

Morphological Changes of Apoptosis in the Human Cholangiocarcinoma Cell Line, HuCCA-1, Induced by Green Pit Viper Venom

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ABSTRACT

The venom of the green pit viper (*Trimeresurus purpureomaculatus*) was used to study the effect on bile duct epithelial cancer cell damage, using the human cholangiocarcinoma cell line, HuCCA-1, as a model. After cell treatment by various concentrations of snake venom at different times, alteration of the cellular structure was observed, especially in the apoptosis of cells. Under a light microscope, many patterns of cellular changes were obvious following treatment with 40 µg/ml snake venom for six h, while the structure of untreated cells was still unchanged. The number of altered cells was significantly higher than in the control ($p < 0.05$). A transmission electron micrograph showed that venom-treated cells had the characteristic of apoptosis to varying degrees. The cells presented plasma membrane blebs, an increased number of cytoplasmic vacuoles, enlargement or deterioration of organelles, reduced nuclear size, irregular nuclear membrane and clumping of nuclear chromatin. Though snake venom in the Viperidae family causes hemorrhagic symptoms by the function of several components, including phospholipase A₂, it was hypothesized that the venom of the green pit viper shared structural similarity with Lys49 phospholipase A₂, which lacked a hemolytic activity, but showed an ability to induce cancer cell apoptosis.

Key words: green pit viper venom, Lys49 phospholipase A₂, cholangiocarcinoma, HuCCA-1, apoptosis

INTRODUCTION

The green pit viper is a venomous snake in the Viperidae family and Crotalinae subfamily. The snake venom from this family has been categorized as hemorrhagic venom or hematotoxin (Jintakune and Chanhom, 1995). Major components found in viperid venom are mostly proteolytic enzymes, including phospholipase A₂ (PLA₂), serine proteases, L-amino acid oxidase and metalloproteinases (Jia *et al.*, 1996; Suhr and

Kim, 1996; Francischetti *et al.*, 2004; Wagstaff and Harrison, 2006). The actions of these enzymes facilitate the snake venom to destroy the blood vascular system. PLA₂ is toxic to the cell membrane and thereby can cause local cell and tissue damage (Nuchprayoon *et al.*, 2001). It affects a variety of pathological processes, including anticoagulation, edema and the inhibition of platelet aggregation (Sai-Ngam *et al.*, 2008). Thrombin-like enzymes, fibrinogenolytic enzymes and a plasminogen activator composed

of serine proteases are also involved in anticoagulation (Rojnuckarin *et al.*, 2006). L-amino acid oxidase induces an apoptotic mechanism in vascular endothelial cells by catalyzing oxidation of L-amino acid and generating hydrogen peroxide (Araki *et al.*, 1993; Suhr and Kim, 1996; Masuda *et al.*, 1997). The snake venom metalloproteinases have been demonstrated to induce hemorrhaging by degradation of the endothelial basement membrane and adhesion proteins, thus weakening the capillary wall and perturbing the interaction between endothelial cells and basement membrane (Jia *et al.*, 1996; Gutiérrez *et al.*, 2005). PLA₂ in hemorrhagic snake venom has been widely studied and three known types of PLA₂ have been isolated. The classical one contains aspartic acid in position 49 (Asp49 PLA₂), which is calcium dependent to maintain active phospholipase activity (Lomonte *et al.*, 2003; Rojnuckarin *et al.*, 2006). All of the PLA₂ isoforms in Thai Russell's viper (*Vipera russelli siamensis*) venom act in this way. This viperid in the Viperinae subfamily expresses highly lethal hematotoxic activity (Jintakune and Chanhom, 1995), consistent with the clinical manifestation of hemolysis, systemic bleeding and the frequent development of renal complications, especially acute renal failure in the victim (Mahasandana *et al.*, 1980; Nuchprayoon *et al.*, 2001). The variants of Asp49 PLA₂ exhibit structural similarity, but contain lysine (Lys49) (Lomonte *et al.*, 2003) or serine (Ser49) (Krizaj *et al.*, 1991) in place of aspartic acid. Such replacement causes these latter two to become enzymatically inactive, since the calcium-binding loop and the catalytic center were structurally altered (Lomonte *et al.*, 2003). The venom of the white-lipped pit viper (*Trimeresurus albolabris*), one of the most poisonous green pit vipers, has been proved to contain Lys49 PLA₂ which does not affect erythrocyte integrity both *in vitro* and *in vivo*, but produces myotoxicity and marked painful edema (Nuchprayoon *et al.*, 2001). The

severe envenoming by *T. albolabris* presents clinical features of local blistering and necrosis, shock, spontaneous systemic bleeding, defibrination, thrombocytopenia and leucocytosis, which are lethal at relatively high doses (Hutton *et al.*, 1990). Lys49 PLA₂ homologue, myotoxin II, was demonstrated to be cytotoxic to various cell lines, but did not depend on membrane phospholipid hydrolysis (Lomonte *et al.*, 1999). In addition, it induced apoptosis of the lymphoblastoid cell line at high concentration (5-25 µg/ml) analyzed by TUNEL assay, whereas cell necrosis occurred at higher concentration (Mora *et al.*, 2005). The 13-mers synthetic peptide derived from the C-terminal region of Lys49 PLA₂ homologues showed rapid cytotoxic effect against B16 melanoma, EMT6 mammary carcinoma, S-180 sarcoma, P3X myeloma and tEnd polyoma virus-transformed endothelial cell lines as indicated by the release of lactate dehydrogenase (LDH) to the supernatant. Moreover, it was able to reduce tumor mass from EMT6 mammary carcinoma cell injection in mice to a similar size of that caused by the administration of the antitumor drug, paclitaxel, in the same time interval (Araya and Lomonte, 2007). However, damage of cancer cell by apoptosis that does not destroy normal tissue and cells is desirable. The characteristics of the apoptotic process prevent cell breakage and leakage of lysosomal hydrolytic enzyme to surrounding tissue. The morphological alterations include cell shrinkage, plasma and nuclear membrane blebbing, organelle relocation and compaction, chromatin condensation and production of membrane-enclosed particles containing intracellular materials known as "apoptotic bodies", which lead to little, if any, activation of the host immune system (Bold *et al.*, 1997).

Cholangiocarcinoma, a cancer of the liver bile duct, has been diagnosed extensively among Thai people, especially in northeast Thailand (Sriamporn *et al.*, 2003). The hidden

cancer mass in the liver parenchyma makes detection difficult by the patients themselves, thus the advanced cancer stage was frequently examined and confirmed at the hospital. Since it was resistant to chemotherapy and radiotherapy, surgical treatment was a choice for cholangiocarcinoma (Gores, 2003). Currently, other alternatives have been discovered to overcome this disease (Reddy and Patel, 2006; Rosen *et al.*, 2008). However, the incidence and mortality rates remained high in Thailand (Sriamporn *et al.*, 2003). As a result, preventive knowledge and treatment that is more effective are required to decrease the number of cholangiocarcinoma patients. The combination of a curative agent with a surgical procedure may be an option. Even though several components in snake venoms were found to inhibit various cancers, the effect of green pit viper venom on cancer cell death has never been studied. The objective of this work was to study whether green pit viper venom could induce cell apoptosis of human cholangiocarcinoma by using HuCCA-1 cell as a model. The venom of the mangrove pit viper (*T. purpureomaculatus*) is as severe as that of the white-lipped pit viper (*T. albolabris*) and shows the same clinical signs in bitten victims. This paper hypothesized that green pit viper venom was composed of Lys49 PLA₂, which at an optimal concentration can destroy HuCCA-1 cells by the apoptotic process. However, advanced studies will be required to develop the green pit viper venom for medical utility.

MATERIALS AND METHODS

Cell culture treatment with snake venom

The lyophilized venom of the green pit viper (*T. purpureomaculatus*) was bought from the Thai Red Cross Society and diluted to a concentration of 5 µg/µl in phosphate buffer solution pH 7.4 and kept at -20°C until used. The cholangiocarcinoma cell line, HuCCA-1, was

previously established (Sirishinha *et al.*, 1991) and generously given by Prof. Stitaya Sirisinha. The cells were cultured in HamF-12 medium containing 10% fetal bovine serum. Until confluent, the cultured cells were trypsinized and 5×10⁵ cells per well were seeded into a 24-well plate and grown for 18 h before treating with venom. The fresh complete culture medium was replaced and then the cells were treated with 1, 5, 10, 20, 40 or 80 µg/ml of venom for 3, 6, 12, 24, 48 and 72 h. Any alterations were observed under phase contrast inverted microscope compared to untreated cells and photographs were taken.

Cell morphology investigation

The treatment produced substantial data on cell morphology. The cells in each 24-well plate were trypsinized and a volume of 10 µl was dropped onto a glass slide, air dried and fixed with methanol for 5 min. The cells were stained with toluidine blue and cell morphology was observed by light microscope (1,000x). The percentage of abnormal cells from various treatments was calculated and statistically analyzed by Student's *t*-test.

The rest of the trypsinized cells were centrifuged and the pellet was collected, prefixed with 2.5% glutaraldehyde, postfixed with 2% osmium tetroxide, dehydrated in an acetone series, infiltrated and embedded in Spurr. All samples were semi-thin sectioned for studying under a light microscope at high magnification and then ultrathin sectioned for studying by transmission electron microscope (TEM).

RESULTS AND DISCUSSION

After various concentrations of green pit viper venom were added into the culture medium, the alteration of cells was studied at different times. Within 3 h, the cells treated with 80 µg/ml of venom were drastically damaged. Most cells presented cytoplasmic condensation and reduced

size, but some cells were ruptured. Extensive focal detachment of cells from the substratum was found (Figure 1). However, the lower concentration did not cause any alteration. At 6 h, the cells treated with 40 $\mu\text{g/ml}$ of venom began to reduce in size. The condensation of cytoplasmic organelles was not obvious and cell rupture was not found. Minute detachment of cells occurred (Figure 2). After 12 h, the cells treated with 20 $\mu\text{g/ml}$ of venom showed similar morphology to the 40 $\mu\text{g/ml}$ treated cells at 6 h, but the normal cells also showed unhealthy signs, such as cell distortion and dullness (data not shown). Since the HuCCA-1 cell was basically an attached epithelial cell line which needed to adhere with neighboring cells and attach to the substratum (Sirisinha *et al.*, 1991), high concentrations or a long period of exposure to

snake venom could destroy cells, visualized by cell detachment and then suspension in the culture medium. However, it was interesting that the confluent HuCCA-1 cells grown for more than 12 h without subculture were also prone to be toxic by metabolic waste excreted into the culture medium. Though the cells were not treated by venom, they exhibited deterioration later on from the effects of toxic metabolic waste.

The cells treated with 1, 5, 10, 20 and 40 $\mu\text{g/ml}$ of venom for 6 h were chosen for further study compared to untreated cells. After being trypsinized and stained, cell morphology was investigated under light microscope. Normal cells showed a round shape, round or indented nucleus, smooth nuclear membrane but small areas of irregular plasma membrane could be found (Figure

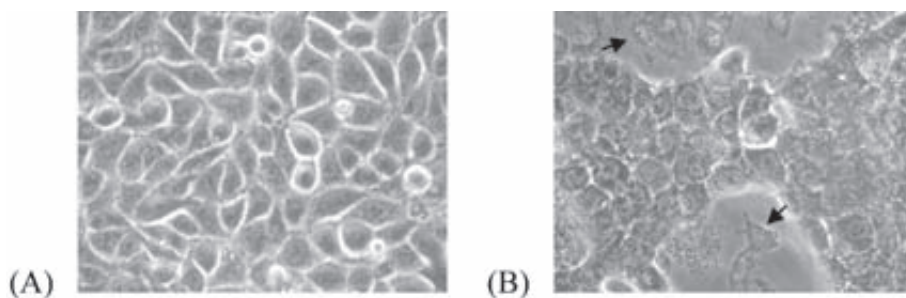


Figure 1 HuCCA-1 cell cultured for 3 h observed under phase contrast inverted microscope (300x). (A) Normal cells; (B) The cells treated with 80 $\mu\text{g/ml}$ of snake venom. Arrows indicate debris of ruptured cells.

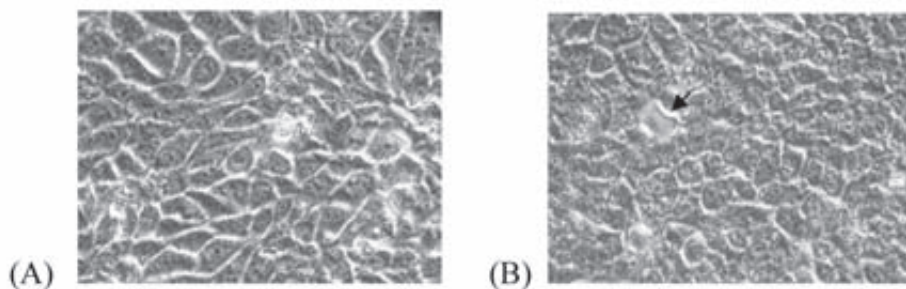


Figure 2 HuCCA-1 cell cultured for 6 h observed under phase contrast inverted microscope (300x). (A) Normal cells; (B) Cells treated with 40 $\mu\text{g/ml}$ of snake venom. Arrow indicates area of cell detachment.

3A), which was correlated to the microvilli or secretory products found on some areas of the cell surface (Sriurairatana *et al.*, 1996). All venom treatments presented various amounts of cell alteration. The most obvious was illustrated in cells treated with 40 $\mu\text{g}/\text{ml}$ venom, which had a smaller size, abnormal cellular and nuclear shapes, plasma membrane blebs and an irregular nuclear membrane. Some cells presented a number of small fragments at the plasma membrane (Figure 3B). These were supposed to be characteristic of apoptotic cells, hence study of the ultrastructure was needed to clarify. The percentage of altered cells (abnormal cells) from all treatments was calculated without consideration of the degree of alteration. The results showed 1.63, 4.61, 6.53, 6.59, 11.30 and 16.57% of abnormal cells in 0, 1, 5, 10, 20 and 40 $\mu\text{g}/\text{ml}$ venom treated cells, respectively. The statistical analysis indicated that the percentage of abnormal cells in all treatments was significantly higher than in untreated cells ($p < 0.05$) (Figure 4).

A semi-thin section of each cell pellet was studied by light microscope (1,000x) and showed that normal cells had adhered to each other. The nucleus with nucleolus was clearly seen. Some granules were also found in cytoplasm (Figure 5A). In contrast, the cells treated with 40 $\mu\text{g}/\text{ml}$ venom lost their adhesion. Many of them presented various degrees of cytoplasmic blebs, irregularity

of nuclear membrane, cytoplasmic vacuolation and degeneration of organelles and nucleus (Figure 5B). It was obvious that cell adhesion was maintained in normal cells, since the plasma membrane was not altered, thus adhesion molecules were functioning properly. Even when trypsinized, they were able to adhere after trypsin removal. In addition, cytoplasmic organelles and nuclei were still intact. In contrast, the venom-exposed cells were separated due to a lack of plasma membrane integrity. Though not ruptured, cellular organelles and nucleus had degenerated, which was apparent from the dissemination of

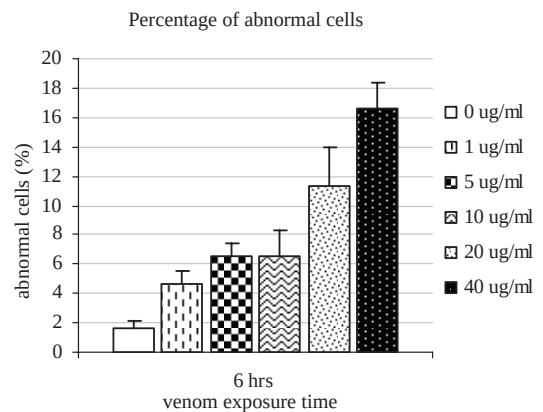


Figure 4 Percentage of abnormal cells compared between cells treated with 1, 5, 10, 20 and 40 $\mu\text{g}/\text{ml}$ of venom and untreated cells.

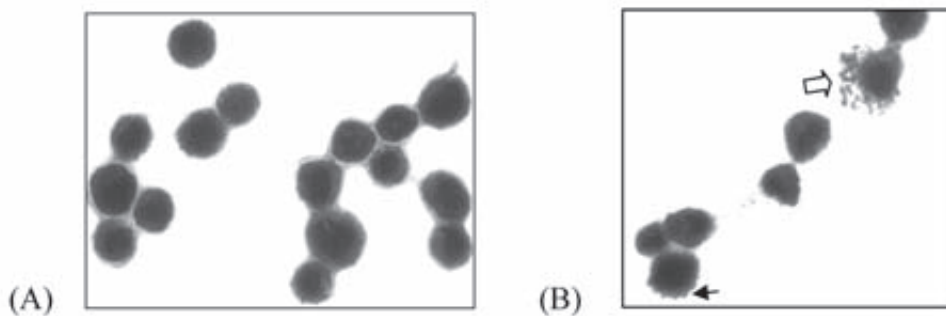


Figure 3 Cell morphology investigated under light microscope (1,000x). (A) Normal cells; (B) Cells treated with 40 $\mu\text{g}/\text{ml}$ of snake venom for 6 h. Arrow indicates plasma membrane blebs and fatter clear arrow indicates small fragments at the plasma membrane.

several vacuoles in the cytoplasm and the deformity of cells and nuclei.

The transmission electron micrograph showed the ultrastructure of normal cells adhering to neighbors. The cell characteristics exhibited a smooth plasma membrane, slightly indented nucleus with euchromatin, and cytoplasm containing organelles and secretory granules (Figure 6A). The cells treated with 40 $\mu\text{g}/\text{ml}$ of venom showed separated cells with plasma membrane blebs, smaller size of nucleus, dilated organelles and increased cytoplasmic vacuoles (Figure 6B). Some cells also showed clumping of the nuclear chromatin and apoptotic bodies (Figure 6C). The transmission electron micrographs presented characteristics of HuCCA-1 cell

apoptosis, which resembled apoptotic HepG2 cells induced by ethanol treatment (Neuman *et al.*, 1999).

Gathering data from the study indicated that optimal concentration and exposure time to green pit viper venom was able to induce cell apoptosis characteristics in HuCCA-1 cells. Though it has not been proven which component exhibited this effect, this paper postulates that the venom of the mangrove pit viper and the white-lipped pit viper share the same structure. The Lys49 PLA₂ contained in green pit viper venom has been suggested to play a role in cancer cell apoptosis (Nuchprayoon *et al.*, 2001; Mora *et al.*, 2005). However, a protein isolation technique is required to confirm this hypothesis.

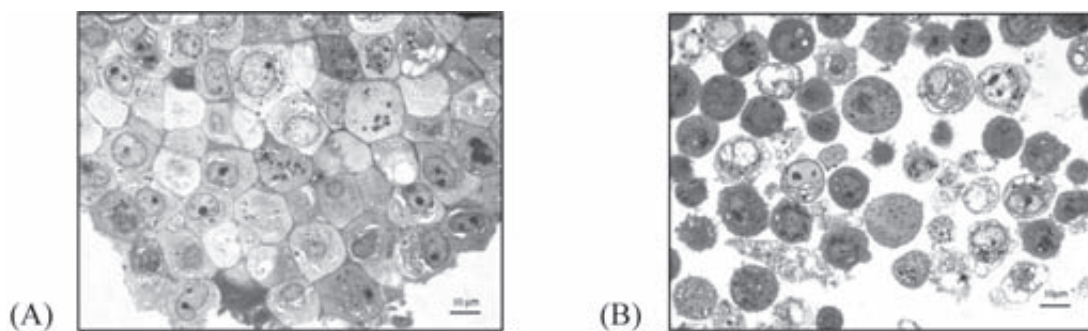


Figure 5 Semithin section in (A) normal cells; and (B) 40 $\mu\text{g}/\text{ml}$ venom-treated cells.

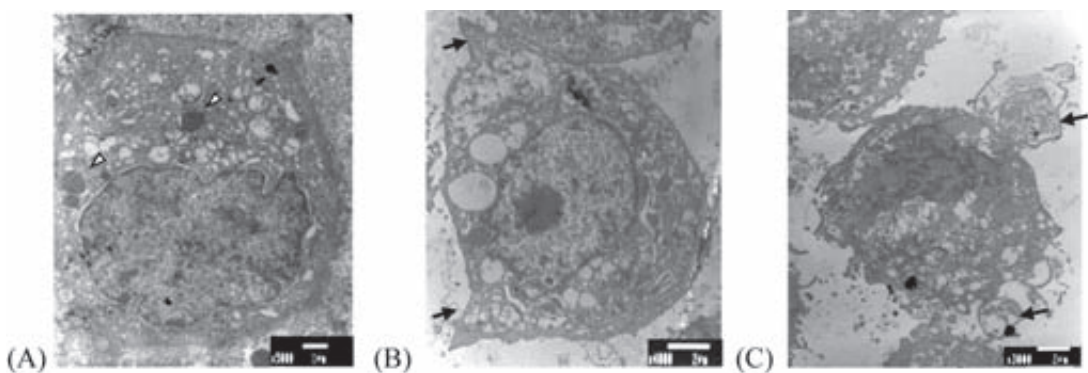


Figure 6 Transmission electron micrographs showing the ultrastructure of HuCCA-1 cells. (A) Normal cell with secretory granules (arrowhead); (B) Cell treated with 40 $\mu\text{g}/\text{ml}$ venom exhibited cytoplasmic blebs (arrow), vacuolation and organelle dilatation; and (C) Nuclear chromatin clumping and apoptotic bodies (arrow) shown in 40 $\mu\text{g}/\text{ml}$ venom treated cell.

CONCLUSION

The morphology investigation was only one of many trials to study cell apoptosis. Even though the electron micrographs showed clear apoptotic characteristics, molecular biological methods, such as TUNEL assay, are also recommended. Nevertheless, this study showed the potential of green pit viper venom to induce cancer cell apoptosis, which should make it possible to develop an anti-cancer agent. To assure patient health security, analysis of active ingredients and *in vivo* study are essential to determine an appropriate dose and eradicate the side effects of venom.

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