Abstract

Backgrounds: Coxsackievirus B3 (CVB3) is a member of the family Picornaviridae, and along with polioviruses, belongs to the Enterovirus genus. The CVB3 genome is composed of a single-stranded RNA encoding polyproteins, which are cleaved to individual functional proteins by 2A and 3C proteases proteins which have been targeted for drug development. Here, we showed that protease activity required to activate a toxic protein may be used to prevent viral infection.

Methods: We modified the MazE-MazF antitoxin-toxin system of Escherichia coli to fuse a C-terminal fragment of MazE to the N-terminal end of toxin MazF with a linker having a specific protease cleavage site for CVB3. This fusion protein formed a stable dimer and was capable of inactivating the mRNA interferase activity of MazF which cleaves the ACA sequence in mRNA substrates.

Results: The incubation of 2A proteases with the fusion proteins induced cleavage between the MazE and MazF fragments from the fusion proteins; the subsequent release of MazF significantly inhibited virus replication. Additionally, we note that, CVB3 infected HeLa cells quickly died through a MazF toxin mediated effect before virus protein expression.

Conclusion: These findings suggest that the MazEF fusion protein has a strong potential to be developed as an anti-virus therapy following CVB3 infection.

Keywords: Coxsackievirus, MazEF, Antitoxin, Cardiotropic, Polyprotein, Proteases

Introduction

Coxsackievirus B3 (CVB3) is a cardiotropic virus that binds to the coxsackievirus adenovirus receptor to infect host cells1-3. CVB3 is a member of the family Picornaviridae, and along with polioviruses, it belongs to the Enterovirus genus. Although most enterovirus infections are subclinical, acute myocardial inflammation triggered by these infections can induce severe arrhythmias and sudden cardiac death or may lead to the development of chronic myocarditis and dilated cardiomyopathy4-10. We previously reported several antiviral drugs developed from small chemical compounds. An inhibitor of enterovirus protease 3C (3CP) showed a particularly strong antiviral effect in a murine viral myocarditis model. It significantly reduced viral replication in the heart and minimized myocardial damage11.

The proteases encoded by RNA viruses, such as Coronavirus, hepatitis A virus (HAV), and CVB3, play an essential role in viral infection, as they are required for the processing of virus-encoded polyproteins12. Most bacteria have the toxin-antitoxin (TA) system which is a mechanism to control the cell growth. Toxin induces cell growth arrest and even cause cell death without the co-expression their cognate antitoxins13,14. Thus, virus proteases have been considered ideal drug targets. In the present report, we attempted to positively use the activity of the viral proteases to activate a latent toxin (MazF) of Escherichia coli from a toxin-antitoxin (MazF-MazE) fusion protein by cleaving off antitoxin MazE fragment. MazF thus released functions as an ACA-specific mRNA interferase15 to eliminate almost all cellular mRNA as well as single-stranded viral RNA in the vi-
rus-infected cells.

The CVB3 genome, consisting of a positive single strand RNA 7400 bases, encodes three major polyproteins (P1, P2, P3) which require processing by the CVB3 protease, 2A. As these viral proteases cleave polyproteins at highly specific amino acid sequences (AXXQ;G), these specific protease cleavage sites for individual RNA viruses may be incorporated into the linker between MazE antitoxin and MazF toxin of the MazE-MazF fusion proteins (MazEF) so that viral proteases induced upon infection cleave the linker to activate MazF as an ACA-specific mRNA interferase. MazF has been shown to be a potent toxin, effectively causing apoptotic cell death in mammalian cells. In E. coli, two dimers of MazF form a stable heterohexamer complex with one MazE dimer in the center.

In this study, we developed a new enterovirus anti-viral system using bacteria to drive the anti-toxin and toxin system. The MazEF toxin significantly inhibited virus replication with viral protease 2A activating the bacterial MazEF toxin. Here, we also note that CVB3 infected HeLa cells were quickly destroyed through a MazEF toxin effect before virus protein expression. These findings suggest that MazEF has the potential to be developed as an anti-viral drug for CVB3 infections.

Materials & Methods

Virus and cell lines

CVB3 was derived from an infectious cDNA copy of the cardio tropic H3 variant of CVB3. Virus titers were determined using the plaque-forming assay in HeLa cells as described previously. HeLa cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, WELGEN Inc, Gyeongsan-si, KOR) supplemented with 10% fetal bovine serum. HeLa-UVM cells were obtained from Dr. ES Jeon (Samsung Medical Center, Seoul, Korea).

Construction of protease 2A cleaved MazEF fusion proteins

We previously generated a MazEF fusion protein with a linker containing a specific cleavage site for HCV proteases (Figure 1A). We have modified the specific cleavage site between C-terminal region of MazE (MazEΔ1-41) and MazF genes from E. coli for CVB3 protease 2A and added four extra residues (Gly-Gly and Gly-Ser at the N-terminal and the C-terminal ends, respectively) (Figure 1B).

In vitro HeLa cell survival assay

We generated a MazEF working system into green fluorescence protein plasmid (pEGFP) for the in vitro cell
survival assay. In brief, HeLa cells were transfected with the MazEF/pEGFP plasmid for 24 hours followed by addition of CVB3 at multiplicity of infection (m.o.i.) of 1 (10 μL) and 2 (20 μL). After 12 hours of infection, we observed the impact on cell morphology and cytopathic effects using fluorescence microscope, and extracted protein for western blot analysis.

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% NP40, 150 mM NaCl, 0.5% sodium deoxycholate). Aliquots (10 μg) of total cell extracts were loaded onto 10% SDS-PAGE gels. Following gel electrophoresis, proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked in 5% non-fat dry milk solution in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and incubated with anti-enterovirus VP1 (1:1000, mouse monoclonal; Novocastra, Lincolnshire, IL, USA), eIF4G1 (1:1000, rabbit polyclonal; Cell Signaling Technology, Danvers, MA, USA), and GAPDH antibodies (1:1000, rabbit polyclonal antibodies; Cell Signaling Technology, Danvers, MA, USA)19.

Statistics

The data are presented as the mean ± SEM. Differences in measured parameters between control and target experimental groups were examined using the Mann-Whitney U test (GraphPad Prism 3.0 for Windows; GraphPad Software, La Jolla, CA, USA). Survival rates were analyzed using the Kaplan-Meier method. P < 0.05 was considered significant.

Results

MazEF induce virus infected cell death

To confirm the antiviral effect of the MazEF toxin, we have overexpressed GFP conjugated MazEF (MazEF/pEGFP) or MazEF only (MazEF/pcDNA3). After 12 hours, CVB3 was added into the cell culture plate and to confirm the cytopathic effect on HeLa cells. As shown in Figure 2A, MazEF-GFP expressing HeLa cells indicate MazEF toxin expression and these cells experienced a greater cytopathic effect in a virus concentration dependent manner compare to GFP overexpressed cells. Western blot analysis revealed that MazEF toxin significantly decreased CVB3 replication in CVB3 infected cells, resulting in a reduction in de-
detectable virus capsid protein VP1 compare with control cells (Figure 2B).

Target inhibition of virus replication
The overexpression of MazEF toxin in HeLa cells resulted in rapid cell death and reduced expression of virus capsid protein VP1 in CVB3 infected cells. Translation initiation factor protein eIF4G1 was cleaved by viral protease 2A soon after CVB3 infection and inhibited host protein production. As shown in Figure 3, cleaved eIF4G1 was detected in CVB3 infected cells. However, viral capsid protein VP1 was significantly decreased in MazEF toxin treated HeLa cells, because cell death is induced by the MazEF toxin before virus replication. These results demonstrated that CVB3 infected HeLa cells were killed by direct overexpression of MazEF toxin before initiation of virus replication.

Figure 3. CVB3 protease 2A induce MazEF cell toxicity. (A) CVB3 protease 2A expressed in CVB3 infected HeLa cell and then cleaved host transcription initiation factor eIF4G1. MazEF-GFP expression reduced virus capsid protein VP1 expression. Virus replication was significantly inhibited by the MazEF toxin. (B) Data are presented as the mean ± SEM from 3 independent experiments (***, P < 0.01).

Figure 4. MazEF inhibit CVB3 genome replication. CVB3 protease 2A activates the MazEF fusion protein which led to rapid death of HeLa cells infected with CVB3. MazEF expression inhibited the CVB3 capsid protein VP1 positive and negative strand RNA amplification. Virus genome amplification was significantly inhibited by MazEF toxin. Data are presented as the mean ± SEM from 3 independent experiments (***, P < 0.01).

MazEF expression inhibit CVB3 genome replication
Amplification of viral RNA amplification is essential for viral replication. However, MazEF toxin resulted in rapid cell death in virus infected cells. We confirmed the CVB3 capsid protein positive and negative strand RNA replication using RT-PCR. In CVB3 infected HeLa cells, CVB3 genome expression was significantly decreased in cells expressing the MazEF fusion protein compared with control cells (Figure 4). CVB3 protease2A modified the structure of the MazE and MazF proteins. MazEF fusion resulted in the rapid death of CVB3 infected cells which subsequently affects virus genome amplification.

Discussion
Xiong et al. have previously demonstrated in vitro that the entero viral protease 2A directly cleaves murine dystrophin in the hinge 3 region in order to determine whether this cleavage plays an important role in
the development of enterovirus-mediated myocarditis, sarcolemma membrane disruption, viral propagation and direct virus-mediated cytopathic effect in vivo.\textsuperscript{21}

In this study, we applied this concept to inhibit virus propagation and systemic side effects accompanying in CVB3 infection. The first stage of viral replication involves processing of the protease 2A into a polyprotein; this is a critical time point to inhibit virus amplification and propagation to the other cells.

Since these viral proteases cleave polyproteins at highly specific amino acid sequences, these specific protease cleavage sites for individual RNA viruses may be incorporated into a linker between the MazE antitoxin and MazF toxin of the MazE-MazF fusion protein so that viral proteases induced upon infection cleave the linker to activate MazF as an ACA-specific mRNA interferase. Activation of MazF in mammalian cells has been result in BAK-dependent apoptotic cell death.\textsuperscript{16} We could regulate CVB3 infected cell death through MazEF fusion protein induction by CVB3 expressed protease 2A. In addition, the fusion protein may potentially be used as a tool for the prevention and/or treatment of RNA virus infections, as cells carrying the fusion protein with a linker cleavable with a specific RNA viral protease may attain resistance to a RNA viral infection.\textsuperscript{18} However, the MazEF fusion protein in vivo systemic effect is not clear and additional research is required to better elucidate this.

These results suggest that the new application for CVB3 protease 2A induction of bacterial toxin protein, MazEF, is applicable to the treatment of many single-strand RNA virus infections, (eg, CVB3 myocarditis, enterovirus71, and influenza virus). MazEF induction was confirmed in CVB3 infected HeLa cells and it was strongly suppressed virus replication and genome amplification. Therefore, the MazEF toxin system could be beneficial in the treatment of cardiomyopathy associated with enterovirus infection.

Conclusion

CVB3 is the main cause of viral myocarditis and meningoitis in childhood. However, effective antiviral drugs have not yet been developed. Our TA system leveraging the MazEF fusion protein, is a potential targeted antiviral system. MazEF activation is regulated by target virus protease expression and may be a potential therapeutic target in human CVB3 infections.

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Conflict of Interest Jung-Ho Park, Jin-Ho Park, Wonho Choi, & Byung-Kwan Lim declares that they have no conflict of interest.

Human and animal rights The article does not contain any studies with human and animal and this study was performed following institutional and national guidelines.

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