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Research Article

Evaluation of *In Vitro* Protective Effect of Nanobody/Intrabody against Bacterial Shiga Toxin: Potential Role of ER Retention Signal (KDEL)

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Abstract

Hemolytic-Uremic Syndrome (HUS) clinically presents as a triad of hemolytic anemia, thrombocytopenia and acute renal failure. Mostly, it is caused by infection with certain strains of Shiga toxin-producing *E. coli*. Recently, a novel class of single-domain antibodies (VHH) or "nanobodies" was raised against Shiga toxin (Stx) and was shown to protect Stx-exposed mice. However, Stx is cleared rapidly from the circulation and is generally not detectable at the time of systemic disease onset. We investigated whether anti-Stx nanobodies (Nbs) can be effective in neutralizing Stx intracellularly. Two Nbs with high affinity to the B subunit of Stx2a were selected to investigate their effect against Shiga toxin. The Nb sequence was cloned in-frame into a backbone vector carrying an Endoplasmic Reticulum (ER) localization and retention signals (KDEL), and Green Fluorescence Protein (GFP). Shiga toxin-sensitive cells (hCMEC/D3, and BxPc3) were transfected with the vector, a cytotoxic dose of the toxin was added after 24 hours and cells viability were determined by resazurin assay at different time points. Intracellular ER-localized expression of anti-Stx nanobodies (JFL-47, JGL-34) also the ER-backbone vectors without nanobody insert protected the cell lines at all timepoints. Control plasmids of other signaling peptides [mem: signal sequence targeting plasma membrane+GFP as positive control] or unrelated vectors [N1: empty vector, GFP alone as negative control] did not show significant differences to wildtype. While inconclusive regarding the protective effect of anti-Shiga toxin nanobody constructs, a novel and significant finding of this study is the observed intrinsic, highly protective effect of the ER-targeting sequence (SEKDEL/KDEL).

Keywords: Hemolytic uremic syndrome; Nanobodies; Shiga toxin; STEC; EHEC; Endoplasmic reticulum; KDEL/SEKDEL

Introduction

Hemolytic Uremic Syndrome (HUS) is a clinical condition characterized by a triad of thrombocytopenia, hemolytic anemia and acute renal impairment [1]. In its diarrhea-associated form (D+HUS), infection by Enterohemorrhagic *Escherichia coli* (EHEC) is considered the most common cause in infants and young children (prevalence is about 70%), with an incidence in developed countries (USA and Western Europe) of 2 to 3 per 100,000 cases per year in children below the age of five years [2,3].

Diagnosis of D+HUS is based mainly on the history and clinical presentation of the patient, who typically presents following 2-5 days of diarrhea (often bloody), accompanied by severe abdominal pain and no fever. Although most cases improve spontaneously without sequela, roughly two thirds of patients will continue to develop HUS after several days [4].

Renal impairment can range from microscopic hematuria to proteinuria to anuria requiring dialysis treatment [4,5]. Apart from renal involvement, HUS may also affect multiple other organs or systems in the body. About 25% of patients experience Gastrointestinal (GI) manifestations [6]. Central Nervous System (CNS) involvement was reported in up to 15% of cases [7]. Although spontaneous recovery rates are high, a third of patients will show manifestations of long term renal impairment like proteinuria, hypertension or chronic renal impairment and up to 10% of these patients will develop end stage renal failure [5].

Shiga toxin (Stx) is a family of functionally and structurally related toxins, produced mainly by *S. dysenteriae* and certain strains of enteropathogenic *E. coli*. The human pathogenic isotypes of Stx (mostly Stx2a) are the main contributing factor to the development of HUS [8]. Structurally, Stx is an AB5-type toxin, consisting of one catalytical A-subunit and an identical pentamer of B-subunits, joined together by a non-covalent bonding. Subunit A consists of two domains linked by a disulfide bond: fragment A1, which is enzymatically active, and a carboxyl terminal A2-fragment. B subunits mediate the toxin binding to the eukaryotic cell receptor Globotriaosylceramide (Gb3).

Although the exact mechanisms by which infection with Shiga toxin producing *E. coli* leads to HUS in humans are not yet fully understood, it is thought that the effects of Stx contribute largely to the disease pathogenesis [9]. As Stx is internalized after the B subunit of the toxin binds to GP3 receptor on the cell surface, it travels

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retrogradely through the several cell compartments to the ER, where the toxin fragment A1 is translocated to the cytosol, triggering several stress response systems [10] and eventually enzymatically inactivating the 28S rRNA, thus inhibiting protein synthesis and leading to cell death [11].

For typical or D+HUS, there is currently no clinically successful specific therapy. While there are promising *in vitro* approaches to develop an efficient therapy for Stx-related diseases like HUS, convincing evidence of their efficacy *in vivo* remains elusive [12,13]. So far, several treatment strategies have attempted to counteract Stx directly by employing specific binders (SYNSORB Pk, STARFISH, Daisy), by Stx receptor mimicry, or anti-Stx antibodies [12-14]. Anti-Stx antibodies (Abs) against Stx1 B subunit or Stx2 A subunit proved effective to neutralize the toxin on Stx-sensitive Vero cells. A number of anti-Stx humanized monoclonal Abs developed against Stx2 and its variants proved to be effective in preventing lethal complications of the toxin in mice models when administered after onset of diarrhea [15].

Nanobodies (Nbs) are single domain, antigen-specific antibody fragment. Nbs were first derived from heavy chain antibodies, so called VHH fragments, found in Camillids, a biological family comprising camels, llama and Vicugna, and some cartilaginous fish, such as sharks. Nbs are soluble molecules, chemically and heat stable, and can easily be cloned and expressed in both eukaryotic and prokaryotic cells. Due to the small molecular weight of the Nbs (12-15 kDa), they are comparatively easy to transform into bacterial cells, and expressed in high levels in microorganisms, different mammalian cell lines and plants [16,17].

The Nb's unique physical and biochemical properties make them favorable to be used for different biomedical purposes [17-19]. Recently, Tremblay et al. have developed a set of specific anti-Shiga toxin nanobodies with high affinity to the toxin, and similar approaches have been demonstrated to be effective in ameliorating Shiga toxin effects in a mouse model of HUS [23].

While presenting a promising therapeutic approach for HUS, clinical relevance likely remains limited by the timing of administration necessary to effectively prevent disease progression [20-22]. At this point, Shiga toxin has typically entered the host target cells and is hardly found at detectable levels in the blood [24]. It follows that a clinically feasible and efficient binder strategy will be most effective targeting the toxin inside the host cell to extend this therapeutic "window of opportunity". Therefore, this study assessed whether specific nanobodies targeted to intracellular compartments may have a protective effect against Shiga toxin.

Methods

Plasmids and proteins

Specific anti-Stx nanobodies clones were kindly provided by the group of Dr. Shoemaker from Tufts University, USA. Nanobodies used in this study are JFL-47 (D10) (GenBank accession no. KF551955), and JGL-34 (G1) (GenBank accession no. KF551956) [23].

Compartment specific plasmids pCMV-myc-ER and pECFP-mempEGFP-N, were kindly provided by Felix Bestvater from the light microscopy core facility in DKFZ, Heidelberg, Germany.

The vector pCMV-myc-ER (Life Technologies) is designed to express proteins in the mammalian cells and target them to the endoplasmic reticulum (ER) [25] and was modified to co-express GFP. It contains an ER signal peptide from mouse muscles immunoglobulin H chain (Vh) and SKEDEL peptide sequence as an ER retention signal [26]. pECFP-mem-pEGFP-N (Clontech) is designed for fluorescent labeling of the plasma membrane of mammalian cells [27]. The pEGFP-N1 (Clontech) is a vector which can be transfected into mammalian cells to express EGFP (enhanced GFP) and was used as a transfection marker [28].

All vectors used in this study were transformed into *E. coli* XL1-blue thermally per supplier standard protocol and cells containing the plasmids were purified using PureLink Quick Plasmid Miniprep Kit per manufacturer's instruction (Thermo Fisher Scientific). The DNA concentration was measured by the Nanodrop Spectrophotometer ND-1000.Plasmids containing anti-Stx nanobody and backbone vectors containing compartment-specific localization and retention signals into which Nbs were sub-cloned were verified by Sanger sequencing.

Bacterial strains and cell lines

E. coli XL1-blue (Agilent) competent bacteria were used for cloning. LB medium was used to grow bacteria and for all molecular biology work.

The Shiga toxin-sensitive cell line hCMEC/D3 (immortalized human cerebral microvascular endothelial cells) is a cell model of the blood brain barrier, frequently used in CNS studies [29]. These cells were kindly provided by the group of Gert Fricker at the Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany. BxPC-3, a cell line of human poorly differentiated primary adenocarcinoma of the pancreas, is known to express Gb3 receptor [30] and have been shown to be sensitive to Stx2.

Antibodies

Rabbit anti-E-tag antibody (RayBiotech, Norcross, GA, US), and anti-rabbit alkaline phosphatase IgG from goat (Sigma-Aldrich, MO, USA) were used to confirm the presence of nanobodies.

Cloning of Nbs into compartment specific vectors and purification

To generate the amounts of plasmid needed for subsequent experimental procedures, the nanobody constructs were thermally transformed into competent *E. coli* XL1-blue, where they were grown, lysed and then prepped to yield plasmid content. The plasmids DNA were purified from *E. coli* cultures using PureLink Quick Plasmid Miniprep Kit and its concentrations were determined by Nanodrop Spectrophotometer ND-1000. Prior to cloning, eight anti-Stx nanobodies provided in pET32b vector-containing His-tag and E-tag were amplified to introduce PstI restriction site using PCR LifeECO Thermal Cycler. The analysis of PCR products showed a single amplified band (about 500 bp) corresponding to the expected Nb size, indicating specific domain amplification. The PCR products were purified using QIAquick PCR Purification Kit and concentration was determined by Nanodrop spectrometry. DNA fragments were separated on 1.5% Agarose gel and were visualized under UV light after being stained with Ethidium Bromide (EtBr).

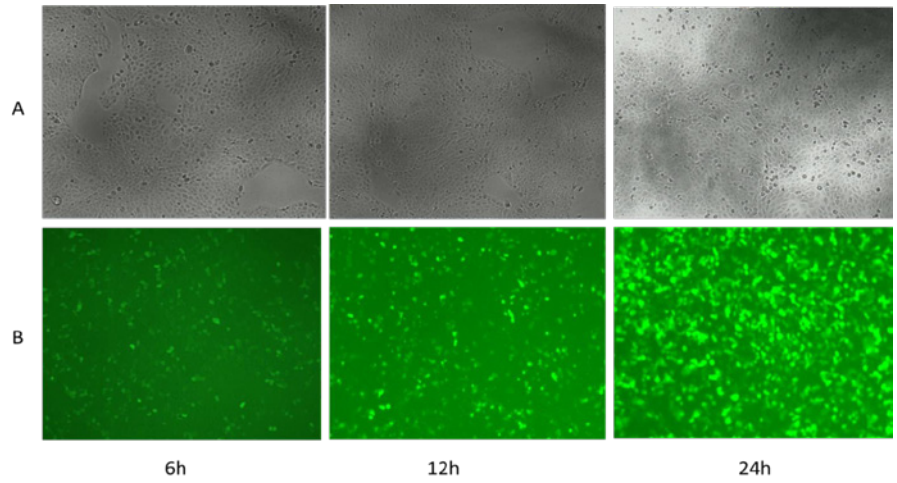

Figure 1: Analysis of treatment efficiency and the impact of transfection on cells at different time points. A) Bright view of cells transfected with GFP-expressing control plasmid pEGFP N1. B) View of the GFP fluorescence confirming the successful BxPC-3 transfection (bright green fluorescence).

For cloning of anti-Stx nanobodies into backbone vectors, a single digestion with PstI were carried out for both nanobody vector and backbone vector. Shrimp Alkaline Phosphatase (rSAP) was added to the backbone vector in order to prevent self-religation. For control purposes, vectors without addition of digestion enzymes were used. Ligation products then were purified using QIAquick PCR Purification Kit. All cloning reagents were bought from New England BioLabs, USA.

The ligation product was transformed into XL1-blue. The transformed bacteria were spread on Agar plates with the appropriate antibiotic and left to grow. Colony PCR were done for eight randomly selected colonies to identify the clones contains the plasmid of interest. DNA sequenced to confirm the orientation of the inserted fragment.

Cell culture

Cell culture work involving Stx, anti-Stx nanobodies and transfection were performed in an S1-safety environment. All cell lines were free of mycoplasma, and cell line contamination was regularly tested by PCR as previously described [31]. BxPC-3 was authenticated by the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

The hCMECcells were grown in EMB-2 medium with 5% fetal bovine serum (FBS), hydrocortisone (1.4 µM), Ascorbic acid (5 µg/ml), 1% Penicillin/Streptomycin (P/S), HEPES (10 mM), Chemically Defined Lipid Concentrate (1/100), and 1 ng/ml of human basic Fibroblast Growth Factor (bFGF). BxPC-3 were grown in Iscove's Modified Dulbecco's Medium (IMDM) with glutamine and HEPES, containing 1% antibiotics (penicillin/ streptomycin), and 10% FBS.

Cells were cultured in flasks of different sizes (from 25 cm^2 to 175 cm^2) at 37°C and 5% CO_2. Medium was changed every 3 days and cell splitting were done when cells reached about 90% confluency.

Cell transfections and viability assay

Two Stx-sensitive cell lines (BxPC-3 and hCMEC/D3) were used in this study. Both hCMEC and BxPC-3 cells ($1.5*10^6$ cells/ml) were seeded in 2 ml of full medium in four of 6-well plates and incubated in 37°C. Transfection was done when cells reached about 70% confluency. For this, 60 µl transfection reagent Lipofectamine 3000 was diluted in 2 ml OPTI-MEM. A transfection mix was prepared by adding 10 µg of plasmid DNA and 15 µl of P3000 to 375 µl of OPTI-MEM per condition. Transfection mix and diluted transfection reagent were mixed in 1:1 ratio and incubated at RT for 15 minthena125 µl of transfection mix/diluted transfection reagent was added in drops to the cells and mixed gently and incubated at 37°C. 24 h after transfection, cells were visualized under fluorescent microscopy to confirm successful transfection. Then, cells of similar experimental condition were pooled together and transferred to four of 24-well plates, 4 wells per condition in each plate, and incubated in 37°C. 48h after transfection, 0.5 ml of 1 µg/l Stx2 was added to two wells per condition in each plate. Each plate had wells with non-transfected cells as control. Viability of cells was assessed with resazurin test at 6 h, 9 h, 18 h and 48 h after adding the Stx.

Data analysis

Raw data of resazurin assays was obtained from reading the plates with a Microplate Reader Infinite 200 PRO, which was then prepared for further analysis in Microsoft Excel. Mean values and standard deviation of each experimental condition were calculated after subtracting background. Percentage of viable cells was determined as ratio of resazurin absorbance of cells treated with Stx to untreated control. For statistical analysis, two-way ANOVA test was performed using Prism to determine how cell viability of each experimental condition is affected by treatment compared to wild type control across several time points.

Results

Cloning and purification of Nbs vectors

Anti-Stx nanobody DNA was amplified by PCR (expected Nb domain size about 500bp) with the introduction of Pst I restriction site to facilitate subsequent subcloning steps. This was followed by standard enzyme digestion, ligation and bacterial transformation protocols that yielded several positive clones, which were screened with colony PCR. Two anti-Stx nanobodies inserts with high binding affinity (Stx2-G1 JGL-34 and Stx2-D10 JFL-47) were successfully

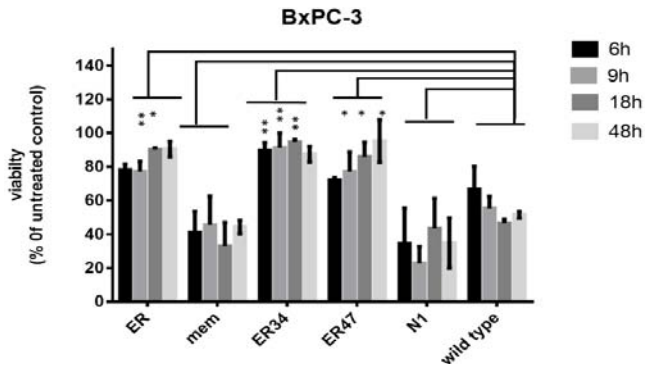

Figure 2: Intracellular effect of anti-Shiga toxin nanobody on BxPC-3. Viability percentage of untreated control after adding 1 µg/l Stx2a to the medium of transfected cells measured at different time points showed significant protective effect of the Nb-ER and ER vectors compared to wild type. (n=3, asterisks indicate significant differences between condition and wild type for each time point, no asterisk: not significant, *: p<0.05, **: p<0.01).

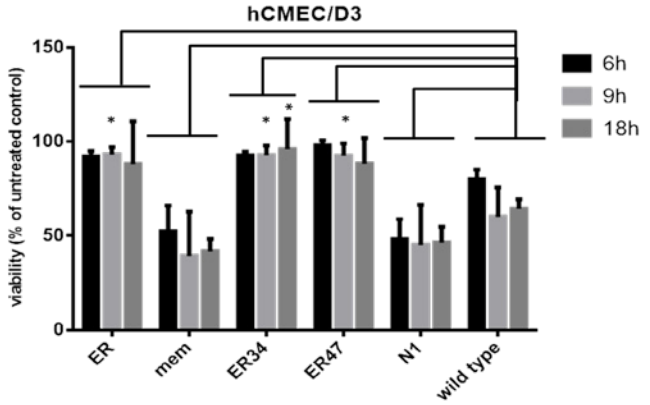

Figure 3: Intracellular effect of anti-Shiga toxin nanobody on hCMEC-D3. Viability percentage of untreated control after adding 1 µg/l Stx to the medium of transfected cells measured at different time points shows significant protective effect of the Nb-ER and ER vectors compared to wild type. (n=3, for N1 n=2, asterisks indicate significant differences between condition and wild type for each time point, no asterisk: not significant, *: p<0.05, **: p<0.01).

cloned in frame into the Pst I site of pCMV-myc-ER vector. The insertion of the Nb in the correct orientation was tested using PCR and confirmed by clone sequencing (results not shown). NBs vectors were routinely purified from *E. coli* according to the manufacturer's plasmid miniprep protocol for further experimental use.

Cell culture and transfection of Stx sensitive cells

Two Stx-sensitive cell lines (BxPC-3 and hCMEC/D3) were transfected with the constructed plasmid, each in four of 6-well plates. In addition to control cells with no nanobody transfection, cells were transfected with an empty vector, which contains only GFP (pEGFP N1), a vector contains membrane signal peptide, and the backbone vector contains ER signal peptide only. GFP was visualized and successful transfection was confirmed. Figure 1 demonstrated GFP fluorescence in BxPC-3 after transfection as an example of regular fruitful DNA uptake by the Stx-sensitive cell lines.

Intracellular neutralization effect of StxNbs

To investigate the intracellular protectivity of the nanobodies, the selected high affinity binding Nbs were sub-cloned in-frame into a vector carrying an ER-localization signal and GFP. Two Stx-sensitive cell lines (BxPC-3 and hCMEC/D3) were transfected with the construct. As nanobody-free controls, cells were also transfected with the following vectors: backbone plasmid with ER-localization signal, backbone plasmid with a membrane localization signal, and an unrelated plasmid with GFP (no localization signal). Non-transfected cells served as additional controls. A 1 µg/l of Stx2 was then added to the cells after 24 h. GFP was visualized and successful transfection was confirmed 24 hours after transfection. Cell viability was determined by resazurin assay after 6 h, 9 h, 18 h and 48 h.

Intracellular ER-localized expression of ER targeted anti-Stx nanobodies JFL-47 and JGL-34 (termed "ER34" and "ER47"), but also ER-backbone vector without nanobody insert protected BxPC-3 at all time points (6 h, 9 h, 18 h and 48 h) compared to wild type. Control plasmids containing membrane localization signal (mem) and empty vector containing only GFP (N1) did not show significant differences to wild type Figure 2. The protective effect of the selected anti-Stx nanobodies were confirmed after transfecting hCMEC/D3. Similar protective effect of Nb cloned in ER vector as well as the ER containing signal alone were seen Figure 3.

There was no significant difference between the effects of the selected anti-Stx nanobodies on the two tested Stx-sensitive cell lines. The average percentage of viable cells determined was similar in both BxPC-3 and hCMEC/D3. After addition of a cytotoxic dose of Stx2 (1 µg/l) for 24 h to the Stx-sensitive cell lines BxPC-3 and hCMEC, cells transfected with either Nb construct (nb47 or nb34) were completely protected from cell death as confirmed by cytotoxicity assay, compared to extensive cell death in wild type or cells transfected with different control vectors.

Discussion

As the onset of systemic symptoms of HUS regularly occurs after clearance of detectable toxin levels from the blood stream,

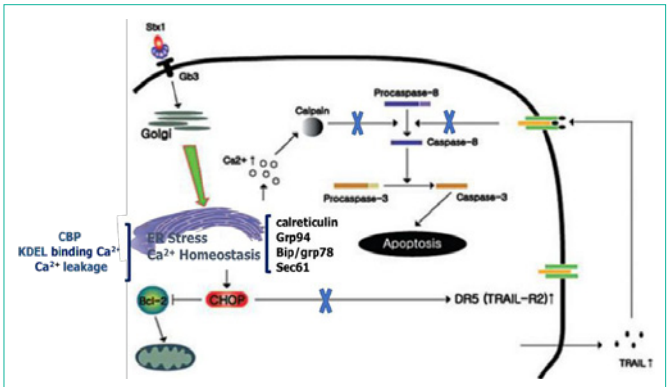

Figure 4: Schematic representation of possible events of cells transfected with KDEL/SEKDEL expressing vector exposed to Shiga toxin. Several mechanisms may apply. 1-Luminal ER calcium exhaustion through Ca^{2+} depletion via KDEL carboxyl groups binding, the increased expression of the Calcium Binding Proteins (CBP), and leakage of Ca^{2+} to the cytosol via the translocon complex resulting in the inability of furin to activate Stx A; 2- In the cytosolic environment KDEL may inhibit the enzymatic activity of the A1-fragment since it mimics protein polynuclotide inhibitors or block apoptotic pathway activation by preventing caspases cascade induction. (X) Indicates inhibition of caspases cascade by SEKDEL tail, Modified from (Lee et al., 2010).

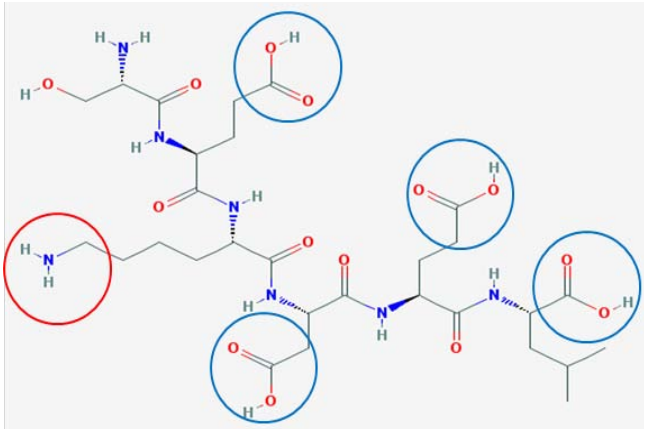

Figure 5: 2D structure of SEKDEL showing multiple free carboxyl groups that can bind Ca^{2+} ions (indicated by blue circles), and a free amino group (indicated by red circle).

we hypothesized that toxin neutralization may be feasible at this intracellular stage of the retrograde toxin transport pathway. A central stage in the pathomechanism of Stx-mediated disease like HUS is the intracellular trafficking and processing of the toxin through the ER, therefore the ER compartment was chosen as a primary deployment target for our intracellular anti-Stx nanobodies. Both constructs with high affinity binders against Stx completely protected the two Stx-sensitive human cell lines.

Contrary to what was expected however, both cell lines transfected with a vector expressing ER signaling peptide (SEKDEL) alone in the absence of Nb also showed a similarly strong protective effect when a cytotoxic dose of Stx was added. Interestingly, the observed protection of Stx-sensitive cells occurred only by overexpression of SEKDEL but not other signaling peptides (e.g., membrane signaling peptide) using the same backbone vector or by transfection of other unrelated control vectors. While we were not able to demonstrate an additional intrinsic benefit of the specific nanobody elements employed, this finding implies a specific mechanism of the SEKDEL sequence interfering with Stx trafficking or processing. Since the ER signaling peptide sequence (SEKDEL) is thought to modify Ca^{2+} homeostasis in the ER, has similar structural properties to known inhibitors of Stx A1 fragment (e.g., protein ploynucleotide inhibitors), and could directly interact with Stx-triggered apoptosis pathways, there are several potential mechanisms by which SEKDEL could interfere with Stx. These are depicted in Figure 4. The two possible scenarios leading to Ca^{2+} disturbance are explained below [inhibition of toxin cleavage by furin and SEKDEL interference with Stx folding and translocation].

Many ER functions as well as Stx trafficking and processing are reliant on shifts in Ca^{2+} homeostasis [32]. The lumen of the ER has the highest concentration of releasable levels of Ca^{2+} in the cell. Changes to ER Ca^{2+} homeostasis including Ca^{2+} depletion lead to disturbance of cell function and stress response initiation and may consequently, depending on the severity and duration of the trigger, lead to cell death [33]. One possible explanation is that the overexpression of the ER-SEKDEL retention signal either interferes with the proteolytic processing of Stx in the ER lumen {here furin, an ER enzyme, which is a calcium-dependent protease needed to process Stx is inhibited. The enzymatic cleaving of the A subunit results in the generation of A1 and A2 fragments, an activation of the toxin with the active component A1 transported to the cytosol [34]} or the translocation of Stx A1 subunit into the cytosol by modulation of calcium homeostasis. In either case, this may lead to Stx being sequestered in the ER and prevented from exhibiting its effect in the cytosol or to Stx being preferentially routed to the ubiquitin protein degradation pathway.

There are several possible mechanisms that may contribute to the depletion of ER Ca^{2+} ions. First, researchers have shown that the binding strength of the carboxylic acid group varies with different metal ions in Arachidic Acid (AA) thin films. Calcium ions have been shown to be the preferential divalent cation for binding [35]. This suggests that the overexpressed SEKDEL and its multiple free carboxyl groups can sequester multiple Ca^{2+} ions [36] and possibly deplete the ER Ca^{2+} store efficiently, hence inhibiting Stx cleaving and activation by furin. Figure 5 shows the 2D structure of SEKDEL where the multiple free carboxyl groups can bind Ca^{2+} ions.

Secondly, the overexpression of SEKDEL coupled with the stress response taxing the protein folding pathway [37] may also lead to leakage of Ca^{2+} ions from the ER membrane, which in turn may be not be sufficiently available for furin to activate Stx. Calcium ion concentration in the ER lumen is normally maintained by a tightly controlled balance of active Ca^{2+} uptake and Ca^{2+} leak [38]. This leak remains the most enigmatic process in Ca^{2+} regulation [39], which may be mediated either by leak channels of yet unknown molecular nature, or *via* the open translocon pore complex of the ER is permeable to ions and neutral molecules [40]. In the latter case, the ribosome-translocon complex has been experimentally shown to mediate calcium leakage from ER stores and is hypothesized to be a major Ca^{2+} leak channel affecting gene expression as well as apoptosis.

Third, it has been demonstrated that Ca^{2+} is buffered inside the ER lumen by multiple proteins that exhibit high Ca^{2+} binding and storage capacity, for example calnexin, calreticulin, calsequestrin, and endoplasmin [38]. Calcium Binding Proteins (CBP) possess a KDEL retention signal and are the most prevalent proteins in the ER lumen [41]. The Ca^{2+} imbalance in the ER triggers stress response, which interferes with CBP expression and leads to stress response initiation. Disturbance of normal ER functions such as Ca^{2+} reduction leads to expression of luminal proteins, including calreticulin and BiP/grp78 [42], which will in turn sequester ER Ca^{2+}. The continued overexpression of SEKDEL leads to the saturation of KDEL receptors causing cellular stress plus availability of SEKDEL carboxyl groups to bind Ca^{2+}, which in turn increases the expression of CBPs [42]. These two mechanisms may act synergistically, where chronic ER Ca^{2+} reduction coupled with recombinant protein overexpression not only initiates stress response in the cell but also upregulates many genes, including non-KDEL CBPs, leading to further intraluminal Ca^{2+} depletion[42]. As a result, depleted Ca^{2+} levels may reduce the activity of furin, a Ca^{2+}-dependent Serine edoprotease, needed to cleave and activate Stx.

Apart from toxin cleaving, SEKDEL expression may also interfere with folding and translocation of Stx. When a protein becomes terminally misfolded, it is ejected into the cytosol and degraded by the proteasome *via* a pathway called ER-Associated Degradation (ERAD). Strikingly, toxins and viruses can hijack elements of the ERAD pathway to access the host cytosol and cause disease [43].

Misfolded proteins are translocated into the cytosol by the ERAD pathway and Sec61 channel, and then degraded by the ubiquitin-proteasome system [44,45]. Free ER Ca^{2+} ions activate the chaperone system as well by interacting with CBPs e.g., calreticulin, calnexin, BiP, and Grp94, to either promote protein folding, or to induce the required degradation components. Since translocation of Stx A1 fragment is a required step in the pathogenesis of Stx, it is conceivable that SEKDEL-inhibition *via* modulation of Ca^{2+} homeostasis may play a role in the observed protective effects.

In conclusion, intracellular neutralizing effect of anti-Stx nanobodies was found to be inconclusive due to ER signaling peptide. However, given the discovery of the robust, specific protective effect of the ER signaling peptide alone against Stx toxicity in human cell lines further studies into this unexpected and promising observation of the KDEL/SEKDEL ER signal is warranted. It appears that Ca^{2+} regulation is a plausible mechanism of the observed ER signal effect. In this regard, a detailed study of Ca^{2+} regulation by SEKDEL may yield the mechanisms behind the observed protection. While transfection of human target cells may prove unfeasible as a clinically applicable treatment strategy, co-opting the mechanisms behind SEKDEL overexpression by other pharmacological means may provide a new therapeutic approach for HUS.

Disclosures

Availability of data and materials

This is an original research article. All materials and data generated or analyzed during this study are retained by SM and the University Hospital Heidelberg as part of her Dr. med degree.

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Authors' Contributions

SM performed experimental planning and execution, literature search, gathered and analyzed data/information, and generated short preliminary write-ups. MWQ supportive framework, draft input, manuscript critical reading and editing. All authors read and approved the final manuscript.

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References

1. Gasser C, et al. Hemolytic-uremic syndrome: bilateral necrosis of the renal cortex in acute acquired hemolytic anemia. Schweiz Med Wochenschr. 1955; 85: 905-909.
2. Salvadori M, Bertoni E. Update on hemolytic uremic syndrome: Diagnostic and therapeutic recommendations. World J Nephrol. 2013; 2: 56-76.
3. Banatvala N, et al. The United States National Prospective Hemolytic Uremic Syndrome Study: microbiologic, serologic, clinical, and epidemiologic findings. J Infect Dis. 2001; 183: 1063-1070.
4. Amirlak I, Amirlak B. Haemolytic uraemic syndrome: an overview. Nephrology (Carlton). 2006; 11: 213-218.
5. Ray PE, Liu XH. Pathogenesis of Shiga toxin-induced hemolytic uremic syndrome. Pediatr Nephrol. 2001; 16: 823-839.
6. Gerber A, et al. Clinical course and the role of shiga toxin-producing Escherichia coli infection in the hemolytic-uremic syndrome in pediatric patients, 1997-2000, in Germany and Austria: a prospective study. J Infect Dis. 2002; 186: 493-500.
7. Boyer O, Niaudet P. Hemolytic uremic syndrome: new developments in pathogenesis and treatment. Int J Nephrol. 2011; 2011: 908407.
8. Melton-Celsa AR. Shiga Toxin (Stx) Classification, Structure, and Function. Microbiol Spectr. 2014; 2: Ehec-0024-2013.
9. Proulx F, Seidman EG, Karpman D. Pathogenesis of Shiga toxin-associated hemolytic uremic syndrome. Pediatr Res. 2001; 50: 163-171.
10. Tesh VL. Activation of cell stress response pathways by Shiga toxins. Cell Microbiol. 2012; 14: 1-9.
11. Mukhopadhyay S, Linstedt AD. Retrograde trafficking of AB (5) toxins: mechanisms to therapeutics. J Mol Med (Berl). 2013; 91: 1131-1141.
12. Scheiring J, Andreoli SP, Zimmerhackl LB. Treatment and outcome of Shiga-toxin-associated hemolytic uremic syndrome (HUS). Pediatr Nephrol. 2008; 23: 1749-1760.
13. Appel GB. Thrombotic microangiopathies: Similar presentations, different therapies. Cleve Clin J Med. 2017; 84: 114-130.
14. Paton AW, Morona R, Paton JC. A new biological agent for treatment of Shiga toxigenic Escherichia coli infections and dysentery in humans. Nat Med. 2000; 6: 265-270.
15. Sheoran AS, et al. Human antibody against shiga toxin 2 administered to piglets after the onset of diarrhea due to *Escherichia coli* O157:H7 prevents fatal systemic complications. Infection and immunity. 2005; 73: 4607-4613.
16. Möller A, et al. Intracellular activation of interferon regulatory factor-1 by nanobodies to the multifunctional (Mf1) domain. J Biol Chem. 2010; 285: 38348-38361.
17. Muyldermans S. Nanobodies: Natural Single-Domain Antibodies. Annual Review of Biochemistry. 2013; 82: 775-797.
18. Hassanzadeh-Ghassabeh G, et al. Nanobodies and their potential applications. Nanomedicine (Lond). 2013; 8: 1013-1026.
19. Van Audenhove, I. and J. Gettemans, Nanobodies as Versatile Tools to Understand, Diagnose, Visualize and Treat Cancer. EBioMedicine. 2016; 8: 40-48.
20. Grisaru S. Management of hemolytic-uremic syndrome in children. Int J Nephrol Renovasc Dis. 2014; 7: 231-239.
21. Melton-Celsa AR, O'Brien AD. New Therapeutic Developments against Shiga Toxin-Producing Escherichia coli. Microbiol Spectr. 2014; 2.
22. Lo AW, et al. The molecular mechanism of Shiga toxin Stx2e neutralization by a single-domain antibody targeting the cell receptor-binding domain. J Biol Chem. 2014; 289: 25374-25381.
23. Tremblay JM, et al. A single VHH-based toxin-neutralizing agent and an effector antibody protect mice against challenge with Shiga toxins 1 and 2. Infect Immun. 2013; 81: 4592-603.
24. Lopez EL, et al. An epidemiologic surveillance of Shiga-like toxin-producing Escherichia coli infection in Argentinean children: risk factors and serum Shiga-like toxin 2 values. Pediatr Infect Dis J. 2012; 31: 20-24.
25. Persic L, et al. An integrated vector system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries. Gene. 1997; 187: 9-18.
26. Sequences of proteins of immunological interest, E.A. Kabat, Editor. 1987,

U.S. Dept. of Health and Human Services, Public Health Service, National Institutes of Health: Bethesda, Md.

27. Moriyoshi K, et al. Labeling neural cells using adenoviral gene transfer of membrane-targeted GFP. Neuron. 1996; 16: 255-260.

28. Inouye S, Tsuji FI. Evidence for redox forms of the Aequorea green fluorescent protein. FEBS Lett. 1994; 351: 211-214.

29. Weksler BB, et al. Blood-brain barrier-specific properties of a human adult brain endothelial cell line. Faseb j. 2005; 19: 1872-1874.

30. Tan MH, et al. Characterization of a new primary human pancreatic tumor line. Cancer Invest. 1986; 4: 15-23.

31. Schmitt M, Pawlita M. High-throughput detection and multiplex identification of cell contaminations. Nucleic Acids Res. 2009; 37: e119.

32. Michalak M, Robert Parker JM, Opas M. Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. Cell Calcium. 2002; 32: 269-278.

33. Mekahli D, et al. Endoplasmic-reticulum calcium depletion and disease. Cold Spring Harbor perspectives in biology. 2011; 3: a004317.

34. Garred O, van Deurs B, Sandvig K. Furin-induced cleavage and activation of Shiga toxin. J Biol Chem. 1995; 270: 10817-10821.

35. Bala T, et al. Interaction of different metal ions with carboxylic acid group: a quantitative study. J Phys Chem A. 2007; 111: 6183-6190.

36. Treves S, et al. Calreticulin is a candidate for a calsequestrin-like function in Ca^{2+}-storage compartments (calciosomes) of liver and brain. Biochem J. 1990; 271: 473-480.

37. Yamamoto K, et al. The KDEL receptor modulates the endoplasmic reticulum stress response through mitogen-activated protein kinase signaling cascades. J Biol Chem. 2003; 278: 34525-34532.

38. Ashby MC, Tepikin AV. ER calcium and the functions of intracellular organelles. Semin Cell Dev Biol. 2001; 12: 11-17.

39. Camello C, et al. Calcium leak from intracellular stores--the enigma of calcium signalling. Cell Calcium. 2002; 32: 355-361.

40. Van Coppenolle F, et al. Ribosome-translocon complex mediates calcium leakage from endoplasmic reticulum stores. J Cell Sci. 2004; 117: 4135-4142.

41. Sandvig K, et al. 10-Shiga toxins: properties and action on cells, in The Comprehensive Sourcebook of Bacterial Protein Toxins (Fourth Edition), J. Alouf, D. Ladant, and M.R. Popoff, Editors. 2015, Academic Press: Boston. 2015; 267-286.

42. Llewellyn DH, Roderick HL, Rose S. KDEL receptor expression is not coordinatedly up-regulated with ER stress-induced reticuloplasmin expression in HeLa cells. Biochem Biophys Res Commun. 1997; 240: 36-40.

43. He K, Ravindran MS, Tsai B. A bacterial toxin and a nonenveloped virus hijack ER-to-cytosol membrane translocation pathways to cause disease. Crit Rev Biochem Mol Biol. 2015; 50: 477-488.

44. Tsai B, Ye Y, Rapoport TA. Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. Nat Rev Mol Cell Biol. 2002; 3: 246-255.

45. Zehner M, et al. The translocon protein Sec61 mediates antigen transport from endosomes in the cytosol for cross-presentation to CD^{8+} T cells. Immunity. 2015; 42: 850-863.

46. Mejías MP, Hiriart Y, Lauché C, Fernández-Brando RJ, Pardo R, Bruballa A, et al. Development of camelid single chain antibodies against Shiga toxin type 2 (Stx2) with therapeutic potential against Hemolytic Uremic Syndrome (HUS). 2016; 6: 24913.