ABSTRACT

Actinoporin Equinatoxin II (EqtII), a sea anemone cytolyisin, has been well known to lyse cells through forming pores on the cell membrane. We show here that an exogenous expression of the EqtII (S105-P142) fragment through gene transferring significantly induced apoptosis in human hepatocellular carcinoma cell line BEL-7404. The EqtII (S105-P142) expression did not alter the cleavage of PARP, as well as levels of Bcl-2 and Bax. Furthermore, the observation under a confocal laser scanning microscope showed that EqtII (S105-P142) colocalized with mitochondria, but not with endoplasmic reticulum and Golgi apparatus. Therefore, the EqtII (S105-P142) fragment was dramatically different from previously reported full length EqtII, which specifically colocalized with Golgi apparatus. The cytotoxicity of EqtII (S105-P142) reported here may provide insights into the utilization of EqtII (S105-P142) as an anticancer agent in future cancer gene therapy.

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Key words: Equinatoxin II; EqtII (S105-P142); Cancer gene therapy

INTRODUCTION

Diversified bioactive compounds have been identified from marine bioreources such as plants, mollusk animals, fishes, and fungi, and were found exhibiting potent anti-fungi and antitumor activity, as well as the capability to cure cardiovascular and cerebrovascular disease[1-4]. Sea anemones produce a variety of toxins which are classified into three types: sea anemone neumtomns, sea anemone cytolysins, and sea anemone potassium channel inhibitors[5]. Sea anemone pore-forming toxins are typical cytolysins, which elicit cytotoxicity through forming pores on target cell membrane[5,6]. Equinatoxin II (EqtII), an actinoporin isolated from the sea anemone Actinia equina, is an archetypal example of α-helical pore forming toxins[6,7]. EqtII specifically binds sphingomyelin[8-10], an important component concentrated in cell membrane lipid rafts, which provide binding targets for a variety of pathogens and toxins to penetrate the plasma membrane. EqtII has become a useful marker to detect subcellular distribution of sphingomyelin[11]. Previously, an intracellular expression of EqtII-GFP fusion proteins was developed to detect the colocalization of EqtII with Golgi apparatus, but not with other organelles including mitochondria, endoplasmic reticulum (ER), and nucleus, revealing the cytoplasmic exposure of Golgi sphingomyelin[12].

Previous studies have shown that the EqtII N-terminal α-helix (amino acid 11-31) is responsible for membrane penetrating, whereas the internal tryptophan-rich region is involved in initial membrane attachment[13,14]. In this work, a sequence encoding the S105-P142 internal region including the tryptophan-rich motif was inserted into plasmid pEGFP-C1 to generate pEGFP-EqtII (S105-P142)-C1. Human cancer cells were transfected with pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1. The exogenous expression, cytotoxicity, and subcellular distribution of EqtII (S105-P142) were analyzed.

MATERIALS AND METHODS

Cell culture, transfection, and flow cytometry

BEL-7404 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). L02 cell line was purchased

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from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution, and 1% L-Glutamine. Appropriate amounts of plasmids were transfected into cells by Thermo Scientific TurboFect Transfection Reagent (Thermo Fisher Scientific Inc, Canada) following the manufacturer’s instruction.

For flow cytometry analysis, BEL-7404 cells were transfected with Plasmids pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1. After 48 h, cells were harvested and stained with Annexin V-PE (BD Biosciences, San Jose, CA, USA) followed by analyzing under a BD FACSAria flow cytometry (BD Biosciences) to detect apoptotic cells. Cells without staining served as the negative control.

Plasmid construction

The plasmid pGH-genes carrying DNA sequences encoding EqtII (S105-P142) were purchased from Shanghai Generay Biotech Co., Ltd, China. The sequences were cut with XhoI, and then inserted into the corresponding site of pEGFP-C1 to form plasmids pEGFP-EqtII (S105-P142)-C1.

Western blotting analysis

The cell extracts were subjected to SDS–PAGE and electroblotted onto the nitrocellulose membrane. The membrane was then blocked with Tris-buffered saline and Tween 20 containing 5% of bovine serum albumin at room temperature for 2 h and incubated with primary antibodies overnight at 4°C. The membrane was washed and incubated with appropriate dilution of IRDye 800 donkey anti-mouse IgG or IRDye 700 donkey anti-rabbit IgG (LI-COR, Inc., Lincoln, NA, USA) for 1 h at room temperature. After washing with Tris-buffered saline, the membrane was then analyzed by an Odyssey Infrared Imaging System (LI-COR, Inc.)

Mouse anti-GFP antibody was purchased from Abmart Inc. (Shanghai, China). Rabbit anti-caspase3 antibody, anti-PARP antibody, anti-Bcl-2 antibody, and rabbit anti-GAPDH antibody were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit anti-Bax antibody was purchased from Epitomics (Burlingame, CA, USA).

Subcellular staining and colocalization study

ER-Tracker Red and Golgi-Tracker Red were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Mito Tracker Red Mitochondrion-Selective Probe was purchased from Invitrogen (Grand Island, NY, USA). Cells were plated on cover glass bottom dish (35 mm) at 1×10^5 per dish one day before transfection with plasmids. Cells were then transfected with plasmids as previously stated for 24 h or 48h. The cells were then stained with ER-Tracker Red, Golgi-Tracker Red or Mito Tracker Mitochondrion Selective Probes followed by observation under a confocal laser scanning microscope (Nikon, Inc., Japan).

RESULTS

EqtII (S105-P142) fragment was capable of being exogenously expressed in human cancer cells

A sequence encoding the S105-P142 region of EqtII was inserted into pEGFP-C1 to generate the pEGFP-EqtII (S105-P142)-C1 plasmid. To determine the exogenous expression of EqtII (S105-P142) in human cells, pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1 plasmids were transfected into L02 and BEL-7404 human cancer cells and the expression of EqtII (S105-P142) was examined by western blotting analysis with an anti-GFP antibody. Results showed that both enhanced green fluorescence protein (EGFP) and EGFP-EqtII (S105-P142) with molecular weights as expected were detected in these cells (Figure 1A), indicating that EqtII (S105-P142) is capable of being expressed in human cancer cells.

EqtII (S105-P142) expression induced apoptosis in BEL-7404 cells

We then examined the effect of EqtII (S105-P142) expression on hepatocellular carcinoma BEL-7404 cells. Plasmids pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1 were transfected into BEL-7404. After 48 h, cells were harvested and stained with Annexin V-PE to detect apoptosis. As compared to pEGFP-C1 transfected cells which showed 0.494 ± 0.0339 of Annexin V-positive cells, upon the EGFP-EqtII (S105-P142)-C1 transfection, the percent of Annexin V-positive cells were significantly promoted to 7.22 ± 1.6140 (Figure 1B). Results indicated that the exogenous expression of EGFP-EqtII (S105-P142) induced apoptosis in BEL-7404 cells. Furthermore, pEGFP-EqtII (S105-P142)-C1 transfection also induced cell death in normal liver cell line L02 (data not shown), suggesting that the cytotoxicity of EqtII (S105-P142) fragment is not cancer specific.

To analyze the mechanism of EqtII (S105-P142)-induced apoptosis, apoptotic signal elements including poly (ADP-ribose) polymerase (PARP), Bax, and Bcl-2 were examined by Western blot. As shown in Fig. 1c, EqtII (S105-P142) did not significantly alter the cleavage of PARP, as well as the levels of proapoptotic factors Bcl-2 and Bax. Our densitometry analysis further confirmed that levels of these proteins were not significantly altered. Results suggested that EqtII (S105-P142) may induce apoptosis in Bel-7404 cells through other apoptotic signaling pathways pending further investigations.

EqtII (S105-P142) colocalized with mitochondria

Full length EqtII has been determined to specifically colocalize with Golgi apparatus [10,12]. To further illustrated the subcellular distribution of EqtII (S105-P142). Plasmid pEGFP-EqtII (S105-P142)-C1 was transfected into BEL-7404. Cells were further stained with ER-Tracker Red, MitoTracker Mitochondrion-Selective Probes, and Golgi-Tracker Red. Results showed that EGFP-EqtII (S105-P142) did not colocalized with endoplasmic reticulum stained with ER-Tracker Red (Figure 2A) and Golgi apparatus stained with Golgi-Tracker Red (Figure 2B). Interestingly, EGFP-EqtII (S105-P142) was shown colocalizing with mitochondria stained with the MitoTracker Mitochondrion-Selective Probe (Figure 2C). Taken together, our data determined that EqtII (S105-P142) located in mitochondria, but not in Golgi apparatus and ER.

DISCUSSION

Previous studies revealed that full length EqtII colocalized with Golgi apparatus, but not with mitochondria and ER[10,12]. However, our data showed that the EqtII (S105-P142) fragment colocalized with mitochondria, but not with Golgi apparatus and ER. Importantly, the expression of EqtII (S105-P142) significantly induced apoptosis. The dramatic locating and functional diversity between full length EqtII and the EqtII (S105-P142) fragment may be due to the deletion of both N-terminal and C-terminal domains from EqtII resulted in a significant conformational change and subsequently exposed cryptic active sites, which showed binding specificity to molecules other than sphingomyelin, a molecule exposed on the Golgi apparatus as detected by full length EqtII[10,12]. In fact, our
Figure 1 The expression and cytotoxicity of EqII (S105-P142). A: The expression of EGFP-EqtII (S105-P142) in human cells. Transformed hepatic cell line L02 and hepatocellular carcinoma cell line BEL-7404 were transfected with pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1 plasmids. Cell lysates were collected after 48h and analyzed by western blot with antibodies against EGFP or GAPDH. B: Hepatocellular carcinoma cell line BEL-7404 was transfected with pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1 plasmids. After 48h, cells were harvested and stained with Annexin V-PE followed by flow cytometry analysis. Shown are representatives from 4 repeats and the statistics (p<0.05). C: Hepatocellular carcinoma cell lines BEL-7404 were transfected with pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1 plasmids. Cell lysates were collected after 24h or 48h and analyzed by western blot with antibodies against Bax, Bcl-2, PARP, and GAPDH.

Figure 2 Localization of EGFP-EqtII (S105-P142). A: BEL-7404 were transfected with pEGFP-EqtII (S105-P142)-C1, and then stained with ER-Tracker Red at 24 h after transfection. The merge image was overlay of EGFP-EqtII (S105-P142) image and ER-Tracker Red stained image. B: BEL-7404 cells were transfected with pEGFP-EqtII (S105-P142)-C1 and stained with Golgi-Tracker Red. C: BEL-7404 cells were transfected with pEGFP-EqtII (S105-P142)-C1 and stained with MitoTracker MitochondriaSelective Probes. All images were taken under 40× oil immersion objective of a confocal laser scanning microscope. Shown are representatives from at least 4 repeats. Bars show 20 μm.
recent finding showing that EqtII (S105-P142) interacted with protein arginine methyltransferase 5 may have provided a further support for this hypothesis\(^{[3]}\).

As a cytolysin, EqtII is capable of lysing cells through inserting the N-terminal α-helix into cell membrane and forming pores\(^{[4]}\). Therefore, systemic administration of EqtII may result in deleterious effects. However, without the N-terminal α-helix, the EqtII (S105-P142) fragment may lose the pore-forming function, resulting in a safer EqtII isotype for medical use. Further tumor-specific controlling of EqtII (S105-P142) expression may provide insights into a novel role of EqtII (S105-P142) as an anticancer agent in future cancer gene therapy.

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CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

REFERENCES


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