cells *in vitro*, inhibiting cell proliferation and inducing apoptosis. To our knowledge, this is the first hCFP that has been shown to target fAChR on RMS cells, such as FL-OH-1 and RD, and thus specifically kills RMS cells.

Materials and methods

Cell lines and culture conditions

The eRMS cell line RD was obtained from the American Type Culture Collection, the human histiocytic lymphoma cell line U937 was obtained from the German Collection of Microorganisms and Cell Cultures and the eRMS cell line FL-OH1 was kindly provided by Ewa Koscielniak (Olgahospital, Stuttgart, Germany). The cell lines were grown in RPMI 1640 medium (Gibco Invitrogen, Carlsbad, USA) supplemented with 10% heat-inactivated fetal calf serum (Biochrom AG, Berlin, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco Invitrogen). The cells were cultivated at 37 °C and 5% CO_2 in humidified air.

Cloning, expression and purification of scFv35-MAP

The DNA sequence of MAP was cloned in the linearized pMT vector already containing the scFv35. For plasmid amplification *E. coli* strain DH5α was used as previously described [40]. The expression was conducted using the pMT plasmid, which is derived from pET27b (Novagen, Wisconsin, USA) [41,42]. The protein was expressed in *E. coli* BL21 (DE3) (Novagen) under stress conditions and purified by immobilized metal-ion affinity chromatography (IMAC) as previously described [43,44]. The protein sample was separated by SDS-PAGE (12% acrylamide) and the gels were stained with Coomassie Brilliant Blue. The concentration of scFv35-MAP was determined by densitometry after gel staining compared to bovine serum albumin (BSA) standards using AIDA Image Analyzer Software v.4.27.039. The separated protein sample was transferred onto a nitrocellulose membrane [40,45] and scFv35-MAP was detected using a murine anti-His primary antibody (Sigma-Aldrich, München, Germany) and an alkaline phosphatase (AP)-labeled mouse IgG-specific secondary antibody (Sigma-Aldrich).

Binding and internalization analysis

The cell-binding activity of scFv35-MAP was determined by flow cytometry. RMS cells were detached by trypsinization, washed with phosphate-buffered saline (PBS) and 4×10^5 cells were transferred to a FACS tube. The cells were incubated for 30 min on ice with 1 µg of either scFv35-MAP or scFv35-ETA', each diluted in 100 µl PBS. After washing with PBS, the bound proteins were detected with an Alexa488-labeled anti-His antibody (Qiagen, Hilden, Germany) diluted 1:100 and incubated for 30 min on ice in the dark. Finally, the cells were washed twice with PBS and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

The internalization of scFv35-ETA' and -MAP was carried out as previously described [46]. Briefly, FL-OH1 and RD cells (2×10^5 per tube) were incubated for 30 min on ice with 1 µg of each protein in 100 µl PBS. After washing with PBS, the cells were incubated in the culture medium at 37 °C in a 5% CO_2 humidified atmosphere for different time periods. The negative control sample (no internalization) was incubated at 4 °C and set as 100% fluorescence intensity in the final analysis. After incubation, the cells were washed with PBS and incubated for 30 min on ice with the ETA'-specific murine antibody TC-1, diluted 1:100 in PBS [47], or tau antibody (HT-7; Thermo Fisher Scientific, Schwerte, Germany), diluted 1:100 in PBS. Bound primary antibody was detected using a phycoerythrin (PE)-labeled anti-mouse antibody (Dianova, Hamburg, Germany) diluted 1:50 in PBS. The cells were washed twice with PBS and analyzed on a FACSVerse flow cytometer (Becton Dickinson).

Cell viability assay

The cytotoxicity of scFv35-ETA' and scFv35-MAP against RMS cell lines RD and FL-OH1 was assessed using a colorimetric XTT cell viability assay as previously described [32]. Briefly, 5×10^3 cells were seeded into 96-well microtiter plates (50 µl medium per well) and incubated for 72 h under standard culture conditions (see above) with 50 µl of serially diluted scFv35-ETA', scFv35-MAP or scFv35-SNAP (O(6)-alkylguanine-DNA alkyltransferase; negative control). Each well was then supplemented with 50 µl XTT (SERVA, Heidelberg, Germany) and phenazine methosulfate (Sigma-Aldrich) in a 100:1 ratio and the cells were incubated for another 4 h at 37 °C and 5% CO_2 as above. The absorbance was then measured at 450 and 630 nm using an Epoch Microplate Spectrophotometer (BioTek, Bad Friedrichshall, Germany). The concentration required to achieve 50% reduction of protein synthesis (IC_{50}) relative to the untreated control cells was calculated using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). All experiments were carried out in triplicates.

Apoptosis assay

The pro-apoptotic activity of scFv35-MAP was determined by Annexin V–propidium iodide (PI) staining using RMS cell lines RD and FL-OH1 and the control cell line U937 as previously described [48]. Briefly, 1×10^5 cells were seeded into 24-well plates and incubated in 300 µl medium containing 350 nM scFv35-MAP for 48 h under standard culture conditions (see above). Cells were also incubated with PBS (negative control) or camptothecin (positive control). The cells were harvested (RMS cells were first detached by trypsinization), washed with 1 × Annexin V binding buffer (15 mM NaCl, 1 mM HEPES, 0.5 mM KCl, 0.2 mM $CaCl_2$, pH 7.4) and stained with 450 µl HEK293T cell supernatant containing Annexin V-eGFP and 50 µl 10 × Annexin V binding buffer. The cells were incubated at room temperature in the dark for 10 min, washed and resuspended in 500 µl 1 × Annexin V binding buffer containing a 1:500 dilution of PI (1 mg/ml). The cells were measured by flow cytometry using a FACSVerse flow cytometer (Becton Dickinson).

Results

Fully human scFv35-MAP can be expressed in E. coli

We previously identified the human cytolytic effector protein MAP, which proved to be effective against cells expressing the epidermal growth factor receptor (EGFR) when coupled to the natural ligand EGF [36]. MAP was shown to be effective when genetically fused to scFvs specific for EpCAM and CD30 [35,37]. We therefore cloned the MAP cDNA in frame with the fully human scFv35 sequence in a pMT vector and expressed the resulting hCFP under osmotic stress [43]. The protein was purified under native conditions by affinity chromatography, separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue (Fig. 1, lane 2). The protein was detected by western blot using a primary antibody targeting the His-tag and a secondary AP-labeled antibody (Fig. 1, lane 3). Although scFv35-MAP holds the potential to be produced as a pure and homogeneous protein, missing optimized downstream processing conditions lead to impurities in the protein