ABSTRACT

The venom of green pit viper (Trimersurus purpureomaculatus) was used to study effect on bile duct epithelial cancer cell damage, by using human cholangiocarcinoma cell line, HuCCA-1, as a model. After cells treatment by various concentration of snake venom for different time points, the alteration of cellular structure was observed, especially an apoptosis of cells. Under light microscope, many patterns of cellular changes were obviously seen by treatment of 40 μg/ml snake venom for 6 hours while the structure of untreated cells was still unchanged. The number of altered cells was significantly higher than in control (p<0.05). By transmission electron micrograph, venom treated cells showed the characteristic of apoptosis in various degrees. The cells presented plasma membrane blebs, increased number of cytoplasmic vacuoles, organelles enlargement or deterioration, reduced nuclear size, irregular nuclear membrane, and clumping of nuclear chromatin. Though an action of snake venom in Viperidae family caused hemorrhagic symptoms by functions of several components including phospholipaseA₂, we hypothesized that the venom of green pit viper shared structural similarity of Lys49 phospholipaseA₂, which lacked hemolytic activity but showed an ability to induce cancer cell apoptosis.

Key words: Green pit viper venom, Lys49 phospholipaseA₂, Cholangiocarcinoma, HuCCA-1, Apoptosis

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INTRODUCTION

The green pit vipers are venomous snake in Viperidae family, subfamily Crotalinae. The snake venom from this family has been known as hemorrhagic venom (Jintakune and Chanhome, 1995). Major components found in viperid venom are mostly proteolytic enzymes including phospholipaseA₂ (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 in destroying blood vascular system. The PLA₂ is toxic to cell membrane thereby can cause local cell and tissue damage (Nuchprayoon et al, 2001). It effects in variety of pathological processes including anticoagulation, edema and platelet aggregation inhibition (Sai-Ngam et al, 2008). Thrombin-like enzymes, fibrinogenolytic enzymes and plasminogen activators composed in serine proteases are also involved in anticoagulation (Rojnuckarin et al, 2006). L-amino acid oxidase induces 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 has been demonstrated to induce hemorrhage by degradation of endothelial basement membrane and adhesion proteins, thus weakening the capillary wall (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 PLA₂ isoforms in Thai Russell’s viper (Vipera russelli siamensis) venom were in such case. This viperid in Viperinae subfamily expresses highly lethal hematotoxic activity (Jintakune and Chanhome, 1995), consistent with the clinical manifestations of hemolysis, systemic bleeding and renal complication especially acute renal failure in biting victims (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 enzymatically inactive since the calcium binding loop and catalytic center were structurally altered (Lomonte et al, 2003). The venom of White-lipped pit viper (Trimeresurus albolabris), one of the most poisonous green pit viper, 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 dose (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 (Lamonte et al, 1999). In addition, it induced apoptosis of lymphoblastoid cell line by high concentration of 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 indicated by the release of lactate dehydrogenase (LDH) to the supernatant (Araya and Lomonte, 2007). Moreover, it reduced tumor from EMT6 mammary carcinoma cell injection in mice to a similar size of that caused by antitumor drug paclitaxel administration, at the same time interval (Araya and Lomonte, 2007). However, damage of cancer cell by apoptosis that did not destroy normal tissue and cells is desirable. The characteristics of apoptotic process prevent cell breakage and leakage of lysosomal hydrolytic enzyme to surrounding. The morphologic alterations include cell shrinkage, plasma and nuclear membrane blebbing, organelle relocalization and compaction, chromatin condensation and production of membrane-bound 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 liver bile duct, was found extensively among Thai people especially in north-eastern part of Thailand (Sriamporn et al., 2003). The cancerous mass in liver parenchyma is difficult to detect by the patients themselves, thus the advanced stage cancer was frequently examined at the hospital. Since resistance to chemotherapy and radiotherapy, surgery was a treatment of choice for cholangiocarcinoma (Gores, 2003). Currently, other alternative treatments were discovered to overcome this disease (Reddy and Patel, 2006; Rosen et al., 2008). However, the incident and mortality rate were still high in Thailand (Sriamporn et al., 2003). As a result, prevention and more effective treatment are required to decrease number of cholangiocarcinoma patient. The combination of curative agent with 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 is to study whether green pit viper venom can induce apoptosis in human cholangiocarcinoma cell by using HuCCA-1 cell as a model. Since the venom of Mangrove pit viper (T. purpureomaculatus) is as severe as White-lipped pit viper (T. albolabris) venom and shows the same clinical signs of biting victim, we hypothesized that Mangrove pit viper venom also composed of Lys49 PLA2 and optimal concentration of this venom can destroy HuCCA-1 cells by apoptotic process.

MATERIALS AND METHODS

Cell culture treatment with snake venom

The lyophilized venom of Mangrove pit viper (T. purpureomaculatus) was bought from the Thai Red Cross Society and diluted in phosphate buffer solution. 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 5x10⁵ cells per well were seeded into 24 well plate, 18 hours before treating with venom. The fresh complete culture medium was replaced and then the venom concentration of 1, 5, 10, 20, 40 or 80 μg/ml was added into each well. Cytopathic effects or alterations were observed under phase contrast inverted microscope at 3, 6, 12, 24, 48 and 72 hours.

Cell morphology investigation

The treatment that showed substantial data was chosen to study cell morphology. The cells in 24 well plate were trypsinized and a volume of 10 μl was dropped on glass slide, air dried and fixed with methanol for 5 minutes. The cells were then stained with toluidine blue and cell morphology was observed under light microscope (1,000x). The percentage of abnormal cell number from various treatments were calculated and statistically analyzed by Student’s t-test.

The rest of trypsinized cells were centrifuged and the pellet was collected, and then prefixed with 2.5% glutaraldehyde, postfixed with 2% osmium tetroxide, dehydrated in acetone series, infiltrated and embedded in Spurr. All samples were semithin sectioned for high magnified light microscope study and ultrathin sectioned for transmission electron microscope (TEM) study.

RESULTS AND DISCUSSIONS

After various concentrations of snake venom were added into the culture medium, the cellular changes were studied at different time points. Within 3 hours, the cells treated with 80 μg/ml of venom were drastically destroyed. Most of cells showed cytoplasmic condensation and reduced size but some were ruptured. The focal detachment of cells from substratum was extensively found (Figure 1).

However, the lower concentration did not cause any alteration. At 6 hours, the cells treated with 40 μg/ml of venom began to reduce size. The condensation of cytoplasmic organelles was not obviously seen and cell rupture was not found. The minute detachment of cells was occurred (Figure 2). After 12 hours, the cells treated with 20 μg/ml of venom showed morphological similarities to the 40 μg/ml treated cells at 6 hours but the normal cells also showed unhealthy sign (data not shown). Since the HuCCA-1 cell was basically attached epithelial cell line which needed to adhere with neighboring cells and attach to the substratum (Sirisinha et al., 1991), high concentration or long period of snake venom incubation could destroy cells, characterized by cell detachment and then suspending in the culture medium. However, it was interesting that the confluent HuCCA-1 cells grown for more than 12 hours without subculture were also prone to be toxic by metabolic waste excreted into the culture medium. Though the cells treated with low concentration of venom (1, 5 or 10 μg/ml) or untreated, they exhibited deterioration later on.
The cells treated with 1, 5, 10, 20 and 40 μg/ml of venom for 6 hours were selected for further study. After trypsinized and stained, cell morphology was investigated under light microscope. Normal cells were round shaped with round or indented nucleus and smooth nuclear membrane but small area of irregular plasma membrane might be found (Figure 3A) which was consistent with microvilli or secretory products found on cell surface (Sriurairatana et al, 1996). All venom treatment presented various degree of cell alteration, significantly in 40 μg/ml. Cell showed smaller size, abnormal cellular and nuclear shapes, plasma membrane blebs and irregular nuclear membrane. Some cells represented small fragments at the plasma membrane (Figure 3B). These findings were supposed to be characteristic of apoptotic cell hence the ultrastructure was needed to clarify. The percentage of altered cells (abnormal cells) from all treatment was calculated, without concerning the degree. The result 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 μg/ml venom, respectively. The statistically analysis indicated that the percentage of abnormal cells in all treatment was higher than in untreated cells significantly (p<0.05) (Figure 4).
Figure 3  Cell morphology under light microscope (1,000x). (A) Normal cells (B) The cells treated with 40 μg/ml snake venom for 6 hours showed plasma membrane blebs (arrow) and small fragments of plasma membrane (clear arrow).

Figure 4  Percentage of abnormal cells treated with 1, 5, 10, 20 and 40 μg/ml of venom compare with untreated cells.

The semithin section of cell pellets was studied by light microscope (1,000x), the normal cells were adhered to each other and nucleus with nucleolus was clearly seen. Some granules were also found in cytoplasm (Figure 5A). In contrast, the 40 μg/ml venom treated cells lost their adhesion. Many of them displayed various degrees of cytoplasmic blebs, nuclear membrane irregularity, cytoplasmic vacuolation and degeneration of organelles and nuclei (Figure 5B). It was obvious that cell adhesion was maintained in normal cells since plasma membrane was not altered, thus adhesion molecules were properly functioned. Even trypsinized, they were able to adhere after trypsin removal. In addition, cytoplasmic organelles and nucleus were still intact. The venom exposed cells, in contrast, were separated due to lacking of plasma membrane integrity. Though not rupture, cellular organelles and nucleus were degenerated which characterized by disseminated cytoplasmic vacuoles and cellular and nucleolar deformity.

Figure 5  Semithin section: normal cells (A) and 40 μg/ml venom treated cells (B)
The transmission electron micrograph showed ultrastructure of normal cell adhering to neighbours. The cell characteristic exhibited smooth plasma membrane, slightly indented nucleus with euchromatin, and cytoplasm containing organelles and secretory granules (Figure 6A). The cells treated with 40 μg/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 nuclear chromatin and apoptotic bodies (Figure 6C). The transmission electron micrographs presented characteristics of HuCCA-1 cell apoptosis which were resembled to apoptotic HepG2 cells induced by ethanol treatment (Neuman et al, 1999).

![Figure 6](image)

**Figure 6** Illustrations of transmission electron micrograph showed ultrastructure of HuCCA-1 cells. (A) Normal cell with secretory granules (arrowhead). (B) Cell treated with 40 μg/ml venom exhibited cytoplasmic blebs (arrow), vacuolation and organelle dilatation. (C) Nuclear chromatin clumping and apoptotic bodies (arrow) shown in 40 μg/ml venom treated cell.

According to data, the optimal concentration and exposure time of Mangrove pit viper venom was able to induce apoptosis in HuCCA-1 cell. Though has not been proved whether which component exhibited this effect, we postulated that venom of Mangrove pit viper and White-lipped pit viper shared the structural similarity. The Lys49 PLA₂ contained in venom was suggested to play role in cancer cell apoptosis (Nuchprayoon et al, 2001; Mora et al, 2005). However, protein isolation technique must be required to confirm this hypothesis.

**CONCLUSION**

The morphology investigation was only one in many trials to study cell apoptosis. Even electron micrographs clearly showed apoptotic characteristics, the molecular biological methods, such as TUNEL assay, were also recommended. Nevertheless, this study showed potency of green pit viper venom to induce cancer cell apoptosis, thus it was possible to be developed as anti-cancer agent. To assure patient health security, analysis of active ingredients and in vivo study were seriously necessary to obtain an appropriate dose and eradicate side effects of venom.
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LITERATURE CITED


