ELSEVIER

Journal of Hepatology 43 (2005) 442–450

Journal of Hepatology

www.elsevier.com/locate/jhep

Protein transduction with bacterial cytosine deaminase fused to the TLM intercellular transport motif induces profound chemosensitivity to 5-fluorocytosine in human hepatoma cells

Annett Hillemann[1], Boerries Brandenburg[1], Ulrike Schmidt[2], Martin Roos[1], Irina Smirnow[2], Marie-Luise Lemken[2], Ulrich M. Lauer[2,†], Eberhard Hildt[1,3,†,*]

[1]*Robert Koch-Institute, Nordufer 20, D-13353 Berlin, Germany*
[2]*Internal Medicine I, University Clinic Tübingen, Tübingen, Germany*
[3]*Institute of Virology, Humboldt-University (Charité), Berlin, Germany*

***Background/Aims*: This study investigates the application of protein based therapeutic suicide enzyme/prodrug approaches providing novel means for both safe and effective local therapeutic regimes in solid tumors.**

***Methods*: Employing a novel cell permeable peptide, known as the translocation motif (TLM) of hepatitis B virus, *E. coli* cytosine deaminase (BCDase) suicide fusion proteins were generated.**

***Results*: TLM fusion proteins formed hexamers (as do parental wtBCDase proteins) and retained the specific enzymatic activity of cytosine conversion to uracil also being comparable to parental wtBCDase proteins. However, only BCDase-TLM fusion proteins, but not TLM-BCDase fusion nor parental wtBCDase proteins were found to be taken up to the cytoplasm of target cells as demonstrated both by confocal laser scanning microscopy and cell fractionation. Uptake of BCDase-TLM worked both efficiently and rapidly and was found to be independent from the endosomal pathway. Since BCDase-TLM fusion proteins completely retained their suicide enzymatic activity in the course of translocation across the plasma membrane their usage as profound inducers of chemo-sensitivity to 5-FC strongly is suggested.**

***Conclusions*: Future therapeutic local application of cell-permeable BCDase-TLM fusion proteins together with a systemic 5-FC prodrug application could result in profound antitumor activities without apparent side effects.**

***Keywords*: Protein transduction; Protein delivery; Cell permeable peptides; Cytosine deaminase; Suicide gene therapy**

1. Introduction

At present, the concept of suicide gene therapy is based mainly on the transfer of genes encoding suicide enzymes of non-mammalian origin which specifically convert an inactive, non-toxic prodrug into a toxic metabolite finally executing the efficient destruction of tumor cells [1,2].

In contrast to this indirect genetically based approach, a direct transfer of the mature suicide protein (i.e. by means of protein transduction) to be applied in selected clinical settings could help to circumvent the manifold risks inevitably associated with therapeutic gene transfer approaches [3–6]. One of the major limitations of protein therapy is the difficulty of efficient intracellular delivery of therapeutic proteins. To overcome this problem, purified CDase of both bacterial as well as of yeast origin (BCDase, YCDase) has been conjugated with antibodies that were able to direct suicide enzymes to tumor cell surfaces [7–9]. Further-more, in vivo implantation of capsules containing YCDase was performed pointing out the potential of CDase protein transduction for both local as well as regional treatments of solid tumors [10].

Received 20 August 2004; received in revised form 12 February 2005; accepted 16 February 2005; available online 4 May 2005

* Corresponding author. Address: Robert Koch-Institute, Nordufer 20, D-13353 Berlin, Germany. Tel.: +49 304547 2528; fax: +49 304547 2328.

E-mail address: e.hildt@rki.de (E. Hildt).

† U.M.L. and E.H. contributed equally to this work.

0168-8278/$30.00
doi:10.1016/j.jhep.2005.02.031

As an alternative, suicide enzymes can be fused to cell-permeable peptides (CPPs) which are defined by their ability to reach the cytoplasmic and/or nuclear compartments in live cells after internalization [11]. In previous studies this has been addressed by generation of viral vectors encoding fusions of suicide genes with the HIV Tat gene [12], the HSV VP22 gene [13–17], or by direct employment either of a (i) malaria circumsporozoite (CS)-BCDase fusion protein [18] or a (ii) HIV-1 Tat basic domain-YCDase fusion protein [19].

Recently, a novel CPP motif was defined as a 12 amino-acid, amphipathic, α-helical peptide, which is derived from the surface protein of hepatitis B virus (HBV) [20]. This translocation motif (TLM) was found to mediate the energy-independent and receptor-independent transfer of peptides, nucleic acids and proteins when fused to them, without affecting the integrity of the peptide transduced cell or interfering with intracellular signal transduction cascades [21–23].

To investigate the potential of cell permeable TLM-based BCDase fusion proteins for direct suicide enzyme applications, we first established eubacterial expression vectors encoding fusion proteins of BCDase and the TLM motif, which was positioned either at the C- or N-terminus. Our findings suggest that cell-permeable BCDase-TLM fusion proteins together with a systemic 5-FC prodrug application could result in profound antitumor activities without apparent side effects.

2. Materials and methods

2.1. BCDase-specific expression constructs

E. coli cytosine deaminase (wtBCDase) was amplified from plasmid pCD2 [28] (kindly provided by R.M. Blaese, NIH, Bethesda, MD) and cloned into pcDNA3 (Invitrogen, Karlsruhe, Germany) [14]. The PreS2-translocation-motif (TLM) from hepatitis-B virus (being defined as the amphipathic α-helix between amino acids 41 and 52 of the PreS2 domain (PLSSIFSRIGDP; subtype ayw; [20])) was introduced as part of a forward primer (5′-CCC ggA TCC Atg CCC TTA TCg TCA ATC TTC TCg Agg ATT ggg gAC CCT ATG TCg AAT AAC gCT TTA CAA ACA (Box 1: BamHI restriction site; Box 2: TLM sequence; Box 3: BCDase nucleotides 1–24); corresponding BCDase amplification primer: 5′-CCC ggA TCC Acg TTT gTA ATC gAT ggC TCC (Box 1: BamHI restriction site; Box 2: BCDase nucleotides 1263–1281)) or a back-ward primer (5′-CCC ggA TCC Agg gTC CCC AAT CCT CgA gAA gAT TgA CgA TAA ggg Acg TTT gTA ATC gAT ggC TTC (Box 1: BamHI restriction site; Box 2: TLM sequence; Box 3: BCDase nucleotides 1263–1281); corresponding BCDase amplification primer: 5′-CCC ggA TCC ATG TCG AAT AAC gCT TTA CAA (Box 1: BamHI restriction site; Box 2: BCDase nucleotides 1–21)).

TLM-BCD amplification products were inserted in both orientations into pcDNA3, yielding TLM-BCDase fusion gene basic constructs pcDNA3-TLM-BCDase or pcDNA3-BCDase-TLM. Finally, insertion of both TLM fusion genes or the wtBCDase gene into the BamHI site of pQe60 (Qiagen, Hilden, Germany) gave rise to C-terminal hexa-his-tag encoding eubacterial expression vectors pQe60-TLM-BCDase, pQe60-BCDase-TLM, pQe60-wtBCDase.

2.2. Protein purification under native conditions

PBS equilibrated Ni-NTA-agarose columns loaded with clarified bacteria lysates were washed with buffer W (20 mM imidazole, 50 mM Tris pH 8.0) removing all weakly binding proteins. Elution was performed by a linear gradient between buffer W and buffer E (250 mM imidazole, 50 mM Tris pH 8.0). Eluate absorbance was monitored at 215, 260, and 280 nm on an AEKTA purifier system (Amersham Biosciences, Freiburg, Germany).

2.3. BCDase-specific antibodies

Rabbits were immunized twice s.c. with 100 μg highly purified BCDase-TLM-6His (day 1: together with Freund′s adjuvant; day 14: together with incomplete Freund adjuvans). 6 weeks later serum was taken, purified and loaded on a N-hydroxy-succinimide (NHS)-activated sepharose column (Amersham Biosciences) containing highly purified immobilized BCDase-TLM-6His protein. Specifically binding proteins were eluted by 0.2 M glycine (pH 2.2).

2.4. Protein analysis

Gel filtration chromatography was performed on a Superose 6 PC 3.2/30 column using an Ettan LC system (Amersham Biosciences) (flow-rate 0.2 ml/min, eluate analysis at 215, 260, and 280 nm). Samples used for SDS-PAGE [29] (20 μg total protein per lane) were adjusted to identical protein concentrations. BCDase-specific rabbit-derived antiserum was instrumental for Western blot analysis. Subcellular fractionation was performed as described recently [20,23]. Membrane reprobing results demonstrated purity of cytosolic (hexokinase-specific antiserum) and microsomal (TNF-RI-specific antiserum) fractions (data not shown).

2.5. Indirect immunofluorescence labelling

Hepatoma cells were grown on cover slides (humidified incubator at 37 °C; 5% CO_2 atmosphere) in Dulbecco's MEM (1000 mg glucose/l plus 10% FCS), incubated with 2 μM purified BCDase-specific fusion proteins and fixed using ice cold DAPI-ethanol. Bound rabbit-derived BCDase-specific antibodies were visualized using Cy3-conjugated goat-derived secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and LSM 510 confocal microscope (63×1.23 objective) (Zeiss, Oberkochen, Germany).

2.6. Quantification of enzymatic activity

Specific activities of purified BCDase proteins were determined by measuring continuously the conversion of cytosine to uracil at 286 nm (starting with 0.5 mM cytosine in 50 mM Tris pH 7.5). Specific activities of cytoplasm translocated BCDase proteins were determined by analytical HPLC (Ettan LC system) using a C18 column (Merck, Darmstadt, Germany) at 254, 267, and 274 nm. Separation of cytosine/uracil was performed at 1.0 ml/min under isocratic conditions using 0.5 M Na-acetate pH 4.6 as eluent. Cell viabilities were determined by SRB assays [14].

2.7. Structure calculation

Employing published 3D structures for BCDase [24], the rasmol program was used for BCDase fusion protein structure calculations.