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Cytotoxicity and hemolytic activity of jellyfish *Nemopilema nomurai* (Scyphozoa: Rhizostomeae) venom

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ABSTRACT

The recent bloom of a giant jellyfish *Nemopilema nomurai* has caused a danger to sea bathers and fishery damages in the waters of China, Korea, and Japan. The present study investigated the cytotoxic and hemolytic activities of crude venom extract of *N. nomurai* using a number of *in vitro* assays. The jellyfish venom showed a much higher cytotoxic activity in H9C2 heart myoblast than in C2C12 skeletal myoblast (LC_{50} = 2 μg/mL vs. 12 μg/mL, respectively), suggesting its possible *in vivo* selective toxicity on cardiac tissue. This result is consistent with our previous finding that cardiovascular function is a target of the venom. In order to determine the stability of *N. nomurai* venom, its cytotoxicity was examined under the various temperature and pH conditions. The activity was relatively well retained at low environmental temperature (≤20 °C) and dramatically lost at high temperature (≥60 °C). In pH stability test, the venom has abruptly lost its activity at low pH environment (pH ≤ 4). Interestingly enough, however, its activity was not significantly affected even at the highest pH environment tested (pH ≤ 12) in the present study. Additionally, hemolytic activity of the venom was examined using the erythrocytes of cat, dog, human, rabbit and rat. Venom concentration-dependent hemolysis could be observed from 10 μg/mL of protein equivalents or higher with variable potencies in different species, among which dog erythrocyte was the most susceptible to the venom (EC_{50} = 151 μg/mL). SDS-PAGE analysis of *N. nomurai* venom showed the molecules of 20–40 kDa and 10–15 kDa appeared to be the major protein components of the venom.

1. Introduction

Many marine invertebrates are included in the phylum Cnidaria, i.e. jellyfish, sea anemones and corals. One of the most distinctive aspects of cnidarian physiology is related to its biologically active components and organelles contained in specialized cells called nematocysts, which are located along the tentacles and body. These organelles are filled with toxins and discharge their content upon an appropriate stimulation. The accidental sting by venomous jellyfish can result in severe local and systemic pathologies, in some cases, can lead to death (Williamson et al., 1996). *Nemopilema nomurai* (also called 'echizen kurage') is one of the largest jellyfishes, with a maximum bell size up to 2 m and weighs up to 200 kg (Yasuda, 2004). Unusual large blooms of *N. nomurai* have occurred for the last years (Yasuda, 2004; Uye, 2008) in Yellow sea, East China Sea, and East Sea.

Biochemical and toxicological studies of jellyfish venoms have been reported by other investigators regarding their hemolytic (Torres et al., 2001), insecticidal (Yu et al., 2005a), cardiovascular (Ramasamy et al., 2005a,b), antioxidant (Yu et al., 2005b), enzymatic (Helmholz et al., 2007), and cytotoxic (Helmholz et al., 2007; Carli et al., 1996) activities. Toxic components of these jellyfish venoms are believed to be a type of proteins. On the other hand, our knowledge about *N. nomurai* jellyfish venom is extremely scarce. Based on a case report study of patients exposed to *N. nomurai* tentacles, the sting was very painful with a strong burning sensation, followed by erythematous eruption with small vesicles (Kawahara et al., 2006). Our previous study demonstrated that *N. nomurai* jellyfish venom had a cardio-depressant effect on rodent animal model, which is accompanied by a marked hypotension (Kim et al., 2006). The toxicological nature of this venom has neither been characterized nor clearly described yet. In the present study, we examined *N. nomurai* jellyfish venom in toxicological perspective using various experimental techniques, including comparative cytotoxicity test. We propose this as a novel method that can be applied for the research of natural toxins.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin sulfate, trypsin, dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-

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2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Alsever's solution were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All other reagents used were of the purest grade available.

2.2. Jellyfish collection and preparation

Mature specimens of *N. nomurai* jellyfish were captured from Korea Strait along the coasts of Tongyoung in September, 2007. The tentacles dissected from the jellyfish were stored in ice and transferred immediately to our laboratory for further preparation. Nematocysts were isolated from the dissected tentacles as described by Bloom et al. (1998) with a slight modification. In brief, tentacles were gently swirled with the addition of distilled water, then stood still for 1–2 h to remove debris and sea water. After decanting the supernatant, tentacles settled down at the bottom were mixed with 2× (v/v) distilled water and shaken vigorously for 3 min. The detached nematocysts were separated by filtering tentacle preparation through 4 layers of medical gauze. This was repeated for two more times with additional distilled water to harvest nematocysts from the tentacles. The filtrates were centrifuged (700 *g*) at 4 °C for 20 min and the pellets (nematocysts) were lyophilized and stored −20 °C.

2.3. Venom extraction and preparation

Venom was extracted from the freeze-dried nematocysts using the technique described by Carrette and Seymour (2004) with a minor modification. In brief, venom was extracted from 50 mg of nematocyst using glass beads (approximately 8000 beads; 0.5 mm in diameter) and 1 mL of ice-cold (4 °C) phosphate buffered saline (PBS, pH 7.4). These samples were shaken in a mini bead mill at 3000 rpm for 30-s intervals for five times with intermittent cooling on ice. The venom extracts were then transferred to a new Eppendorf tube and centrifuged (22,000 *g*) at 4 °C for 30 min. This supernatant was used as *N. nomurai* jellyfish venom for the present study. Protein concentration of the venom was determined by the method of Bradford (1976) technique (Bio-Rad, CA, USA) and the venom was used based on its protein concentration.

2.4. Cell culture and cytotoxicity assay

C2C12 (skeletal myoblast) and H9C2 (heart myoblast) cells were used for assessing the cytotoxic activity of the venom. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C with 5% CO_2. The cells were seeded in 24-well plates at a density of 10^4 cells/well and cultured for 24 h. Non-adherent cells were removed by gentle washing with fresh culture medium and *N. nomurai* jellyfish venom was treated at the indicated concentrations. After incubation (24 h), the treated cells were examined and photographed under phase contrast microscopy. Cytotoxicity was assessed by measuring mitochondrial dehydrogenase activity, using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, 100 μL of MTT solution (5 mg/mL) was added to each well and incubated for another 3 h at 37 °C. After removing the supernatant, the formazan crystal generated was dissolved by adding 150 μL/well of dimethyl sulfoxide (DMSO) and the absorbance was detected at 540 nm using a spectrophotometric microplate reader (BioTek Instruments, Inc., Winooski, USA).

2.5. Stability of N. nomurai jellyfish venom

Freshly prepared venom was incubated at the conditions of various temperature and pH, and then the retained cytotoxic activity of the venom was measured in H9C2 cells. For thermal stability study, each aliquot of the venom was incubated at the temperatures of 4, 20, 40, 60 and 80 °C for 60 min, respectively. The treated venom was added to the culture medium of H9C2 cells at the indicated concentrations for 24 h. The venom stability was then assessed by measuring its residual cytotoxic activity as described above. In order to determine the heating time-dependent venom stability, it was incubated at 40 °C for the periods of 0, 10, 30 min and 2, 6 h, respectively. The venom was then evaluated for its residual activity. We have also examined pH-dependent stability of the venom using various pH buffered solutions (Marino et al., 2004) with a little modification. Briefly, each aliquot of the venom extract was adjusted its pH for the indicated level by addition of 0.5 M of acetate (pH 2 and 4), 0.1 M of phosphate (pH 6, 7, and 8) and 0.5 M of glycine–NaOH buffers (pH 10 and 12), respectively. They were incubated for 1 h on ice, and their residual cytotoxic activity was assessed as described above.

2.6. Hemolysis assay

Hemolytic activity of the venom was tested using the erythrocytes of cat, dog, human, rabbit and rat. In brief, freshly collected blood samples were immediately mixed with anticoagulant, Alsever's solution (pH 7.4) to prevent blood coagulation. To obtain a pure suspension of erythrocytes, 1 mL of whole blood was then made up to 20 mL in phosphate buffered saline (PBS, pH 7.4), and centrifuged at 1500 *g* for 5 min at 4 °C. The supernatant and buffy coats were then removed by gentle aspiration, and the above process was repeated two more times. Erythrocytes were finally resuspended in PBS to make 1% solution for hemolysis assay. For this, various concentrations of jellyfish venom (0.01–2 mg/mL) were added to the suspension of red blood cells obtained from five different species (cat, dog, human, rabbit and rat). The venom–erythrocyte mixtures were incubated at 37 °C for 30 min in water bath and then centrifuged at 1500 *g* for 5 min at 4 °C. The supernatants were transferred to 96-well microplates and the absorbance at 545 nm was determined by using a spectrophotometric microplate reader (BioTek Instruments) to measure the extent of red blood cell lysis. Positive control (100% hemolysis) and negative control (0% hemolysis) were also determined by incubating erythrocytes with 1% Triton X-100 in PBS and PBS alone, respectively.

2.7. SDS-PAGE

Electrophoresis was carried out according to Laemmli method (Laemmli, 1970) using 16% polyacrylamide gel with 4% stacking gel. Samples were resuspended in SDS-PAGE sample buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue) and incubated at 95 °C for 5 min, then stored at −20 °C until use. Jellyfish venom protein (250 μg) was electrophoresed for 90 min at 100 V constant voltage at room temperature, using Tris–glycine running buffer. The molecular size marker, 3.5–260 kDa (Novex Sharp pre-stained protein standards, Invitrogen, CA, USA), was run parallel with venom sample for molecular weight estimation. Protein bands were visualized by Coomassie R-250 (Sambrook and Russell, 2001).

2.8. Statistical analysis

The results are expressed as a mean ± standard deviation (S.D.). A paired Student's *t*-test was used to assess the significance of differences between two mean values. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Cytotoxicity of N. nomurai jellyfish venom

Heart myoblast H9C2 and skeletal myoblast C2C12 cells were incubated for 24 h with various concentrations of *N. nomurai* jellyfish venom. A comparison of the relative cytotoxicity on the cells was