Anti-cancer effect of ICD-85(venom derived peptides) on MDA-MB231 cell line (*in vitro*) and experimental mice with breast cancer (*in vivo*)

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Abstract: Breast cancer has become common in developing and developed countries. Alarming increase in this disease as a leading cause of death in women is a concern of all. Despite the significant improvements in the management of breast cancer, the survival rate is not more than 20% - 25%. For this study, MDA-MB231 cell line was used and the effect of ICD-85 (biologically active peptides from venomous animals) was assayed by measuring the activity of the cytosolic enzyme lactate dehydrogenase(LDH) released into the culture medium after membrane legions. Morphological changes of cells were checked in control and cells incubated with ICD-85 as chemo preventive agent. Results showing in test groups - incubation with 10µg/ml dose of ICD-85 had decreased cytoplasmic branch. Some cells were ruptured and lost the continuity of their surrounding membranes, and number of cells was decreased. On the other hand, when ICD-85 was used as an anticancer treatment of mice with breast cancer, it was shown that ICD-85 can prevent the growth of breast tumor, increase the life duration of experimental mice and inhibit angiogenesis in breast tumor as compared with control group. It seems that ICD-85 acts at the membrane level and can prevent the cell growth as well as producing apoptosis (cell suicide) for breast cancer cells.

Key words: anticancer, breast cancer, ICD-85, mice.

Introduction

Breast cancer has become common in developing and developed countries. Alarming increase in this disease as a leading cause of death in women is a concern of all. Despite the significant improvements in the management of breast cancer, the survival rate is not more than 20% - 25%. Most common death in breast cancer patients is due to metastatic spread of cancer cells which invade into angiogenic blood vessels growing into the tumor (Gupta *et al*., 2003). The identification of efficacious and safe agents, biomarkers of efficacy and risk, and suitable cohorts for clinical intervention are critical of progress in chemoprevention. Basic research in negative growths regulation of cell lines, as a cancer from *in vitro* model, has identified many genetic legions and other cellular constituents associated with the initiation and progression of malignancy (Macarithy *et al*., 1985).

Since 1987 chemoprevention testing programs, more than 1000 agents and agent combinations have been selected and evaluated in preclinical studies of chemopreventive activity, ranging from *in vitro* mechanistic assays and cell-based transformation assays to carcinogen-induced and transgenic animal models (Antman *et al*., 2001; Fenaux *et al*., 2000; Wang *et al*., 2001). New agents are continually considered for development as chemopreventive drugs, with selection based on preliminary efficacy data, mechanistic considerations, and potential for

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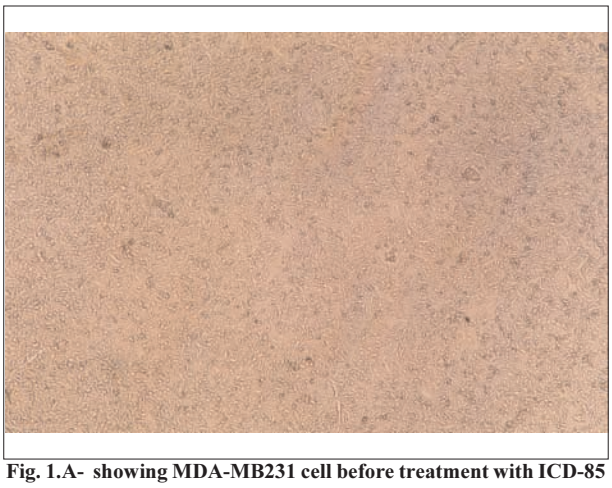

Fig. 1.A- showing MDA-MB231 cell before treatment with ICD-85 elongated cells with high density of cells.

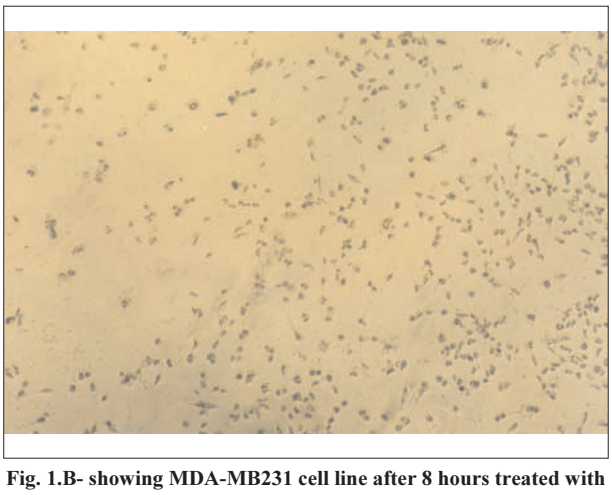

Fig. 1.B- showing MDA-MB231 cell line after 8 hours treated with ICD-85. The density of cells are reduced and the shape of cells changed from elongated to round cells.

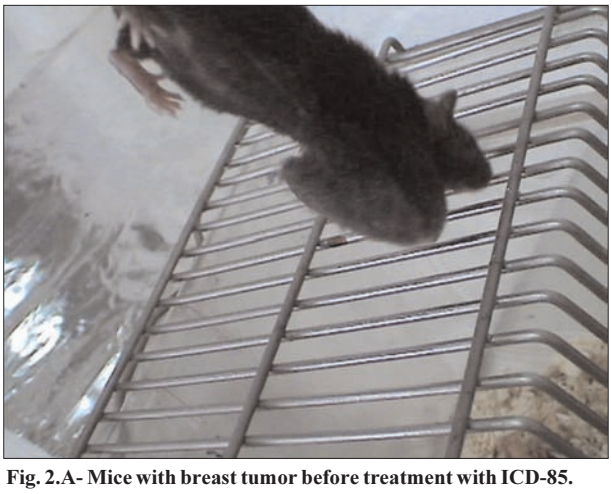

Fig. 2.A- Mice with breast tumor before treatment with ICD-85.

Fig. 2.B- Mice with breast cancer on day 45 after treatment with ICD-85

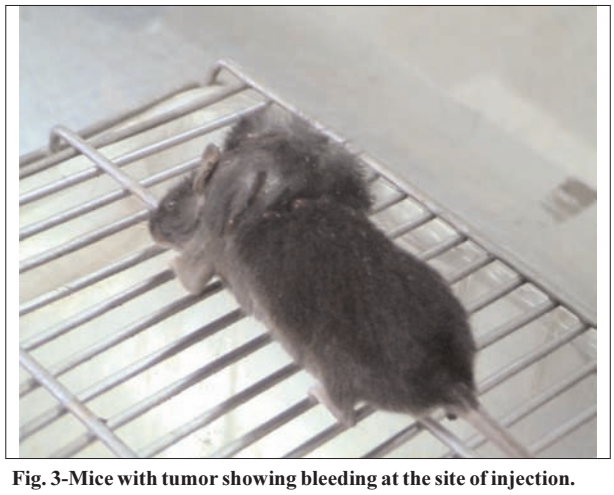

Fig. 3-Mice with tumor showing bleeding at the site of injection.

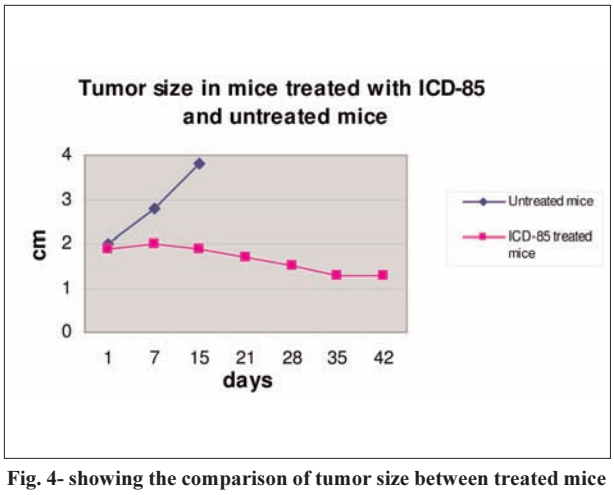

Fig. 4- showing the comparison of tumor size between treated mice and untreated mice with ICD-85. All untreated mice died within 15 days of experiment.

improved chemopreventive (therapeutic) index (Arjmand *et al*., 2004).

There are reports showing the cytotoxic activity of various snake venoms *in vitro* and *in vivo*, using melanoma and chondrosarcoma cells (Chaim-Matyas *et al*., 1987). It was further demonstrated that

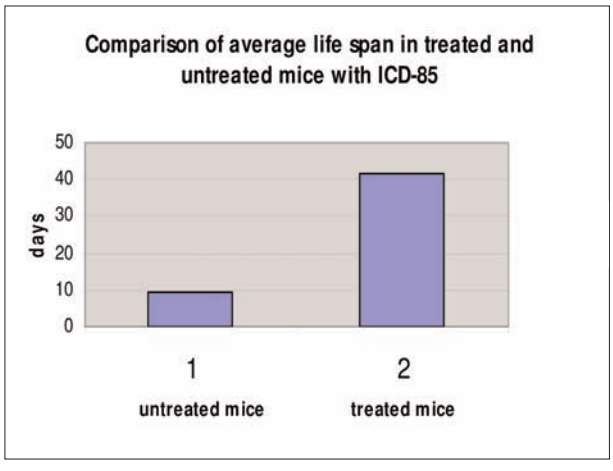

Fig. 5- showing the average of life span of both the groups of mice

a purified protein from cobra venom was selectively cytotoxic to cancer cells (Braganca *et al*., 1967). Silva *et al*., 1996 evaluated the action of the venoms of Crotalus durissus terrificus and Bothrops jararaca on Ehrlich ascitic tumors and found that both venoms act directly on tumor cells (Silva *et al*., 1996). Stephin et al recently reported that use of liposomal delivery system for snake venom disinterring (a peptide derived from snake venom) has a significantly prolonged circulatory half life compare with native CN, LCN is passively accumulated in the human breast tumor and limits the progression (Stefen, 2004).

In this study we aimed to determine characterization of biological and pharmaceutical activities of ICD-85 on MDA-MB231 cell line as well as *in vivo* effect of ICD-85 on experimental breast cancer induced mice, in order to gain a better understanding of the cytotoxic, apoptotic, and / necrotic effects of this compound as a chemopreventive agent.

Materials and Methods

ICD-85: The active fraction of ICD-85 is a combination of 3 peptides ranging from 10000 to 30000 dalton derived from the Iranian brown snake and yellow scorpion venom which is formulated and provided by correspondent author.

Cell line: The MDA-MB 231 breast cancer cell line was kindly provided by Dr Mohammad kazem Koohi from the University of Tehran. MDA-MB 231 cells were plated out in suitable culture flasks in Dulbecco's minimal essential medium (DMEM) (Gibco) supplemented with 10% of fetal calf serum(J.D. Iran) and 1%L-glutamin (sigma) plus 0.5%penicillin/streptomycin (sigma) and incubated at 37°C in an atmosphere containing 5% Co_2 (Koohi *et al*., 2005; Koohi *et al*., 2007).

ICD-85 cytotoxicity: Cell used in this study was plated in 3(12-well) plates at 100000 cells /well. An incubation condition was as mentioned above. After 24 hours of incubation, the cells were washed two times with PBS and incubated with one Ml serum free medium (SFM) supplemented with 10µg/ml of ICD-85 and checked control culture well was treated with an equal amount of SFM(SFM was used to avoid the possible interaction between ICD-85 and FBS) (Koohi *et al*., 2005; Koohi *et al*., 2007).

Toxicity was assayed by measuring the activity of the cytosolic enzyme lactate dehydrogenase (LDH) released into the culture medium after membrane legions. Samples from clarified medium of treated and untreated control wells were taken after 30 min of incubation and the LDH activity measured using the cytotoxicity assay cytotox 96® in conjunction with a fully automated microplate reader photometer. Cell damage and morphological change to cell line was investigated using a light phase contrast microscope and photos were taken at each time point.

***In vivo* studies:** Mice with breast cancer (Razi Mice) were provided by the department of laboratory animals, Razi Vaccine and Serum Research Institute, Karaj Iran. About 30-35 % of these mice are naturally getting breast cancer at the age of 7-9 months.

Fourteen mice were selected on the base of the size of tumor developed in their breast. Effort was taken to choose the mice that had a tumor size of 2±0.5 cm. in the breast tissue. They were divided into two groups. Group 1 received saline and group 2 received ICD-85 (10 µg /mice) intra-tumoral injection every weak once up to 42 days and then the live mice were scarified for pathological studies.

All the animals were observed every day and the size of tumor in each animal breast were determined every weak by cullies.

Results

***In vitro*:** The MDA-MB231 cells exhibited polygonal shape with distinct boundaries and homogenous or slightly granulated cellular contents. They were thin and elongated with many tapering ends as cytoplasmic branch (metastatic branch). In group 2, cells incubated with the dose of 10µg/ml of ICD-85 showed swelling feature, and decreased cytoplasmic branch. Some cells were ruptured and lost the continuity of their surrounding membranes, and number of cells was decreased (figure 1B). Almost all the cells exhibited rounded appearance rather than elongated configuration. Within 8 hours of incubation of MDA-MB231 cells with 10 µg ICD-85, the growth was almost completely inhibited. But when the MDA-MB231 cells were left without addition of ICD-85 after 24 hours, the few remained cells started to grow again and returned back to the same condition as normal MDA-MB231 cell line.

Results obtained from the ICD-85 stability test revealed that ICD-85 is stable during the incubation time period (24h.) with the culture medium, and its cytotoxic effects on the MDA-MB231 cell line were significant. Lactate dehydrogenase released from cultured cells exposed to a biomaterial provides a sensitive and accurate marker for cellular toxicity. ICD-85 induced identical response effect on MDA-MB231 cells, releasing, 20±4 IU LDH after 30 min. at used concentration (10µg/ml) compared to untreated MDA-MB231 cells with ICD-85 which was 9±3 IU of LDH.

***In vivo*:** The average size of tumor in both groups of mice was 2±0.5 cm. However two mice had tumor size of greater than 3.5cm that died after 5 days and excluded from this study. Graph No 1 shows the changes in breast tumor size in both groups. Group 1 mice that received saline showed a significant ($p>0.001$) increase in the size of tumor (4.2±0.5) and all of them died within 15 days of experiment (Figure 2A). However in group 2 only one of the animals that received ICD-85 died in day 25 after ICD-85 treatment, and the rest of animals survived till the end of the experiment (Figure 5). Significant ($p<0.01$) reduction in size (1.3±0.3) of tumor was observed in ICD-85 treated animals (figure 2B). All the animals in this experiment showed bleeding at the site of injection (tumor site) when experiment started (figure 3). In group 1 (saline receiving animals) bleeding at tumor site was increasing till their death, while in group 2 (ICD-85 treated animals), although at the start of experiment, showed bleeding at the site of injection (tumor site), but within 21 days after treatment, none of them showed bleeding during ICD-85 injection till the end of experiment.

Discussion

The *in vitro* study of inhibitory effect of ICD-85 compound on MDA-MB 231 cell line clearly reveals that it effectively inhibits the growth of these cells. The MDA-MB 231 cell line was used in this study, because it had been shown to be a highly invasive breast cancer cell line (Koohi *et al*., 2005; Koohi *et al*., 2007). This investigation shows that the lesion induced to cell line by ICD-85 is direct. The cytotoxicity was remarkable and cell survival was highly reduced at the concentration of 10?g/ml within 8 hours of incubation. The LDH activity was measured using the cytotoxicity assay. This assay method for detection quantifying LDH release from cell can detect whether cell and apoptotic bodies remain enclosed by intact plasma membrane, and thus, can be used to discriminate between apoptosis and necrosis (Kuwana *et al*., 1998; Nihhawan *et al*., 2000). It seems that ICD-85 at used concentration acts at the membrane level, this allows the passage of ions down their concentration gradient, resulting in osmotic changes in organelles followed by several unidentified mechanisms leading to cell death. This might be one of the reasons that produce apoptosis (cell suicide) for cell line as a result of treatment with ICD-85 (Nihhawan *et al*., 2000). In this respect, death and apoptosis could be set in motion by diverse stimuli such as chemical toxicity, radiation, or genotoxic damage. ICD-85 kills cancer cells by various mechanisms. The membrane lesions are more prominent and clear in the early stages of toxicity, while other forms of cell damage such as swelling, rupture, and/or necrosis occurred in the later stages.

Based on the results obtained by *in vitro* studies,

we determined the anticancer effect of ICD-85 on mice in which cancer was naturally developed in their breast. Local delivery of ICD-85 to breast tumor targets during the experiment showed inhibitory effect on the growth of tumor. As shown in the graph 1, the size of tumor increased significantly in untreated mice with ICD- 85 while in contrast the size of tumor in mice treated with ICD-85 showed no change or decrease (figure 4). Expectancy of life increased from average of 15 days after the tumor reached to the size of 2 cm (untreated animals) to more than 45 days in ICD-85 treated animal. It seems that the ICD-85 at the injected dose acts as an anti-angiogenesis. Because in all the animals that received ICD-85 as treatment, bleeding at the tumoral site of injection stopped within 21 days of treatment (figure 3). Anti-angiogenic therapy is a promising alternative for treatment of cancer. It may also be used as a maintenance therapy to prevent the metastasis or recurrence (Gupta *et al*., 2003).

Evidence from basic researches and from experiments on animal models and cell culture systems supports the concept that many compounds, as natural products may offer protection against organ site carcinogenesis (Black *et al*., 1998; Shaikh *et al*., 2006; Wu *et al*., 2001). Such compounds as biological products or natural phyto-chemicals, either as single agents or as adjuvant, may represent valuable lead compounds for preventive therapeutic intervention (Chih-hsin *et al*., 2004).

Crude Snake venom as well as isolated peptides from snake venom are shown to be effective as inhibitor of cancer cells (Fenaux *et al*., 2000; Lipps *et al*., 1999). Despite the great advances in understanding the morphological and biochemical alterations associated with substances and chemicals induced cell injury, it has to be realized that the knowledge acquired is still insufficient to establish which of changes lead to cell death and which secondary disturbances are. This study attempts to clarify some of the cytotoxic effects of ICD-85 which occurs on cultured cell line.

Hence based on the results obtained in this study, the active components of ICD-85 are able to act as anti growth factor against invasive breast cell line (MDA-MB 231), *in vitro* and prevent further growth of breast tumor and expand the life duration of mice with breast cancer, *in vivo*.

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تاثیر ضد سرطانی ICD-85 (پپتید مشتق از سَم) بر روی سلول‌های MDA- MB231 و سرطان پستان موش

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چکیده

سرطان پستان در کشورهای در حال توسعه و پیشرفته از جمله بیماری‌های شایع می‌باشد. افزایش شیوع این بیماری در جمعیت زنان موجب نگرانی در سطح جامعه انسانی شده است. علیرغم بهبود قابل ملاحظه در مدیریت سرطان پستان، میزان زنده ماندن افراد مبتلا به بیش از ۲۵-۲۰ درصد نمی‌رسد. هدف از این مطالعه تعیین تأثیر ICD-85 در جلوگیری از بروز سرطان پستان در شرایط *In vitro* و *In vivo* است. برای این منظور سلول‌های MDA-MB231 مورد استفاده قرار گرفتند و تاثیر ICD-85 (پپتیدهای فعال بیولوژیک که از سم حیوانات حاصل می‌شوند) بر روی آسیب غشایی از طریق سنجش فعالیت آنزیم لاکتات دهیدروژناز سیتوزلی مورد سنجش قرار گرفت. همچنین تغییرات مورفولوژیکی سلول‌ها در گروه‌های کنترل و درمانی با ICD-85 مورد ارزیابی قرار گرفت. نتایج حاکی از آن است که غلظت ۱۰µg/ml از ICD-85 موجب کاهش cytoplasmic branch شده است. همچنین برخی از سلول‌های دچار پارگی شده، پیوستگی غشاء محاط خود را از دست داده و تعداد سلول‌ها نیز کاهش پیدا کرد. از طرف دیگر در این مطالعه نشان داده شد که ICD-85 موجب جلوگیری از رشد تومور پستانی در موش‌ها شده است. بنظر می‌رسد ICD-85 از طریق تاثیر بر سطح سلول می‌تواند موجب جلوگیری از رشد سلول و بدنبال آن مانع از مرگ برنامه‌ریزی شده سلول‌های سرطان پستان شود.

واژه‌های کلیدی: ضد سرطان، سرطان پستان، ICD-85، موش.

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