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The single-chain immunotoxin MCSP-ETA', targeting melanoma-associated chondroitin sulfate proteoglycan, is a potent inducer of apoptosis in cultured human melanoma cells

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Abstract

A recombinant immunotoxin was constructed by fusing a single-chain Fv (scFv) antibody fragment, specific for the melanoma-associated chondroitin sulfate proteoglycan (MCSP), to a truncated variant of *Pseudomonas* Exotoxin A (ETA'), carrying a C-terminal KDEL peptide for improved intracellular transport. The resulting immunotoxin, MCSP-ETA', induced antigen-specific, potent apoptosis in the cultured human melanoma-derived cell lines A2058 and A375M, and treatment with a single dose of the agent eliminated up to 80 % of these cells within 72 h. The dose needed for half-maximum killing (EC50) was approximately 1 nM for both cell lines. MCSP-ETA' also displayed cytotoxic activity against cultured primary melanoma cells from patients with advanced disease, with net cell death reaching up to 70 % within 96 h after treatment with a single dose of 14 nM. MCSP-ETA' induced cell death synergistically with Cyclosporin A (CsA), both in established human melanoma cell lines and cultured primary melanoma cells. The distinctive antigen-restricted induction of apoptosis and the synergy with CsA justify further evaluation of this novel agent with regard to its potential applications for the treatment of melanoma and other MCSP-positive malignancies.

Keywords

immunotoxin; MCSP; melanoma; Exotoxin A; Cyclosporin A

Introduction

Malignant melanoma represents the most common form of fatal skin cancer and accounts for 1-3 % of all neoplasias. Its incidence has grown world-wide over the past decades, especially

among the Caucasian population with intense sun exposure [17]. The 5-year survival rate drops from 95 % for patients with a maximum tumor thickness of 1 mm lacking metastases (pathologic stage I) to below 10 % for patients with visceral metastasis (pathologic stage IV) [3]. If distant metastases are present, disease progression is much faster, and life expectancy is commonly only 6 to 9 months [36]. Metastatic melanoma is largely resistant to known treatment modalities. In particular the application of chemotherapeutics is not effective, due to the resistance of melanoma cells to systemic treatment with anti-cancer agents [21]. Therefore, novel therapeutic options are needed, and immunotherapeutic approaches, targeting melanoma-associated antigens with restricted distribution in normal tissues, offer a promising possibility.

The melanoma-associated chondroitin sulfate proteoglycan (MCSP), also known as high molecular weight-melanoma associated antigen (HMW-MAA), is highly expressed on over 85 % of human melanoma tissues, with a limited degree of intra- and interlesional heterogeneity [8]. MCSP is also expressed in malignant lesions of non-melanocytic origin, such as basal cell carcinoma, glioma, neuroblastoma and sarcoma [8]. Despite its lack of expression on normal hematopoietic cells, MCSP is expressed on a subtype of leukemic blasts derived from patients with acute myeloid and acute lymphoblastic leukemia (AML, ALL). Its expression correlates with poor prognosis and balanced translocations into the mixed-lineage leukemia (MLL) gene [44,49]. MCSP expression remains unaffected by chemotherapeutic treatment [16]. Because of its high expression on tumor cells and its restricted distribution in normal tissues, this antigen has been used as a target for antibody-based immunotherapy in patients with melanoma. Furthermore, toxins and chemotherapeutic agents chemically conjugated to MCSP-specific monoclonal antibodies (mAb) have been shown to control tumor growth both *in vitro*, and in animal model systems [7,35,41,22]. An antigen-independent cytotoxicity of these conjugates may be caused at least in part by the limited stability of the link between the antibody and the toxin, and in part by the binding of the immunotoxin to antigen-negative, Fc-receptor-positive non-malignant target cells.

To overcome the limitations associated with the use of toxins chemically linked to mAb, we have linked a toxic component to a single chain Fv fragment (scFv) of the MCSP-specific mAb 9.2.27 in the present study, to eliminate non-specific, Fc-receptor-mediated uptake by non-malignant cells. Moreover, in our design, we have utilized recombinant DNA technology to link the toxin to the antibody fragment to increase stability of the bond and to reduce non-specific uptake of the toxin resulting from premature breaking of the bond in the extracellular space. Furthermore, we have selected the truncated version of Exotoxin A (ETA') from *Pseudomonas aeruginosa*, which lacks domain Ia and consists only of domains Ib, II, and III [30]. Lastly, in our molecule the endogenous C-terminal REDLK-peptide was replaced by the KDEL-peptide to improve intracellular retrograde transport [39].

Other groups have previously reported on scFv-ETA'-fusion immunotoxins of similar format specific for CD22, CD25, CD7, CD64, CD33, CD19 and more antigens for the treatment of hematological malignancies [25,26,32,46,37,38] and others, specific for the Lewis Y antigen, IL-13, the EGF-receptor, ErbB2/HER2, EpCAM, and MUC1 for the treatment of solid tumors [33,29,34,2,13,40]. These scFv-ETA' immunotoxins have not yet been approved for routine clinical use, but in preclinical and early-stage clinical studies they have demonstrated encouraging results. Especially for solid tumors, an immune response against the bacterial toxin is discussed to be the major limiting factor of ETA'-derived immunotoxins. Novel findings in this field disclosed the B-cell epitopes of the ETA' component, and point-mutations of these epitopes did not lead to a reduced cytotoxic activity but displayed a decrease in the production of ETA'-neutralizing antibodies in an animal model system [28].

Taken together, scFv-ETA' immunotoxins have been found to be useful in early-phase clinical trials for the elimination of malignant cells from a variety of hematologic malignancies [25, 31], but only limited experience exists in the treatment of cells derived from solid tumors [33]. Therefore, we investigated in preclinical cell-culture studies whether this format may also offer favorable properties for the potential treatment of human melanoma.

Materials and methods

Bacterial strains

Escherichia coli XL1-Blue and BL21 (DE3) were purchased from Stratagene, Amsterdam, The Netherlands and from Novagen, Inc., Madison, WI, USA, respectively.

Culture of eukaryotic cells

The human melanoma cell line A2058 [14] was cultured in DMEM-Glutamax-I medium (Invitrogen, Karlsruhe, Germany), containing 10 % fetal calf serum (FCS) (Invitrogen), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Invitrogen). The human melanoma cell line A375M and M14 [9], the lymphoblastoid cell line CEM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, German Collection of Microorganisms and Cell Lines, Braunschweig, Germany), and the hybridomas 9.2.27 [6] and 14G2a (both provided by Dr. Ralph A. Reisfeld, Scripps Research Institute, La Jolla, CA, USA) were maintained in RPMI 1640-Glutamax-I medium (Invitrogen), containing 10 % FCS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. The stably transfected M14-MCSP cell line [8] was cultured in RPMI 1640-Glutamax-I medium, containing 10 % FCS, 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 400 µg/mL Geneticin (Invitrogen).

Patient-derived melanoma cells

Primary human melanoma cells were obtained by surgical excision of solid metastatic tissues, which were mechanically disrupted to small pieces, using a *Cell strainer* 40 µm nylon membrane (Becton Dickinson, Heidelberg, Germany), and cultured in RPMI 1640-Glutamax-I medium, containing 20 % FCS, 100 units/mL of penicillin, 100 µg/mL of streptomycin and 40 µg/mL Gentamycin (Sigma, Taufkirchen, Germany).

Construction and expression of scFv-ETA'-fusion proteins

The MCSP-directed scFv was sub-cloned from the hybridoma 9.2.27 as previously described [32]. The sequence coding for the MCSP-specific scFv was inserted as an SfiI-cassette into the vector pASK6-linker, containing the coding sequences for the N-terminal STREP- and hexa-histidine-tag, and the 20 amino acid linker (G₄S)₄, which will connect the scFv to the truncated ETA'. The resulting vector, pASK6-MCSP-linker, was digested with NotI and XbaI, and the fragment containing the two tags, the MCSP scFv, and the linker was cloned into the expression vector pet27b(+), upstream of the coding sequence for a truncated ETA'-REDLK variant [32]. The vector pet27b-STREP-His-MCSP-ETA'-REDLK was digested with XhoI and XmaI, for the exchange of the coding sequence for the C-terminal REDLK motif, against the sequence coding for the KDEL motif. The insert, containing the KDEL motif, was excised from the vector pet27b-STREP-His-CD33-ETA'-KDEL [37] by digestion with XhoI and XmaI. Ligation created the expression vector pet27b-STREP-His-MCSP-ETA'-KDEL.

The scFv-ETA'-fusion proteins MCSP-ETA', CD7-ETA' [32], and CD33-ETA' [37] were expressed under osmotic stress conditions [4]. Cultures were harvested 20 h after induction. The bacterial pellet from 1 L of culture was resuspended in 200 mL of periplasmatic extraction buffer (100 mmol/L Tris, pH 8; 500 mmol/L sucrose; 1 mmol/L EDTA) for 4 h at 4°C. The

scFv-ETA'-fusion proteins were enriched by affinity chromatography using streptactin agarose beads (IBA, Goettingen, Germany) [43] according to the manufacturer's instructions.

Flow cytometric analysis

Adherent cells were harvested by incubation with 5 mM EDTA in PBS for 15 min at 4°C. Following washings with phosphate-buffered bovine albumin (PBA) buffer, containing PBS, 0.1 % bovine serum albumin, and 7 mmol/L sodium azide, 3×10^5 cells were incubated on ice for 60 min with 25 µL of an immunotoxin solution at the concentration of 5 µg/mL. The unrelated immunotoxins CD7-ETA' and CD33-ETA', served as controls for background staining of MCSP-ETA' and CD7-ETA', respectively. Cells were washed in PBA and incubated on ice for 60 min with 25 µL of murine penta-His antibody (Qiagen, Hilden, Germany). Afterwards cells were washed in PBA, and 25 µL of phycoerythrin (PE) conjugated goat-anti-mouse-IgG antibody (DAKO Diagnostica, Hamburg, Germany) was added, and incubation was continued for additional 60 min on ice. After a final wash, cells were analyzed using a FACSCalibur instrument with CellQuest software (Becton Dickinson, Mountain View, CA, USA).

To test primary melanoma cells for MCSP expression, adherent cells were harvested using the TrypLE Express harvesting reagent (Invitrogen). Following washing with PBA, 3×10^5 cells were incubated on ice for 60 min with 25 µL of a murine mAb 9.2.27 solution at the concentration of 5 µg/mL. An unrelated murine IgG2a isotype (DAKO Diagnostica) served as control for background staining. Cells were then washed with PBA and stained with PE-conjugated anti-mouse-IgG as described above.

For each sample 10^4 events were collected, and analysis of whole cells was performed using appropriate scatter gates to exclude cellular debris and aggregates. QIFIKIT® (DAKO Diagnostica) was used for quantitative determination of MCSP expression on A2058 and A375M cells, according to the manufacturer's instructions. Equilibrium constants were determined using a flow cytometric assay as described before [5].

Measurement of cytotoxic activity of immunotoxins and Cyclosporin A (CsA)

For dose-response experiments, 3×10^4 cells/mL were seeded in 24-well plates in a final volume of 500 µL, and immunotoxin was added at varying concentrations between 0.01 and 2.5 µg/mL. Every sample measured represented one well, containing cells in the supernatant and adherent cells, which were removed by the TrypLE Express reagent. Cell death was measured by staining nuclei with a hypotonic solution of propidium iodide (PI) according to standard procedures. The extent of cell death was determined by measuring the fraction of nuclei with subdiploid DNA-content. For each sample, 10^4 events were collected and analyzed for subdiploid nuclear DNA content.

To determine whether cell death occurred via apoptosis, cells were seeded at 3×10^4 /mL in 24-well plates in a final volume of 500 µL, and immunotoxin was added at a concentration of 1 µg/mL. After incubation, whole cells were stained with FITC-conjugated Annexin V [47] (BD Pharmingen, San Diego, CA, USA), and PI in PBS according to the manufacturers' instructions. For blocking experiments, the parental mAb 9.2.27, and the non-related isotype matched mAb 14G2a (targeting the ganglioside GD2), respectively, were added in 10-fold molar excess to the culture, 1 h before addition of the immunotoxin.

CsA (Sigma) was dissolved in EtOH at a concentration of 50 µg/mL, and used immediately or stored at -20°C. For CsA experiments, cells were seeded at 3×10^4 /mL in 24-well plates in a final volume of 500 µL, and CsA was added at the indicated concentrations, immunotoxin at 0.1 µg/mL, and mAb 9.2.27 at 0.22 µg/mL. Following a 72 h incubation at 37°C, cell death

was quantified by staining nuclei with a hypotonic PI-solution as described above. Synergistic cytotoxic effects induced by combined treatment of cells with MCSP-ETA' and CsA were determined by evaluating the cooperativity index (c_i), defined here as the quotient of the sum of the percentages of cell death obtained with single agent treatment and the percentage of cell death induced by combination treatment; c_i values < 1 were considered to indicate synergism.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed by standard procedures. Gels were stained with Coomassie brilliant blue R250 (Sigma). Western blots were performed with secondary antibodies coupled to horseradish peroxidase (Dianova, Hamburg, Germany). Enhanced chemiluminescence reagents (Amersham Pharmacia, Freiburg, Germany) were used for detection. ScFv-ETA'-fusion proteins were detected with a murine anti-penta-his antibody (Qiagen), or with a polyclonal rabbit anti-*Pseudomonas* ETA serum (Sigma). Full-length poly ADP-ribose polymerase (PARP) and its specific cleavage product were detected using a mouse anti-human-PARP antibody (BD Pharmingen).

Statistical analysis

Statistical analysis was performed using Microsoft EXCEL software. Group data are reported as average $\pm$ SD. P-values were obtained using t-tests with a confidence interval of 95 % for evaluation of the statistical significance compared with the control.

Results

Construction, expression, and purification of the recombinant immunotoxin

The MCSP-directed scFv was sub-cloned from the hybridoma 9.2.27 by phage display as described [32], and fused to the coding sequence for truncated *Pseudomonas* ETA. To improve retrograde transport through the trans-Golgi network, the REDLK-motif was replaced by the endoplasmic reticulum retention motif KDEL of eukaryotic cells. Variable light (v_L) and heavy (v_H) chain domains were connected in this order by a 20 amino acid flexible linker (G_4S)₄. The same linker was used to connect the scFv moiety to the ETA' polypeptide. For purification and specific detection, an N-terminal STREP-tag and a hexa-histidine-tag were added (Fig. 1a). The resulting construct was cloned into the bacterial expression vector pet27b, and expressed in *E. coli* BL21 (DE3) under osmotic stress conditions. The recombinant immunotoxin was highly enriched after a single purification cycle with streptactin beads (Fig. 1b). In Western-transfer experiments, the immunotoxin was bound by antibodies specific for the ETA'-moiety (Fig. 1c) and the hexa-histidine-tag (Fig. 1d), respectively. The yield of enriched recombinant protein was approximately 20 - 30 μ g/L of bacterial culture.

Antigen-specific binding of MCSP-ETA'

To determine whether the described scFv-ETA'-fusion protein is capable of specific binding to MCSP, the immunotoxin was incubated with M14-MCSP human melanoma cells, a cell line stably transfected with MCSP c-DNA, and as a control with the parental MCSP-negative cell line M14. MCSP-ETA' showed effective binding to M14-MCSP cells (Fig. 2a), whereas no binding was detected to the parental MCSP-negative M14 cells (Fig. 2b). Additionally, MCSP-ETA' binds to MCSP-positive A2058 (Fig. 2c) and A375M (Fig. 2d) melanoma cells. For comparison, the similarly constructed CD7-specific immunotoxin [32] was tested for binding to CD7-positive CEM-cells. The CD7-specific immunotoxin did bind to these cells (Fig. 2e), but did not bind to MCSP-positive, CD7-negative A2058 cells (Fig. 2f). Antigen-specificity of the binding by MCSP-ETA' to intact cells was further demonstrated by blocking experiments with 10-fold molar excess of the mAb 9.2.27 in cytotoxicity experiments (see below). In summary, the data show that MCSP-ETA' did bind to MCSP in an antigen-specific

manner. The measured equilibrium constants of the 9.2.27 parental antibody, the subcloned scFv and the fusion protein MCSP-ETA' were 5 ± 0.5 nM, 43 ± 8 nM and 134 ± 3 nM, respectively (data not shown).

Dose-dependent and antigen-specific cytotoxic activity of MCSP-ETA'

To determine cytotoxic activity of MCSP-ETA', the agent was incubated with MCSP-positive cultured human melanoma cells A2058 and A375M. These cells expressed $168 \pm 21 \times 10^3$ and $195 \pm 21 \times 10^3$ antigenic sites, respectively, as determined by quantitative analysis of MCSP-antigen molecules per cell (data not shown). As control, MCSP-negative human melanoma cells M14 were used. Cell death was quantified by PI staining and flow cytometry 72 h after treatment. MCSP-ETA' specifically mediated cell death of the MCSP-positive A2058 and A375M cells in a dose-dependent manner, but had no detectable effects on MCSP-negative human melanoma cells M14 (Fig. 3). The determined concentration of the agent (applied as a single dose) needed to reach half-maximum cytotoxic effect (EC50) following 72 h of incubation at 37°C, was approximately 60 ng/mL (0.9 nM) for A2058 cells and 80 ng/mL (1.1 nM) for A375M cells. Statistically significant cell death of MCSP-positive cells ($p < 0.05$), compared to untreated cells, was obtained at MCSP-ETA' concentrations of ≥ 10 ng/mL (0.14 nM) (Fig. 3). In an additional experiment, 1 μ g/mL of immunotoxin was given at time zero, and additional doses of 1 μ g/mL were added after 24 and 48 h. This did not lead to an increased maximum cytotoxicity for both cell lines after 72 h of incubation (data not shown).

The cytotoxic effect was antigen-specific since pre-incubation of the A2058 (Fig. 4a) and A375M (Fig. 4b) cells with the MCSP-specific parental mAb 9.2.27 in a 10-fold molar excess inhibited cytotoxicity by the immunotoxin. The inhibition was specific, as preincubation of the cells with the GD2-specific isotype matched mAb 14G2a at a similar molar excess, failed to block the cytotoxic effect of the immunotoxin. It is noteworthy, that the isotype matched mAb used as a single agent had no detectable cytotoxic effect on melanoma cells. As a further control, the CD7-ETA' immunotoxin of similar format displayed no antigen-independent cytotoxic potential against CD7-negative melanoma cells (Fig. 4). In conclusion, MCSP-ETA' acted in a highly antigen-specific and dose-dependent manner and showed cytotoxic potential against MCSP-positive human melanoma cells in a low nanomolar concentration range.

Antigen-specific induction of apoptosis by MCSP-ETA'

To evaluate whether cell death was attributable to apoptosis, cells were analyzed by Annexin V and PI double staining in flow cytometric studies. MCSP-ETA'-treated MCSP-positive A2058 (Fig. 5a) and A375M (Fig. 5b) cells displayed Annexin V-positive and PI-negative staining. This subpopulation reflects cells in an early apoptotic stage. Ninety-six hours after treatment, 40 % of the A2058 cells were in the early apoptotic stage and additional 20% were dead (Fig. 5a). Seventy-two hours after treatment, 32 % of the A375M cells were in the early apoptotic stage and additional 38 % were dead (Fig. 5b). This cytotoxic effect was blocked by pre-incubation of target cells with the parental mAb 9.2.27 in a 10-fold molar excess (Fig. 5a and b). The apoptotic nature of cell death was further demonstrated by the cleavage of PARP. MCSP-ETA' promoted the cleavage of intact PARP (116 kDa) to its characteristic 85 kDa proteolytic fragment in A2058 (Fig. 5c) and A375M (Fig. 5d) cells 48 h after treatment. Thus, two independent methods have shown that MCSP-ETA' induced apoptosis in long-term cultured human melanoma cells in an antigen-specific manner.

MCSP-ETA' induces cell death of cultured primary melanoma cells

Due to the fact that long-term cultured cancer cells only inaccurately represent the features of the parental tumor cells, the immunotoxin was tested on cultured primary melanoma cells. Therefore, melanoma cells from metastatic lesions, surgically removed from 10 patients with advanced disease (stage IIIC and IV), were treated with a single dose of 1 μ g/mL MCSP-ETA'

or 1 µg/mL of a similarly constructed unrelated control toxin. At indicated time-points, cell death was determined by PI staining of nuclei and flow cytometric analysis. The MCSP-ETA' agent produced cytotoxic effects for primary human melanoma cells from some of the tested patients (Table 1 and Fig. 6). Specific cell death (net above background) in cells from patients #3, #4, #7, and #8 occurred in a time-dependent manner and ranged from 44 to 69 % following a 96 h incubation at 37°C, with a background varying between 17 and 23 % (Fig. 6). In the 10 samples, the percentage of MCSP-positive cells, representing the melanoma cells, ranged from 14 to 99 %, as determined by flow-cytometric analysis of cells stained with mAb 9.2.27 prior to incubation with the immunotoxin. The mean fluorescence intensity (MFI) of the different primary cell samples after staining with the mAb 9.2.27 displayed no direct correlation between MCSP-expression level and susceptibility to the immunotoxin. Table 1 summarizes the data obtained with the cell samples from the 10 melanoma patients. Thus, MCSP-ETA' displayed a cytotoxic effect also against primary melanoma cells.

MCSP-ETA' cooperates with the anti-tumor effect of CsA in a synergistic manner

Beside its immunosuppressive properties, CsA has been shown to induce apoptosis in human melanoma cells [10]. To examine synergistic effects of CsA and MCSP-ETA', the human melanoma cell line A2058 (Fig. 7a) was incubated for 72 h at 37°C with varying concentrations of CsA as a single agent, or with CsA in combination with a constant concentration of MCSP-ETA', and cell death was evaluated by PI staining of nuclei and flow cytometry. When used as a single agent, CsA induced cell death in A2058 cells at concentrations ranging from 10 to 25 µg/mL (Fig. 7a). When used in combination with 100 ng/mL MCSP-ETA', cell death was markedly enhanced. The cooperativity index (c_i) for 1, 5, and 10 µg/mL CsA and 100 ng/mL MCSP-ETA' ranged from 0.56 to 0.66, indicating a strong synergistic effect of these two agents (Fig. 7a). To assess the role of intracellular signaling induced by antibody-binding to MCSP in the synergistic effect of CsA and MCSP-ETA', A2058 cells were incubated with varying concentrations of CsA in combination with the unmodified parental mAb 9.2.27 used in a concentration, equimolar to MCSP-ETA' (Fig. 7a). No increase in cytotoxicity was detected, excluding a major role of the binding to MCSP in the synergistic effect of the combination of CsA with MCSP-ETA'.

A similar synergistic cytotoxicity was found when melanoma cells from patient #4 were used as targets (Fig. 7b). In the latter case, cells were incubated for 48 h at 37°C with CsA as a single agent at 5 and 10 µg/mL, or with CsA in combination with 1 µg/mL MCSP-ETA'. Cytotoxicity was not detected when cells were incubated with CsA alone, but was increased, when cells were incubated with CsA in combination with MCSP-ETA'. This effect was also synergistic, as indicated by a cooperativity index of 0.65 (Fig. 7b).

Taken together, these results show that MCSP-ETA' and CsA synergize in their cytotoxic effects for both long-term established melanoma cell lines and primary melanoma cells.

Discussion

The purpose of this study was to determine whether the format of a fusion-protein consisting of a tumor-antigen specific single chain antibody fragment and a truncated *Pseudomonas* ETA' toxin exhibits cytotoxic potential against melanoma-derived cells. The results we obtained were: (a) the MCSP-ETA' fusion protein was capable of efficiently eliminating both established melanoma-derived cell lines and cultured primary melanoma cells from melanoma patients, (b) the cytotoxic effect was highly antigen-specific, (c) cell death occurred via apoptosis, and (d) the agent cooperated with the cytotoxic potential of CsA in a synergistic manner.

The agent eliminated the two MCSP-positive human melanoma cell lines A2058 and A375M with similar efficacy. For both lines, the EC50 dose was approximately 1 nM, which is a low effective concentration comparable with other immunotoxins of similar format [46,25]. An immunotoxin, consisting of an intact MCSP-antibody, chemically linked to wild-type ETA, has already been published [22]. This agent eliminated human glioblastoma-derived cells in an animal model, and, therefore, the MCSP-antigen must be sufficiently well internalized after binding of a bivalent antibody to present a favorable target for immunotoxins. Nevertheless, it was not known so far, that monovalent binding to MCSP is sufficient to induce receptor mediated internalization. Furthermore it had to be determined whether a similar immunoconjugate, or a format derived from it, would also be effective for melanoma cells.

It might be expected that our molecule displays less side effects in a potential clinical application, compared to the published MCSP-directed immunotoxin containing the whole mAb chemically linked to wild-type ETA [22]. The published compound would probably be resorbed by human Fc-receptors on non-malignant, antigen-negative cells, due to the presence of the murine IgG2a Fc-part, thus possibly causing severe side effects. Moreover, the existence of wild-type ETA, including its natural binding domain I, would allow extra-cellular cleaved toxin to enter MCSP-negative, non-malignant cells. Furthermore, the tissue penetration, which is an important property of immunotoxins targeting solid tumors, is expected to be improved due to the reduced size of our scFv-ETA' immunotoxin (approx. 66 kDa), compared to the published immunoconjugate (approx. 216 kDa).

The survey of metastatic tissue samples of 10 melanoma patients with advanced disease (Table 1) demonstrated that cells from some patients were more susceptible to the agent than others, such as cells from patients #5 and 6. The lack of sensitivity of cells from these patients cannot be attributed to the low percentage of MCSP-positive cells in these cultures, because in both cases, > 90 % of the cells were antigen-positive. We suspect that the different response rates reflect heterogeneity in the pattern of genetic alterations that is specific for every tumor and even for sub-clonal lines within the tumor of every patient. Although these cells probably are, as it is the case for most solid tumors, clonal descendants of one original malignantly transformed cell, they probably represent different sub-clonal lineages present in the tumor tissue. Each of these sub-clones, in addition to carrying the initial set of genetic alterations that led to malignant outgrowth, would carry a sub-clone-specific pattern of additional mutations, which may influence their relative susceptibility for our agent. In a recent cancer-genomics study of primary breast and colon carcinoma, a surprisingly high number of mutations were reported, on the order of 100 mutations per cell, with several dozen mutations per cell in known oncogenes and tumor-suppressor genes [42]. If a similar pattern was applicable for melanoma cells, then it would not be surprising to find differences in the susceptibility to the novel agent for different sub-clonal lines within different patients or even the tumor tissue of the same patient. However, we do not consider it a discouraging result that 6/10 patient samples showed a response to our agent, seeing the fact, that all patients already received standard chemotherapeutic treatment before surgical excision of the metastatic tumor cells used in these studies. Presumably some cells already gained further resistance to apoptosis inducing agents due to the prior treatment of the patients with chemotherapeutics. Taken together, the positive result of our study was that tumor cells from 6/10 patients showed a clear response to the agent, plus another 2/10 showed marginal responses above background of spontaneous cell death. Under these conditions, and given the limited success of chemotherapeutic agents for the treatment of melanoma, the obtained results have to be considered positive.

The new agent would probably present some shortcomings, when considered as an anti-cancer drug for the treatment of solid tumors. One source of concern would be the use of the ETA' moiety as the toxic component. Upon multiple administration of immunotoxins containing bacterial toxins, neutralizing antibodies have often been observed in a fraction of the patients

precluding further use of the agent [48]. In spite of this known disadvantage, ETA'-based immunotoxins are currently under consideration for further development for the treatment of selected types of solid tumors, including breast cancer and head-and-neck cancer [2,13]. Such agents may, therefore, also be useful for the treatment of melanoma. To reduce the immunogenicity of the bacterial toxin, several investigators have considered a combination-treatment of the patients with immunosuppressive agents, such as CsA. Interestingly, CsA not only functions as an immunosuppressive agent, but also has an intense ability to induce apoptosis in certain types of tumor cells, including gliomas, melanomas and leukemias [27, 10,24]. CsA acts as a modulator of the mitochondrial permeability transition pore to induce apoptosis [12], and a similar mode of action has also been reported for *Pseudomonas* ETA [1]. Moreover, CsA synergizes with other anti-tumor agents, that act on mitochondria, such as resveratrol, at least for leukemic cells [50]. Published reports suggest that CsA binds to cyclophilins and thereby inhibits their peptidyl-propyl-cis-trans-isomerase (PPIase)-like activity [19]. PPIase-like enzymes act as molecular chaperones to support protein folding, intracellular transport, and the maintenance of stability of multiprotein complexes [20]. Apart from its interaction with cyclophilin D inside the mitochondrial membrane [11], and the ensuing direct effect on apoptosis, CsA may therefore also produce additional indirect pro-apoptotic effects, which may synergize with ETA. Regarding both agents influence protein synthesis, it would be easy to conceive synergistic pro-apoptotic effects for the combination of ETA and CsA. Therefore, we were gratified to find, that our MCSP-ETA' molecule showed synergistic effects with CsA in the elimination of cultured melanoma cells. This property makes our agent particularly attractive for further development, because it would simultaneously suppress the neutralizing response against the toxin and achieve a synergistic anti-tumor effect. Current research in the field of ETA'-derived immunotoxins show further promising results. The mutation of characterized B-cell epitopes inside the polypeptide chain of ETA' reduces the production of neutralizing antibodies without losing its cytotoxic potential [28].

Interestingly, MCSP expression is often correlated with an immature character of the affected cell. This property has been reported for melanoma cells with stem cell-like properties [15], and for leukemic blasts with chromosomal rearrangement of the MLL gene, which often have a stem cell-like phenotype [23,45,49]. In addition, MCSP is expressed on epidermal and hair follicle progenitor cells [18], which also have stem cell-like properties, but not on the corresponding mature cells. Finally, MCSP may also be expressed on some breast cancer stem cells (S. Ferrone, unpublished data). Taken together, these observations indicate that MCSP may be expressed on cells with stem cell-like properties and possibly on melanoma tumor stem cells [15]. Therefore, MCSP is a particularly attractive target, because the elimination of tumor stem cells should be a high-ranking objective for every cancer therapy.

In summary, the study presented here demonstrated that an immunotoxin consisting of an MCSP-specific scFv fused to a truncated *Pseudomonas* Exotoxin A (ETA'), effectively eliminated both MCSP-positive melanoma cell lines and primary cells from melanoma patients with advanced disease in low nanomolar concentrations, and that the agent showed a striking degree of synergism with CsA. Based on available knowledge in the field, this format might be expected to offer significant benefit in the treatment of malignant melanoma, as a single agent or in combination therapy, and therefore deserves further characterization in view of its advancement towards preclinical and clinical investigation.

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Abbreviations List

MCSP, melanoma-associated chondroitin sulfate proteoglycan; ETA’, truncated variant of *Pseudomonas* Exotoxin A.

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Figure 1.

Design and purification of the immunotoxin MCSP-ETA'. **(a)** Design of MCSP-ETA'. S, N-terminal Strep-tag; H, hexa-histidine-tag; V_L and V_H, variable region light and heavy chains of the scFv; (G₄S)₄, 20 amino acid linker composed of 4 repeats of the Gly₄Ser unit. Exotoxin A', truncated *Pseudomonas* Exotoxin A fragment consisting of domains Ib, II and III of the toxin; K, endoplasmic reticulum retention motif KDEL. Molecular masses of the fragments in kDa were calculated from their amino acid sequence. **(b)** The purity of the enriched immunotoxin after bacterial expression and elution from streptactin beads was evaluated by SDS-PAGE and staining with Coomassie brilliant blue; lanes 1, 2: elution fractions 1, 2. **(c)** The toxin was detected in a Western-transfer experiment with an antibody specific for the ETA' component and **(d)** specific for the hexa-histidine-tag.

Figure 2.

Specific binding of recombinant immunotoxins to antigen-positive cells. Cells were incubated with the scFv-ETA' fusion protein (*black*) or a non-related scFv-ETA' fusion protein (*white*) at identical concentrations and analyzed by flow cytometry. **(a)** MCSP-ETA' was incubated with the MCSP-positive human melanoma cell line M14-MCSP, **(b)** the parental cell line M14, **(c)** melanoma cell line A2058 and **(d)** melanoma cell line A375M. **(e)** The control toxin CD7-ETA' was incubated with CD7-positive CEM-cells, and **(f)** with MCSP-positive, CD7-negative A2058 human melanoma cells.

Figure 3.

MCSP-ETA' induces cell death of MCSP-positive A2058 and A375M human melanoma cells. A2058 cells (grey bars), A375M cells (white bars) and M14 cells (black bars) were incubated for 72 h with MCSP-ETA' at the indicated concentrations and then evaluated for the percentage of cell death by PI staining and flow cytometry. Cells showing subdiploid DNA content were considered dead. Data points are mean values of 3 independent experiments, and error bars reflect the SD. Values reaching statistical significance ($p < 0.05$) compared with untreated cells are indicated by an asterisk.

Figure 4.

Killing of MCSP-positive A2058 and A375M human melanoma cells by MCSP-ETA' is antigen-specific. **(a)** A2058 cells and **(b)** A375M cells were incubated with MCSP-ETA' (1 $\mu\text{g/mL}$) (white bars), CD7-ETA' (1 $\mu\text{g/mL}$) (black bars), MCSP-ETA' (1 $\mu\text{g/mL}$) and the parental mAb 9.2.27 (22 $\mu\text{g/mL}$) (grey bars), MCSP-ETA' (1 $\mu\text{g/mL}$) and the isotype matched control mAb 14G2a (22 $\mu\text{g/mL}$) (diagonally hatched bars), or the isotype matched control mAb 14G2a (22 $\mu\text{g/mL}$) alone (vertically hatched bars). The percentage of cell death was determined by PI staining and FACS analysis following 48 h and 72 h of incubation at 37°C. Data points are mean values from 3 independent experiments and the SD is indicated by error bars.

Figure 5.

Elimination of MCSP-positive A2058 and A375M human melanoma cells by MCSP-ETA' occurs via apoptosis. **(a)** A2058 cells and **(b)** A375M cells were incubated at 37°C with MCSP-ETA' (1 µg/mL) or with MCSP-ETA' (1 µg/mL) and the parental mAb 9.2.27 (22 µg/mL). Cells were stained with Annexin V and PI at the indicated time points and analyzed by flow cytometry. Numbers in the right quadrants of each plot represent the percentage of cells in the early and late apoptotic stage, respectively. The data are representative for 3 separate experiments. **(c)** A2058 cells and **(d)** A375M cells were incubated for 48 h at 37°C with MCSP-ETA' (1 µg/mL) and analyzed for cleavage of poly ADP-ribose polymerase (PARP) by Western-transfer experiments. (→): specific cleavage product of 85 kDa; lanes 1, 2: cells treated with PBS or MCSP-ETA', respectively.

Figure 6.

MCSP-ETA' induces cell death in melanoma cells isolated from surgically removed metastases. Melanoma cells prepared from patients #3, 4, 7, and 8, containing 64 %, 95 %, 99 % and 99 % MCSP-positive cells, respectively, were incubated for 96 h at 37°C with MCSP-ETA' (1 µg/mL) (grey, *square*) or unrelated scFv-ETA' control toxin (1 µg/mL) (black, *diamond*). The percentage of cell death was determined by PI-staining and flow cytometry at the indicated time points. Triplicate samples were measured for each time point and error bars represent the SD. Figures indicate the percentage of cell death following 96 h of incubation at 37°C.

Figure 7.

Synergistic cytotoxic effect of MCSP-ETA' and Cyclosporin A (CsA) on MCSP-positive cultured melanoma cells. **(a)** A2058 cells were incubated for 72 h at 37°C with CsA (black bars) at varying concentrations, with CsA in combination with MCSP-ETA' (100 ng/mL) (grey bars), or with CsA in combination with the parental mAb 9.2.27 (220 ng/mL) (hatched bars). **(b)** Primary melanoma cells from patient #4 were incubated for 48 h at 37°C with MCSP-ETA' (1 µg/mL) (white bars), with CsA (5 and 10 µg/mL) (black bars), or with both agents in combination (grey bars). Percentage of cell death was measured by PI staining and flow cytometry. Percentage specific cell death is defined as percentage cell death net above background. Data points are mean values of 3 independent experiments and standard deviations are indicated by error bars. Figures indicate the values of the cooperativity index (c_i) calculated from the data.

Table 1
MCSP-ETA' eliminates primary patient derived melanoma cells

patient	sex	age	stage*	distant metastasis*	% MCSP §	ΔMFI #	24h	% specific cell death ‡	72h	96h
1	f	32	IV	M1c	94	84	2	16	14	21
2	f	77	IV	M1b	98	320	6	8	14	25
3	f	40	IV	M1c	64	182	15	26	26	45
4	f	71	III C	-	95	329	12	27	39	44
5	m	30	IV	M1c	98	475	3	4	3	8
6	m	30	IV	M1a	94	425	5	4	7	-
7	f	67	III C	-	99	504	12	19	35	55
8	m	40	IV	M1c	99	627	6	39	57	69
9	f	61	IV	M1c	14	11	2	11	14	17
10	f	59	IV	M1c	97	507	3	4	20	-

* according to [2]

§ percentage of MCSP-positive cells at the beginning of treatment

‡ percentage of cell death above background

mean fluorescence intensity above background