Reporter Gene Assay for Detection of Shellfish Toxins[1]

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Objective To explore the potential reporter gene assay for the detection of sodium channel-specific toxins in shellfish as an alternative for screening harmful algal bloom (HAB) toxins, considering the fact that the existing methods including HPLC and bioassay are inappropriate for identifying HAB toxins which poses a serious problem on human health and shellfish industry. **Methods** A reporter plasmid pEGFP-c-fos containing c-fos promoter and EGFP was constructed and transfected into T24 cells using LipofectAMINE 2000. Positive transfectants were screened by G418 to produce a pEGFP-c-fos-T24 cell line. After addition of increasing neurotoxic shellfish poison (NSP) or GTX2,3, primary components of paralytic shellfish poison (PSP), changes in expression of EGFP in the cell line were observed under a laser scanning confocal microscope and quantified with Image-pro Plus software. **Results** Dose-dependent changes in the intensity of green fluorescence were observed for NSP in a range from 0 to 10 ng/mL and for GTX2,3 from 0 to 16 ng/mL. **Conclusion** pEGFP-c-fos-T24 can be applied in detecting HAB toxins, and cell-based assay can be used as an alternative for screening sodium channel-specific HAB toxins.

Key words: Shellfish poisoning; Green fluorescent protein; Harmful algal bloom toxins

INTRODUCTION

One of the determinants of harmful algal bloom (HAB) is HAB toxins produced by some algae, which accumulate in shellfish and subsequently result in poisoning of humans and animals that consume shellfish. In general, HAB toxins include paralytic shellfish poison (PSP), diarrhetic shellfish poison (DSP), neurotoxic shellfish poison (NSP), amnesia shellfish poison (ASP) and ciguatera, *etc*.[1] Due to the lack of convenient yet sensitive screening assays, poisoning by HAB toxins poses a serious problem on human health and shellfish industry. Mouse bioassay and HPLC analysis have been used extensively for assaying HAB toxins such as PSP in the world[2-4]. However, the wide range of known and undiscovered toxin analogues indicates that chromatographic methods are not always reliable indicators of toxicity. Its use for mouse bioassay is increasingly questioned on ethical grounds[5]. As a result, considerable efforts to develop simple, fast and more socially acceptable screening assays for HAB toxins have been made in recent years[6-7]. A number of alternative technologies have been proposed for screening assays of HAB toxins, such as antibody-based methods[8], binding assays involving rat brain sodium channels[9], desert locust bioassay[10], biosensor[11-12], voltage sensitive fluorescent assay[13], receptor binding assay[14], neurophysiological assay[7], intra-neuronal free [Ca^{2+}] analysis[13] and reporter gene assay[15]. Among these methods, cell culture-based assay has attracted much more attentions due to their relative accuracy in detection of toxicity of shellfish samples[3, 16-19].

PSP and NSP toxins have the sodium channel protein as their sole molecular target and binding with high affinity to specific sites on the α subunit. While PSP is a sodium channel blocker, NSP toxin functions as a sodium-channel activator. Fatal PSP intoxication represents the most serious threat of marine origin worldwide, with a prominent public health and economic impact in Asia, Europe, North America, and South America[18]. c-*fos* is an immediate response gene that has been used as a sensitive biomarker to assess the effects of algal derived toxins[20]. Fairey has developed a cell-based assay for brevetoxins, saxitoxins, and ciguatoxins

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using a stably expressed c-*fos*–luciferase reporter gene[15], demonstrating that reporter gene assay can detect sodium channel-specific HAB toxins. Because green fluorescent protein (GFP) can be used to monitor gene expression with ultraviolet or blue light without addition of substrates or cofactors in eukaryocytes, it has been extensively used as a convenient protein marker molecule in gene regulation studies and as a reporter in measuring environmental pollutants and other stimuli[21-22]. In this study, luciferase was replaced with enhanced green fluorescence protein (EGFP) to construct a pEGFP-c-fos-T24 cell line in order to provide a new alternative for screening sodium channel-specific HAB toxins.

MATERIALS AND METHODS

Materials

HF443 plasmid was provided by Professor Toshio, Tokyo University (Tokyo, Japan). pEGFP-N1 was provided by Professor HE, Jinan University (Guangzhou, China). *E. coli* DH5-α was provided by Professor LI, Jinan University (Guangzhou, China). Human bladder transitional cell carcinoma T24 cells were purchased from the Cell Bank in Academia Sinica (Shanghai, China). NSP （PbTx-2）was purchased from Sigma Co, Ltd. GTX2,3 was provided by Professor JIANG, Jinan University (Guangzhou, China).

Amplification of c-fos Promoter

c-fos promoter was amplified from HF443 plasmid with the following primers: 5′ -TAT AAT TAA TTT CTC ATT CTG CGC CGT TCC CG-3′ (upstream) and 5′-AAA GAA TCC CCG CCG GCT CAG TCT TGG CTT-3′ (downstream), which were designed according to the sequence of human *c-fos* gene. Amplification was performed for 30 cycles on a PTC200 PCR system under the following conditions: denaturation at 94 ℃ for 40 s, anneal at 60 ℃ for 1 min, extension at 72 ℃ for 1 min, and a final extension at 72 ℃ for 7 min. The PCR products were identified by agarose gel electrophoresis and then recovered. After digestion with VspI and *Eco*RI, the products were purified with a PCR purification kit.

Construction of pEGFP-c-fos Plasmid Vector

c-fos promotor with a cohesive terminus was ligated with plasmid pEGFP digested with the same restriction enzymes at 16 ℃ for 30 min. The resulted reaction mixture was transformed into competence *E. coli* DH5-α. Recombinant plasmid pEGFP-c-fos was identified by enzyme digestion and confirmed by sequencing.

Screening of pEGFP-c-fos-T24 Strain

Adherent human bladder transitional carcinoma T24 cells were cultured in DMEM containing 10% newborn calf serum at 37 ℃ in 5% CO_2. Recombinant plasmid pEGFP-c-fos was transfected into T24 cells through LipofectAMINE 2000. Positive transformants were screened by G418. To further screen positive pEGFP-c-fos-T24 strains sensitive to HAB toxins, the fluorescence intensity of T24 cells was observed under a laser scanning confocal microscope (FluoViewTM FV1000) at 12 h after 50 ng/mL NSP was added.

Detection of NSP and GTX2, 3 with pEGFP-c-fos T24 Strain

pEGFP-c-fos T24 cells were dispensed into a 96-well plate to a final density of 70%-80% confluence. After complete cell adhesion, NSP toxin was added to each well. The wells with 0.1% alcohol were used as vehicle controls. Fluorescence intensity was recorded with a camera under a laser scanning confocal microscope after 16 h, and analyzed by Image-pro Plus image analysis software (Media Cybernetics). To detect GTX2,3, T24 cells were treated with increasing concentrations of GTX2,3 from 0 to 16 ng/mL, and then 10 ng/mL NSP at 4 h. The cells treated with 0.1% alcohol were used as negative controls.

RESULTS

Identification of pEGFP-c-fos

The recombinant plasmid pEGFP-c-fos was identified by enzyme digestion and confirmed by sequencing. The fragments of plasmid pEGFP-c-fos digested with VspI and EcoRI are shown in Fig. 1. A 550 bp product consistent in size with the *c-fos* promoter was inserted. The sequence of promoter was the same as reported in GenBank, indicating that the recombinant plasmid, *pEGFP-c-fos*, was successfully constructed.

Time of EGFP Expression Induced by NSP in pEGFP-c-fos-T24 Cells

The time of fluorescence intensity of pEGFP-c-fos-T24 cells after the addition of 50 ng/mL NSP is shown in Fig. 2. The fluorescence intensity of pEGFP-c-fos-T24 cells was elevated after 10 h and

lasted 30 h. The EGFP expression reached its peak at 16 h. Therefore, the fluorescence intensity was detected at 16 h after the addition of NSP. In contrast, the fluorescence intensity of pEGFP-c-fos-T24 cells in the absence of NSP or in the presence of 0.1% alcohol was weak, suggesting that NSP but not alcohol induced the expression of EGFP by *c-fos* promoter, and that pEGFP-c-fos-T24 cells are suitable for the screening and detection of NSP.

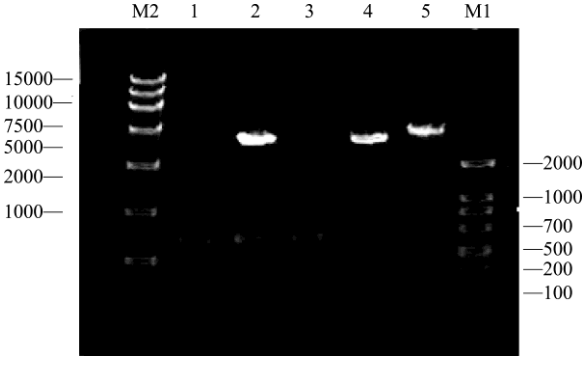

FIG. 1. Recombinant plasmid pEGFP-c-fos identified by enzyme digestion. M2: Marker DL15000; lane 1: c-fos promoter; lane 2: recombinant plasmid pEGFP-c-fos digested with VspI and EcoRI; lane 3: PCR products of the recombinant plasmid pEGFP-c-fos; lane 4: pEGFP plasmid digested with VspI and EcoRI; lane 5: pEGFP plasmid digested with EcoRI; M2: Marker DL2000.

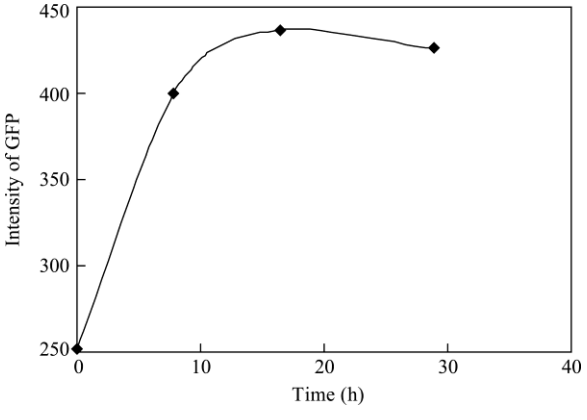

FIG. 2. Changes in fluorescence intensity with time in pEGFP-c-fos-T24 cells.

Changes of EGFP Expression in pEGFP-c-fos-T24 Cells Induced by HAB Toxins

The relation between the fluorescence intensity of pEGFP-c-fos-T24 cells and the concentration of NSP toxin is demonstrated in Fig. 3. The fluorescence intensity of pEGFP-c-fos-T24 cells increased with increasing concentrations of NSP toxin in a dose-dependent manner with an EC50 of 1.99 ng/mL, which is consistent with the reported data[15].

The GTX2,3-induced changes in fluorescence intensity of pEGFP-c-fos-T24 cells are shown in Fig. 4. GTX2,3 was able to cause a concentration-dependent inhibition of NSP-enhanced EGFP expression in a range of 0-16 ng/mL. IC50 was about 3.68 ng/mL, comparable to that of cytotoxicity and cell-based assay using luciferase[15].

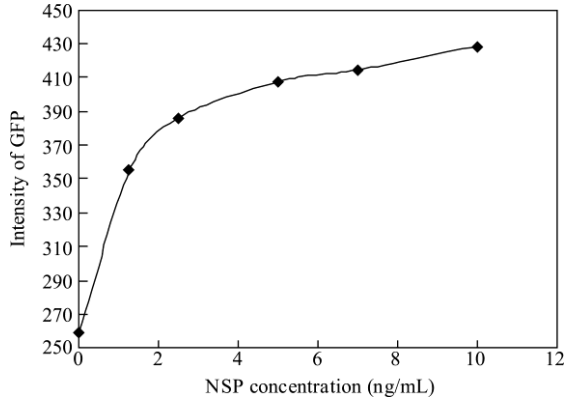

FIG. 3. Relation between fluorescence intensity and concentration of NSP toxin.

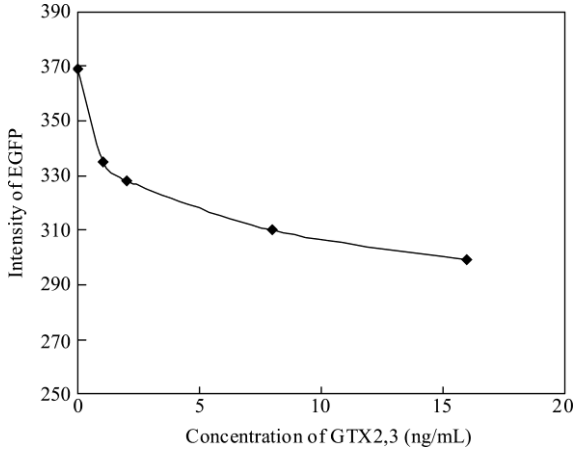

FIG. 4. Relation between fluorescence intensity and concentration of GTX2,3.

DISCUSSION

The fluorescence intensity in pEGFP-c-fos-T24 cells was correlated with the concentrations of NSP and GTX2,3 in this study, indicating that this technique can be used to measure the toxin levels. The detection limit of this method was at least 2 ng/mL for NSP and PSP, which was less than that of the traditional mouse bioassay, suggesting that the method can screen sodium channel-specific toxins

with a considerably higher sensitivity than mouse bioassay.

In conclusion, fluorescence-based GFP quantification in T24 cells containing the *c-fos* promoter-EGFP reporter-gene can be established as a potentially useful indicator of sodium channel-specific HAB toxins. However, this assay is still in its preliminary form. Many problems, such as loss of exogenous gene, regulation of EGFP gene expression and quantification of fluorescence intensity, need to be solved. At present, many assays are available for quantifying the fluorescence intensity of GFP, such fluorescence microscopy, laser scanning confocal microscopy, flow cytometry[22-24]. These methods are expensive, limiting their practical application in cell-based assays for HAB toxins. Further study is needed to find a fast and convenient method for measuring the fluorescence intensity of GFP.

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