

RESEARCH REPORT

Characterization of crude *Echis carinatus* venom-induced cytotoxicity in HEK 293T cells

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ABSTRACT

Echis carinatus (saw-scaled viper) produces potent hemorrhagic venom that causes the development of apoptotic and necrotic tissues. In this study, we used polyethyleneimine (PEI) to enhance cellular adherence, and to determine whether the substrate attachment influenced the survival of cells treated with crude *E. carinatus* venom. Human embryonic kidney (HEK) 293T cells were grown for 18hr in tissue culture plates with or without polyethyleneimine (PEI), and were then stimulated with crude *E. carinatus* venom for 3 or 12hr. HEK 293T cells grown without PEI displayed a robust oxidative response to corresponding substrate detachment, loss of plasma membrane integrity and decreased cell viability. Cells grown on PEI adsorbed substrates demonstrated prolonged substrate attachment resulting in significantly higher cell viabilities. These observations suggest that the cytotoxicity of crude *E. carinatus* venom is dependent upon cellular detachment.

KEYWORDS: Anoikis, *Echis carinatus*, polyethyleneimine, apoptosis, cytotoxicity

INTRODUCTION

E. carinatus venom consists of a heterogeneous mixture of bioactive molecules, which degrade tissue structure and promote hemorrhaging (Sunitha et al, 2011). The most widely studied proteins that induce tissue damage in *E. carinatus* venom include metalloproteases, phospholipases and disintegrins. Snake venom metalloproteases (SVMP) exhibit a range of hemostatic activity including, inhibiting platelet aggregation by binding to glycoprotein IIb-IIIa receptors on platelets, enzymatically degrading basement membrane (e.g., laminin, fibronectin, and collagen IV), hydrolyzing endothelial cell adherence proteins (e.g., integrins and cadherins) and activating pro-thrombin. Several SVMPs isolated from *E. carinatus* venom (e.g., ecarin, multisquamase, carinactivase-1) have been characterized as pro-thrombin activators (Petrovan et al, 1997; Yamada and Morita, 1997; Yamada and Morita, 1999). These activators consume clotting factors and promote coagulation, resulting

in the development of microvascular thrombosis among other vascular related pathologies (Terra et al, 2009).

Polgár et al (1996) characterized the secretory phospholipase A₂ (sPLA₂) ecarpholin S, from *E. carinatus* venom. Other groups have isolated proteins and protein complexes with phospholipase A₂ activity from the same species (Kornalik and Blombäck, 1975; Kemparaju et al, 1994; Nagpal et al, 1999). Secretory sPLA₂ enzymes can induce their pharmacological effects dependent or independent of their enzymatic activity. Enzymatic activity involves Ca²⁺-dependent hydrolysis of the sn-2 ester bond of phosphoglycerides. Non-enzymatic mechanisms may involve agonistic or antagonistic activities (Kini and Evans, 1989). As a part of their mechanism, sPLA₂ enzymes bind to specific protein targets on cells or tissue (Lambeau and Lazdunski, 1999). For example, N-type and M-type receptors were identified as sPLA₂-binding proteins using a sPLA₂ from *Oxyuranus scutellatus scutellatus* (Rouault

et al, 2006; Kovacic et al, 2007). Receptor binding and other ancillary activities induces neurotoxic, myotoxic and hemorrhagic effects. Although a variety of pharmacological effects are induced by sPLA₂ enzymes, not all produce the same response.

E. carinatus venom also contains disintegrins. Disintegrins are examples of monodimeric, homodimeric or heterodimeric non-enzymatic proteins, which are released from the proteolytic processing of SVMP class II metalloprotease precursors. Disintegrins may contain KGD, MVD, MLD, VGD, ECD, MDG or RGD sequences which bind to integrin receptors, resulting in competitive inhibition of integrin binding to extracellular matrix proteins. The heterodimeric disintegrin EC3, isolated from *E. carinatus* venom, carries the VGD (α -subunit) and MLD (β -subunit) sequences (Marcinkiewicz et al, 1999). Another example includes echistatin, which inhibits fibrinogen-dependent platelet aggregation initiated by ADP, thrombin, epinephrine, collagen, or platelet-activating factors. Staiano et al (1997) demonstrated that echistatin induces FAK-mediated cellular detachment followed by alterations in cytoskeletal structure and apoptosis. Thus disintegrins are capable of activating integrin-signaling pathways and not merely passive integrin-blocking agents.

Metalloproteases, phospholipase A₂, and disintegrins from *E. carinatus* venom promote apoptosis as a part of their pathological mechanisms. For example, *In vitro* studies using adherent cell cultures have shown exposure to purified disintegrins or SVMP induce cellular detachment (Rucavado et al, 1999; Trummel et al, 2005). Interestingly, removal of the stimulants enables cellular reattachment and cellular survival, suggesting that disintegrins and SVMP induces a substrate dependent form of cell death, termed anoikis. Anoikis is important for maintaining tissue homeostasis and plays an essential role in the prevention of dissemination of cells to inappropriate sites, most notably during the metastatic process (Matter and Balda, 2007).

The activity of isolated metalloproteases, phospholipases, disintegrins and other bioactive proteins may be different in crude venom samples. Some PLA₂ enzymes express their pharmacological effects at full potency only when they form a complex with other protein factor(s). For example, β -bungarotoxin components are held together by a covalent bond, whereas crotoxin, mojave toxin, taipoxin and textilotoxin involve noncovalent interactions (Arocas et al, 1997; Krizaj et al, 1997). Although cellular detachment is observed with several isolated venom proteins, it is less clear whether crude *E. carinatus* venom induces anoikis, or if cellular detachment is the end result of cell death programming. Here, we investigate the relationship between cell adherence and crude *E. carinatus* venom-induced cytotoxicity. The ability of the extracellular matrix to influence cell survival is well documented and because cellular adhesion is influenced by the surface charge, density and texture, of the substratum, we employed PEI to enhance substrate binding (Ramsey et al, 1984; Vancha et al, 2004). We hypothesize that crude *E. carinatus* venom cytotoxicity is dependent on substrate detachment.

MATERIAL AND METHODS

Cell culture

HEK 293T cells were purchased from American Type Culture Collection (ATCC). The cells were cultured in 100mm tissue culture petri dishes with DMEM 1X containing 4.5g/l glucose, L-glutamine, sodium pyruvate (Mediatech, Inc. Herndon, VA) and 10% (v/v) heat-inactivated fetal calf serum (Mediatech, Inc. Herndon, VA). Cells were passaged every 3-4 days.

Cell viability assay

Twenty-four well plates were pre-incubation for 5hr with 0 (no PEI), 0.25 or 2.5 μ g/ml polyethyleneimine (PEI) (Vancha et al, 2004). Excess PEI was removed and 0.6ml of HEK 293T cell suspension was added at 1x10⁵ cells/ml. After 18hr, confluent HEK 293T cells were stimulated with 100 μ g/ml crude *E. carinatus* venom (Sigma, St Louis, MO) (100 μ g/ml) for 3 or 12hr. Controls included non-stimulated cells and cells incubated with 10 μ M staurosporine (Sigma St Louis, MO) for 3 or 12hr. This concentration of staurosporine is known to induce cell death in cellular models (Hwang et al, 2011). Next, 100 μ l of CellTiter 96[®]AQ_{ueous} One Solution Reagent (Promega, Madison, WI) was added to each well, incubated at 37°C for 1.5hr, then read at 490nm. The MTS tetrazolium (Owen's reagent) is bio-reduced by cells into formazan (dark blue).

Detection of reactive oxygen species

Carboxy-dichlorofluorescein (carboxy-H₂DCFH) (Invitrogen, Carlsbad, CA) was used to monitor intracellular ROS levels according to the manufacture's instruction. Briefly, HEK cells were plated at a concentration of 5x10⁵ cells/ml in 6-well plates with or without 2.5 μ g/ml PEI, and then incubated for 18hr at 37°C. The cells were stimulated with 100 μ g/ml crude *E. carinatus* venom for 50min. After 50min, the media was removed and the cells were washed with Hank's balanced salt solution (HBSS). Next, 2ml of 25 μ M carboxy-dichlorofluorescein (carboxy-H₂DCFH) was added for 30min. Following incubation, each well was washed twice with HBSS and then observed using an Olympus IX71 microscope containing FITC filter sets coupled to an Olympus DP71 charged coupled device (CCD) camera.

Adherence assay

Established procedures used for cytotoxicity analysis were adapted for the adherence assay (Albrecht et al, 2011). Briefly, six well plates were pre-incubation for 5hr with 0 (no PEI), 0.25 or 2.5 μ g/ml polyethyleneimine (PEI). Excess PEI was removed and 2ml of HEK 293T cell suspension was added at 5x10⁵ cells/ml. After 18hr, confluent HEK 293T cells were stimulated with 100 μ g/ml crude *E. carinatus* venom (Sigma, St Louis, MO) (100 μ g/ml) for 3 hr, then washed with PBS to remove non-adherent cells. Then MTS reagent was added to each well for 1.5hr and the absorbance was taken at 490nm.

Propidium iodide assay

Sixty millimeter tissue culture petri dishes were pre-incubation for 5hr at 37°C with 0.25, or 2.5 μ g/ml PEI, and then excess PEI was removed. Non- PEI (0 μ g/ml) groups were processed in parallel. Cells were plated at 3x10⁵

cell/ml and incubated at 37°C for 18hr. Monolayers for each PEI substrate were stimulated with 100µg/ml *E. carinatus* venom for 12hr, and then the media was removed. Cells were harvested by adding trypsin containing 0.01% (w/v) EDTA, for 5min. Media was added to neutralize trypsin. The cells were collected by centrifugation at 1000rpm, and the pellet was resuspended in 2ml of phosphate buffered-saline (PBS) and was kept on ice. Then, 0.5µl propidium iodide (250µg/ml) was added to 200µl of cell suspension and incubated for 10min in the dark. Ten microliters of propidium iodide stained cells were placed on a hemocytometer prior to imaging. This protocol was also used for non-stimulated cells that were processed in parallel with the stimulated groups. Bright-field (total cell count) and fluorescent images (PI positive cells) were acquired using an Olympus IX71 microscope containing TRITC filter sets coupled to an Olympus DP71 CCD camera. Fluorescent and bright-field images were imported in to Image J software (freeware: <http://rsbweb.nih.gov/ij/index.html>), inverted and analyzed, to determine the percent of propidium iodide positive cells.

Western blotting

HEK 293T cells were plated into 100mm petri dishes at a concentration of 5×10^3 cells/ml, and incubated at 37°C under 5% (v/v) CO₂ for 18hr. The media was removed and the cells were washed with PBS, placed on ice and lysed using NP40 buffer (50mM Tris-HCl pH 8.0, 1%, v/v, NP40) containing protease inhibitors (Roche, Mannheim, Germany). The lysate was collected with sterile cell scrapers and sonicated. The resulting cell lysate was centrifuged at 13,500xg for 10min and the supernatant was saved. The protein concentration was estimated using a BioRad protein colorimetric assay (Hercules, CA). The cell lysates were fractionated in a 10% (w/v) sodium dodecyl sulfate poly acrylamide gel (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked for 1hr with 5% (w/v) non-fat dry milk dissolved in TBST (1M Tris pH 8, 1M NaCl, 0.1%, v/v, Tween20). A dilution of 1:1000 anti-human mouse antibodies to β1 integrin, B-cell lymphoma 2 (Bcl2) and X-linked inhibitor of apoptosis protein (XAIP) (Cell Signaling Technology, Danvers, MA) was used for immunoblotting. After overnight incubation at 4°C, the membranes were washed three times with TBST and incubated with secondary anti-mouse rabbit coupled to horseradish peroxidase for 2hr at room temperature. The membranes were then washed twice with TBST followed by an additional wash with TBS. The immune-reactive bands were visualized using chemiluminescence western blotting detection agents (Biorad, Hercules, CA) according to the manufacturer's directions.

Biosensor assay

Biolayer interferometry (BLI) uses optical biosensing methods similar to surface plasmon resonance (Hall K and Aguilar MI, 2010). All (BLI) measurements were made on a ForteBio (Menlo Park, CA) Octet QK biosensor using amine reactive biosensors. All volumes were 200µl and measurements were made at 25°C. Sensors were first activated by dipping into a mixture of 50mM EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and 200mM NHS (N-hydroxysuccinimide) for 300sec. Sensors were then moved to PEI in 100mM MES pH 5.0 for 300sec. Crosslinking was quenched in 1M

ethanolamine, pH 8.5 for 300sec. Baseline measurements were made in PBS for 300sec. Sensors were then dipped into FCS in 150mM NaCl. Association response was monitored for 300sec after which the sensors were moved to buffer only for monitoring of dissociation for 300sec. Non-specific binding was measured by dipping sensors without crosslinked PEI into the highest concentration of FCS. Non-specific binding was negligible (Figure 1A, dash line).

Statistical analysis

Statistical analysis of MTS and propidium iodide assays was carried out using a two-sided *t* test and Microsoft Excel software.

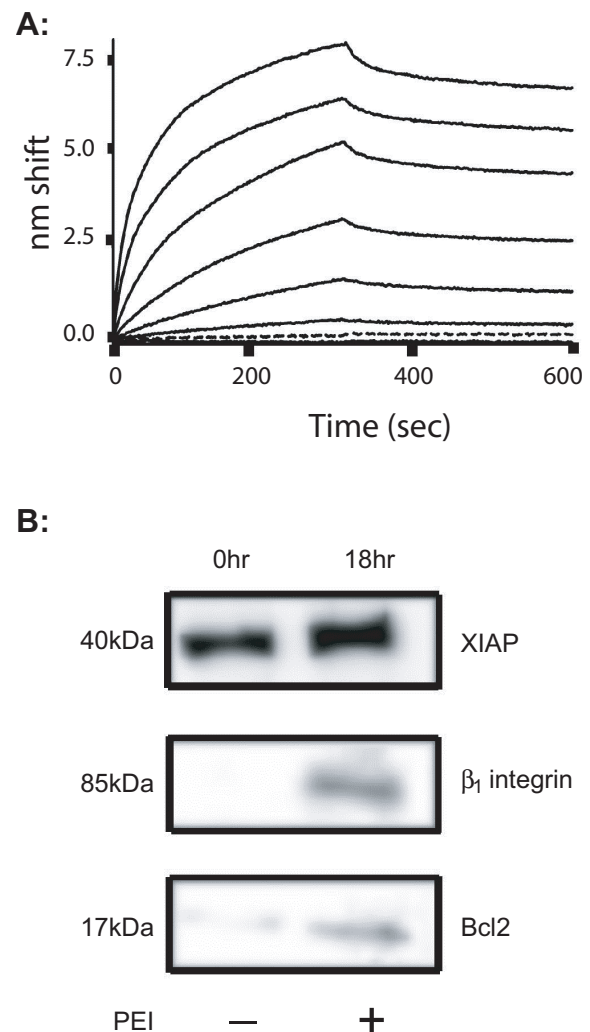


Figure 1. PEI promotes FCS protein binding and increased β1 integrin and Bcl2 protein expressions. **A.** Biolayer interferometry demonstrates specific binding of FCS to PEI. FCS was used as analyte vs immobilized PEI. FCS concentration (v/v) was 0.2%, 0.1%, 0.05%, 0.025%, 0.013%, 0.006% and 0%, respectively, in descending order. Binding of 0.2% (v/v) FCS to a sensor without PEI is shown as a dashed line and demonstrates negligible binding. Association phase was from 0-300sec, after which sensors were moved to buffer only (300-600sec) to observe dissociation. **B.** Western blotting to detect expression of XAIP protein, β1 integrin and Bcl2. Cell lysates were collected after 18hr growth on 2.5µg/ml PEI. Control lysates were collected from cells grown in the absence of PEI.

RESULTS

PEI-coated substrates increased soluble protein binding, and $\beta 1$ integrin and BCL2 expression

The poly-cation PEI is known to enhance cellular attachment, therefore, it was employed to examine the relationship between cellular adherence and *E. carinatus* venom cytotoxicity (Vancha et al, 2004). Initial experiments used BLI and western blotting techniques to better describe the effect of PEI in our model system. Because fetal calf serum (FCS) in HEK 293T media contains a heterogeneous mixture of negatively charged proteins, such as fibronectin and fibrinogen, BLI was used to examine the interactions between immobilized PEI and FCS (Figure 1A). Binding was complex; a two-state model produced good fits (data not shown). Binding of FCS to sensors without PEI was negligible, indicating that observed binding was specific. Furthermore, association was rapid and stable (though a small fast-off component was present). This suggests PEI-coated plates attract FCS protein binding partner(s) providing a more favorable surface for the cellular attachment, compared to non-PEI coated culture ware (Figure 1A).

HEK 293T cells express at least five $\beta 1$ integrin-containing subunits (Bodary and Mclean, 1990; Erguang et al, 2001) several are implicated in substrate dependent cell death programming (Xia et al, 2004). Therefore, we monitored the expression of $\beta 1$ integrin, and two other anti-apoptotic proteins (Bcl2) and (XIAP) in non-stimulated HEK 293T cells grown in tissue culture plates (at 18hr) adsorbed with 2.5 μ g/ml PEI or without PEI. Western blot analysis consistently showed an increase in expression of $\beta 1$ integrin and Bcl2 (Figure 1B). This is consistent with several previous studies, which have shown that extracellular matrix components alter the phenotypic expression of adherence receptors, such as $\beta 1$ integrins (Curtis and McMurray et al, 1986; Ertel et al, 1991; Keselowsky et al, 2005). The expression of XIAP protein remained unchanged.

Crude *E. carinatus* venom cytotoxicity correlated with loss of cell spreading

Early cellular cytotoxicity events (<3hr) include decrease in metabolic activity. Therefore, initial experiments examined cell viability and morphological changes of HEK 293T cells after exposure to crude *E. carinatus* venom for 3hr. Cell viability was measured using a colorimetric MTS assay, where changes in absorbance are directly proportional to the population of viable cells. Figure 2A suggests a dose-dependent relationship between *E. carinatus* venom concentration (2.5-100 μ g/ml) and cell viability. Cell stimulated with 100 μ g/ml of crude *E. carinatus* venom significantly decreased cell viability by $63.2 \pm 7.0\%$, as compared with non-stimulated controls. This concentration was then used in subsequent experiments to determine whether the addition of PEI to the substratum would influence the cytotoxic effects of crude *E. carinatus* venom. Figure 2B shows the morphological changes to HEK 293T cells grown without PEI or on substrates coated with 0.25 or 2.5 μ g/ml PEI, and then stimulated with 100 μ g/ml *E. carinatus* venom. Non-stimulated (control) HEK 293T monolayers maintained the same appearance when grown with or without PEI substrates (Figure 2B). Stimulation with 100 μ g/ml crude *E. carinatus* venom for 3hr caused significant detachment from non-PEI

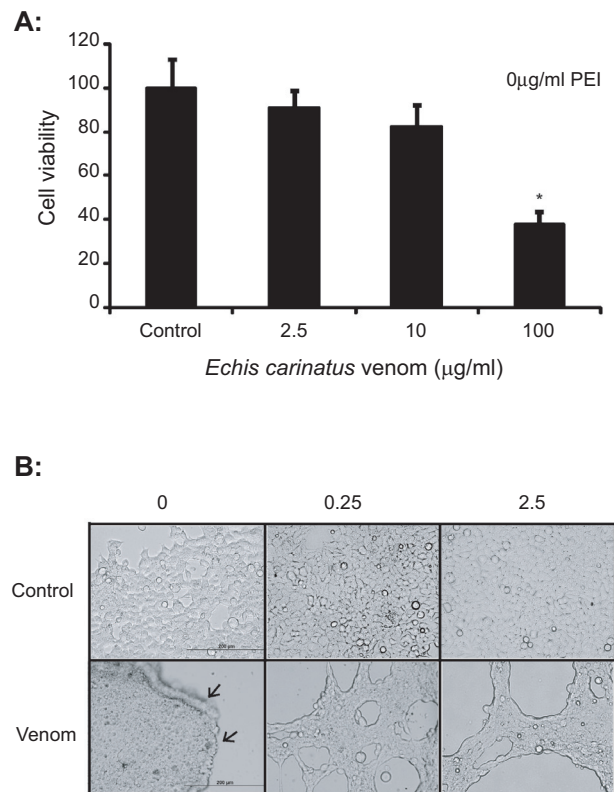


Figure 2. Dose response curve for *E. carinatus* venom. **A.** MTS assay. HEK 293T cells were grown on non-PEI adsorbed tissue cultureware (0 μ g/ml) and stimulated with *E. carinatus* venom (0-100 μ g/ml) for 3hr. * $P < 0.01$ vs 0 μ g/ml PEI non-stimulated control **(B)** Representative brightfield images (200x magnification) of HEK 293T cells grown without PEI (0 μ g/ml), 0.25 or 2.5 μ g/ml PEI, then stimulated with 100 μ g/ml crude *E. carinatus* venom for 3hr.

adsorbed substrate. The addition of adsorbed PEI resulted in increased cellular adherence, which appeared to be directly proportional to PEI concentration. Cells grown on 0.25 PEI showed areas of cell adhesion and cellular retraction, combined with intact monolayers that were completely removed from the substratum. Cells grown on 2.5 μ g/ml PEI maintain significant cellular spreading that was punctuated with non-cellular gaps (Figure 2B). In contrast, cells grown in the absence of PEI were consistently removed from the substratum as cellular sheets (indicated with arrows in Figure 2B).

PEI adsorbed substrates protect against *E. carinatus* venom cytotoxicity

We utilized MTS and propidium iodide-based assays to further characterize the cytotoxic response induced by crude *E. carinatus* venom. MTS assay data, displayed in Figure 3A and B, depicts the viability of HEK 293T cells grown with (0.25 or 2.5 μ g/ml) or without (0 μ g/ml) PEI then stimulated with *E. carinatus* venom for 3 and 12hr. As depicted in Figure 3A, only 37.0 ± 5.4 percent of the cells grown without PEI substrates remained viable when stimulated with crude *E. carinatus* venom for 3hr. Cells growing on 2.5 μ g/ml PEI were significantly more viable ($p < 0.01$) compared to non-PEI groups after stimulation, maintaining an average viability of $95.2 \pm 2.3\%$. During experimentation we utilized staurosporine, a known inhibitor of PKC, as a positive control to induce substrate independent apoptosis.

Stimulating cells with 10 μ M staurosporine for 3hr did not cause a detectable loss of cell viability in non-PEI and PEI groups (Figure 3A). Thus, after 3hr of stimulation, only cells exposed to venom on non-PEI absorbed substrate showed detectable losses in viability, which were directly proportional to the concentration of absorbed PEI.

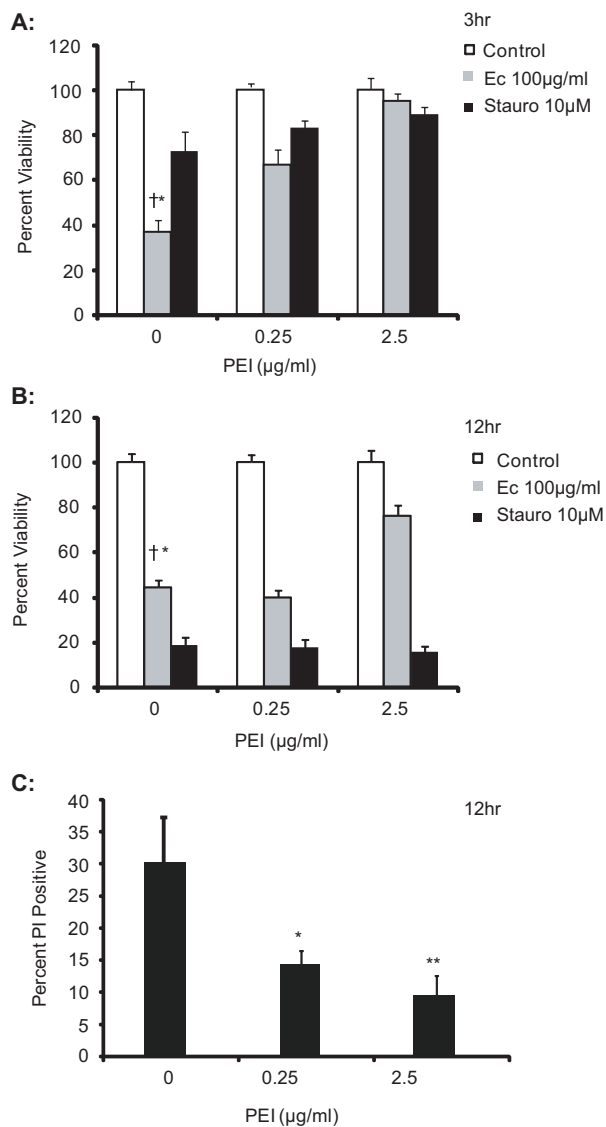


Figure 3. PEI reduces crude *E. carinatus* venom cytotoxicity. **A.** MTS assay. HEK 293T cells were grown without PEI (0 μ g/ml) or with 0.25 or 2.5 μ g/ml PEI absorbed substrates for 18hr, and then stimulated with 100 μ g/ml crude *E. carinatus* (Ec) (grey) venom or 10 μ M staurosporine (black) for 3hr. * P <0.01 vs 0 μ g/ml PEI non-stimulated control (black). [†] P <0.01 vs 2.5 μ g/ml PEI with 100 μ g/ml EC venom. **B.** MTS assay. HEK 293T cells were grown without PEI (0 μ g/ml) or with 0.25 or 2.5 μ g/ml PEI for 18hr, and then stimulated with 100 μ g/ml EC venom for 12hr. * P <0.01 vs 0 μ g/ml PEI non-stimulated control (12hr). [†] P <0.01 vs 2.5 μ g/ml PEI with 100 μ g/ml EC venom (12hr). Data are \pm SE of three separate experiments. **C.** Propidium iodide assay. Substrate dependent loss of plasma membrane integrity of HEK 293T cells stimulated with crude *Echis carinatus* venom. HEK 293T cells were grown in dishes pre-treated with 0.25 or 2.5 μ g/ml PEI and then stimulated with 100 μ g/ml *E. carinatus* venom for 12hr. Venom treated and non-stimulated control groups were stained with PI then counted using a hemocytometer and fluorescent microscopy. * P <0.05 vs 0 μ g/ml PEI. Data are \pm SE of three separate experiments.

Extending crude venom stimulation to 12hr resulted in increased cytotoxicity for venom and staurosporine-treated cells grown with or without PEI-absorbed substrates (Figure 3B). However, the presence of adsorbed PEI significantly increased cell survival. For example, HEK 293T cells grown on 2.5 μ g/ml PEI, then stimulated with 100 μ g/ml *E. carinatus* venom for 12hr were approximately 31.7% more viability compared to cells grown without PEI (Figure 3B). All staurosporine-treated groups exhibited significant reductions in cell viability ranging from 15.1 \pm 2.7 to 19.1 \pm 3.4 percent, suggesting staurosporine toxicity was independent of PEI concentration (Figure 3B). Thus, the pattern of protection (higher cell viability) was similar for 3 and 12hr time points.

Next, we used propidium iodide, a cell impermeable fluorescent dye, to examine plasma membrane integrity during *E. carinatus* venom exposure (Figure 3C). Cells were stimulated with *E. carinatus* venom for 12hr, stained with PI, and counted using fluorescent microscopy. Quantitative results showed 30.3 \pm 7.0 percent of the cells grown in the absence of PEI stained positive for PI, compared to only 9.6 \pm 3.0 percent when grown on 2.5 μ g/ml PEI. This demonstrates that an inverse relationship exists between PEI concentration and plasma membrane damage (Figure 3C). Although venom stimulation produced PI positive cells inversely proportional the concentration of PEI, each group maintain significant populations of cells with intact plasma membranes. Combining this result with MTS data (Figure 3A and B) suggests that the presence of PEI-absorbed substrates reduces dehydrogenase dysfunction and protects against plasma membrane damage.

Increase in ROS corresponds to loss of adherence

To determine whether the cyto-protective effect of absorbed PEI correlated with increased cellular adherence we monitored ROS and cellular adherence during venom stimulation. Figure 4A, represents micrographs of HEK 293T cells stimulated for 50min with 100 μ g/ml *E. carinatus* venom, then incubated with carboxy-H₂DCFH to detect ROS. After 50min, cellular detachment was evident for cells grown on non-PEI-containing substrates and this coincided with elevated ROS levels, indicated by the fluorescent probe. Cells grown on PEI, then stimulated with *E. carinatus* venom showed cortical retraction and reduced spreading. The areas retracting were punctuated with fluorescence (Figure 4A).

We utilized a MTS-based adherence assay to measure cellular detachment (Figure 4B). Cells were grown on 0, 0.25 or 2.5 μ g/ml PEI for 18hr, and then stimulated for 3hr with 100 μ g/ml *E. carinatus* venom. After stimulation, non-adherent cells were washed away with PBS and then MTS reagent was added. Thus, the percent cell viability is directly proportional to the number of remaining cells, capable of converting MTS tetrazolium to formazan (colored species). Our results suggest stimulating cells for 3hr with *E. carinatus* venom promoted a loss of adherence proportional to the concentration of the PEI absorbed substrate (Figure 4B). Cells grown on substrate without PEI, stimulated with 100 μ g/ml *E. carinatus* venom, and then washed with PBS had an average cell viability of only 9.0 \pm 2.5 percent. Microscopic examination of culture wells confirmed the absence of the cells after washing. In

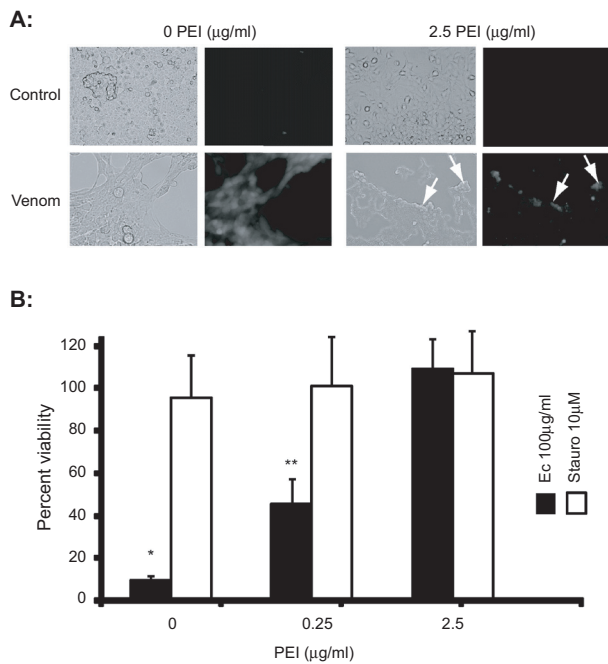


Figure 4. A. Carboxy- H_2DCFH assay. Brightfield and fluorescent micrographs were acquired at 200X magnification. Fluorescence indicates intracellular ROS. HEK 293T cells were stimulated with 100μg/ml crude *E. carinatus* venom for 50min showed fluorescence localized to areas of cellular retraction (arrows). **B.** MTS adherence assay. HEK 293T cells were grown on 24-well plates pre-treated with 0.25, 2.5μg/ml PEI or without PEI (0μg/ml) for 18hr. Cells were stimulated with 100μg/ml crude *E. carinatus* (EC) venom (black) or 10μM staurosporine (white) for 3hr then washed with PBS to remove non adherent cells. * $P < 0.05$ vs 0.25μg/ml PEI (100μg/ml EC venom). ** $P < 0.05$ vs 2.5μg/ml PEI (100μg/ml EC venom). Data are \pm SE of three separate experiments.

contrast, cell grown on 2.5μg/ml PEI and stimulated under identical conditions, remained adherent, registered cell viability values comparable to non-stimulated controls. As expected, cells stimulated with staurosporine for 3hr did not display a significant decrease in cell adherence. Thus, cells grown without PEI rapidly detach when exposed to crude *E. carinatus* venom. This event corresponded to elevated ROS and dehydrogenase dysfunction. In contrast, cells grown on 2.5μg/ml PEI remained adherent, but displayed elevated levels of ROS where cellular retraction was present. Moreover, the prolonged cellular attachment significantly protected against dehydrogenase dysfunction as noted by higher cell viability percentages (Figure 4B).

DISCUSSION

E. carinatus venom, like venom from other Viperidae species, induces an acute inflammatory reaction involving disruption of the hemostatic system and tissue destruction. Valuable information has been gathered to describe the biochemical activity of hemorrhagic snake venom components and their involvement in cell death; however, crude venom activity is less resolved. Because crude *E. carinatus* venom contains a variety of proteins which induce cellular detachment, it is plausible that loss of substrate contact is the main event promoting cytotoxicity that leads to cells death. This is supported by several studies investigating purified SVMPs.

For example, BaP1, a SVMP from *Bothrops asper*, causes rapid detachment followed by DNA fragmentation in endothelial cells (Rucavado et al, 1995). Another SVMP, jararhagin, promotes cell death involving the disruption of focal adhesion signaling pathways (Zigrino et al, 2002). In both instances, cellular detachment preceded apoptotic/anoikis programming.

In this study, PEI was used to enhance cellular adherence. Biosensor data demonstrated rapid binding events between PEI and soluble FCS proteins. Thus during plating, PEI-coated substrates rapidly attracted FCS protein providing more ligands for integrin and other adherence receptors to bind (Figure 1A). In addition, it is likely that the positively charged amino groups of PEI electrostatically attract the negatively charged residues associated with the glycocalyx. Interestingly, we found cells grown on tissue cultureware without PEI did not express a measurable amount of $\beta 1$ integrin, but did display a modest amount of Bcl2. PEI-coated substrates induced an increased expression of $\beta 1$ integrin and Bcl2 proteins in cells prior to experimentation (Figure 1B). The signal transduction events involved was not resolved; however this is in agreement with other studies showing that biomaterial consisting of amino functional groups increase $\beta 1$ integrin expression (Lee et al, 2006). The parallel increase in $\beta 1$ integrin and Bcl2 expression has been reported in Chinese hamster ovary (CHO) cells and these phenotypic changes improved cellular survival when challenged with pro-apoptotic stimuli (Zhang et al, 1995). Schwartz and Frisch (1994) showed that cell attachment by integrins was essential to suppress anoikis. In addition, overexpression of Bcl2 inhibited anoikis, indicating that mitochondrial membrane permeabilisation (MMP) was a part of the mechanism (Meredith et al, 1993; Frisch and Schwartz, 1994).

Using this cellular model, crude *E. carinatus* venom was employed as complex mixture, to determine if enhanced cellular adherence influenced cytotoxicity. Initial experiments determined the cytotoxic range for HEK 293T cells grown without PEI then stimulated with crude *E. carinatus* venom (Figure 2A). Microscopic observations showed that cells grown on adsorbed PEI (0.25-2.5μg/ml) remained attachment during venom stimulation. This suggested that the changes induced by PEI (*i.e.*, increase $\beta 1$ integrin, Bcl2) may be contributing to prolonged cellular attachment during exposure to the venom.

Next, we extended our study using MTS assays to determine if PEI enhanced cellular adherence correlated with increased cellular survival overtime. The MTS directly measures the conversion of MTS tetrazolium to formazan by dehydrogenase enzymes. Thus, cell viability may be correlated to the dysfunction of mitochondrial, endoplasmic reticulum and cytosolic dehydrogenase enzymes. HEK 293T cells were grown on culture plates adsorbed with or without PEI, and then stimulated with crude *E. carinatus* venom for 3hr or 12hr. After 3hr of venom stimulation PEI significantly ($p < 0.01$) improved cell viability, which was proportional to the concentration of adsorbed PEI. Similarly, cells stimulated with staurosporine for 3hr demonstrated a modest improvement in viability when grown on PEI-adsorbed substrates. Stimulating for 12hr caused further reduction

in cell viability, but the same pattern (increased viability) was observed for cells grown on adsorbed PEI. As expected, cells stimulated with staurosporine exhibited a dramatic reduction in cell viability on PEI and non-PEI-adsorbed substrates. These experiments demonstrated that crude *E. carinatus* venom causes a progressive decrease in viability overtime, but the presence of PEI-adsorbed substrates significantly increased cell viability. If the crude venom contained bioactive molecule(s) in sufficient concentration to induce significant toxicity, a pattern similar to the staurosporine treated cells would have emerged. Since this was not the case, the data suggested that PEI-adsorbed substrates inhibited cytotoxic mechanism(s) leading to cell death.

In order to further characterize crude *E. carinatus* venom cytotoxicity, we examined plasma membrane integrity. Because SVMP have been shown to induce plasma membrane damage at later time intervals (hours) (Wang et al, 2003), we predicted that weakly attached cells (grown without PEI), may be more susceptible to plasma membrane damage. Our propidium iodide results supported this prediction, demonstrating that crude venom disrupts a greater percent of membranes when grown without PEI-adsorbed substrates (Figure 3C). Notably, the majority of the cells assayed did not stain positive for propidium iodide, indicating membrane damage was not a primary characteristic of crude venom toxicity in our experimental time-frame. Recent investigations aimed at understanding phospholipase activity have correlated hydrolysis rates with structural membrane changes that occur during late apoptotic programming (Chioato et al, 2007; Olson et al, 2010). This suggests that calcium-dependent hydrolysis of phosphoglycerides may require membrane changes in order to gain access to phospholipid substrates.

The production of reactive oxygen species (ROS) during integrin detachment is well documented (Li et al, 1999). Several lines of evidence suggest this involves the small GTPase Rac-1, where integrin detachment induces Rac-1-dependent production of ROS from NADPH oxidase, 5-LOX and mitochondria (Chiarugi et al, 2003; Taddei et al, 2007). Therefore, rapid production of ROS may serve as early indicators of anti-apoptosis processes.

To determine if crude *E. carinatus* induces excessive ROS production, carboxy-H₂DCFH was used to monitor ROS. Our experimental groups consisted of HEK cells grown without PEI or on substrates adsorbed with 2.5µg/ml PEI, then exposed to crude *E. carinatus* venom for 50min. Each condition caused excessive intracellular ROS compared to non-stimulated controls. However, cells without PEI were completely detached, similar to Figure 1B results, and display robust whole cell fluorescence. In contrast, PEI substrate groups remained attached, but expressed areas of cellular retraction that correlated with fluorescence (Figure 4A). Carboxy-H₂DCFH does not distinguish between ROS species. Thus, ROS generated in response to crude *E. carinatus* venom may be derived from arachadonic metabolism, lipoxygenase, cytochrome P450, mitochondrial electron transport system, NAD(P)H oxidase (Nox), or xanthine oxidase sources (Klaunig et al, 2011; Cho et al, 2011). We used MTS based adherence assays to confirm there was a loss of cellular adherence after 3hr of crude venom stimulation.

Higher viabilities are directly proportional to the number of adherent cells that remain after the washing step. Only 9.0 ±2.5% remained adherent after 3hr of stimulation on non PEI adsorbed substrates. Therefore, it appears that crude venom cytotoxicity involves early loss of substrate contact (>1hr, 100µg/ml) that coincides with excessive reactive oxygen species and metabolic dysfunction. In contrast, Alimentia et al (2004) demonstrated that the purified disintegrin, echistatin, promotes cytotoxicity in GD25 cells expressing β1 integrins prior to cellular detachment, suggesting that integrin mediated death (IMD) is the primary mechanism involved. In our study, increased β1 expression did not cause HEK cells to be more prone to cell death. In fact, we observed that cell attachment was central to cellular survival. This difference may involve extracellular matrix composition. Echistatin more selectively binds α_vβ₃ compared to α_vβ₁ integrins (Kumar et al, 1997). In our model, PEI attracted FCS proteins including fibronectin, which binds to α_vβ₁ integrins. Thus, the presence of echistatin in crude venom may not be as effective in detaching the cells in our model system.

CONCLUSIONS

- The addition of PEI to the substrate prolonged cellular attachment during crude *E. carinatus* stimulation resulting in reduced metabolic dysfunction, membrane damage, and ROS generation.
- Crude *E. carinatus* venom induces substrate-dependent cytotoxicity.

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STATEMENT OF COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

FCS; fetal calf serum
 ROS; reactive oxygen species
 PEI; polyethyleneimine
 HEK; Human Embryonic Kidney
 Bcl2; B-cell lymphoma 2
 XIAP; X-linked inhibitor of apoptosis protein
 MTS; 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

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