Brief Report

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Toad skin extract cinobufatini inhibits migration of human breast carcinoma MDA-MB-231 cells into a model stromal tissue

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Summary **Toad skin extract cinobufatini study has been focused on anticancer activity, especially apoptosis-inducing activity by bufosteroids. The present study examined effect of the toad skin extract on cancer cell migration into model stromal tissues. Human breast carcinoma cell line MDA-MB-231 was incubated in the presence or absence of toad skin extract on a surface of reconstituted type I collagen gel as a model stromal tissue allowing the cells to migrate into the gel. Frozen sections were microscopically observed after azan staining. Data showed a decrease of cell number in a microscopic field and shortening of cell migration into the model stromal tissue in a dose dependent manner. This suggests that toad skin extract may possess migration-preventing activity in addition to cell toxicity such as apoptosis-inducing activity. The multifaceted effects including apoptosis-inducing and cancer cell migration-preventing activities would improve usefulness of toad skin extract cinobufatini as an anticancer medicine.**

Keywords: Toad skin extract cinobufatini, cell migration, type I collagen, cancer

1. Introduction

An aqueous extract from the skin of toad *Bufo bufo* gargarizans Cantor is used as a source of the Chinese traditional medicine cinobufacini (*1*). The toad skin extract has been found to possess anticancer activity (*2-4*). Although the detailed nature of the ingredients contained in the extract remains unknown, recent studies have revealed that a series of bufosteroids such as bufalin, cinobufagin, and regibufogenin shows apoptosis-inducing activity against cancer cells *via* some cell signaling pathways (*2,5-7*).

Carcinoma cells that begin in epithelial tissues first destroy basement membranes and start to infiltrate and invade into stromal tissues (*8,9*). In this process, various types of metalloproteinases are involved in degradation of matrix proteins such as collagens (*10,11*). Since cell infiltration and invasion is the first step of metastasis, it should be important to inhibit this step to control cancer.

Our previous study reported a method for assessment of cancer cell invasion into reconstituted a type I collagen gel as a model stromal tissue that includes processes of freeze sectioning and azan staining (*12*). The method permits us to observe the distribution of the cells migrating into the collagen gels from the gel surface and to evaluate invading ability of cancer cells and inhibiting activity of compounds of interest against cancer cell invasion. The present study applied this method to analyze the effect of toad skin extract on cell migration into the model stromal tissue. We describe that toad skin extract may possess cancer cell migration-preventing activity in addition to cell toxicity such as apoptosis-inducing activity.

2. Materials and Methods

2.1. *Reagents*

Toad skin extract cinobufatini was kindly provided by

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Anhui Jinchan Biochemical Co., Ltd., Anhui, China. Bovine skin collagen type I was purchased from (Koken, Tokyo, Japan) and diluted to 1 mg/mL in 0.05 M acetic acid before use. Reconstitution buffer used to prepare reconstituted type I collagen gels was 1 M Hepes buffer, pH 7.4, containing 10 M $NaHCO_3$. All the chemicals used were of analytical grade.

2.2. *Cells*

Human breast carcinoma cell line MDA-MB-231 was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) supplemented with penicillin-streptomycin and 2 mM glutamine at 37°C in a 5% CO_2 atmosphere. The cells were harvested after preincubation in serum-free medium for 24 h at 37°C and subjected to experiments.

2.3. *Bilayer reconstituted type I collagen gel*

Bilayer reconstituted type I collagen gels were prepared in wells of an 8-well chamber slide (Nalge Nunc, Naperville, IL, USA) as described previously (*12*) with minor modifications. Briefly, lower gels were prepared by mixing 100 μL of 0.1% collagen solution with 10% FCS, DMEM, and reconstitution buffer (8:1:1, v/v) in an 8-well chamber slide. Two-hundred μL of type I collagen solution without FCS was mixed with or without 1 μL of toad skin extract diluent and incubated on the lower gel at 37°C to form the upper gel.

2.4. *Histochemical observation of cell distribution in the gel*

Cell suspensions (10^5 cells/mL) were preincubated in the presence or absence of toad skin extract diluent for 30 min at 37°C. The suspension (250 μL each) was loaded onto the reconstituted type I collagen gel in a chamber slide and incubated for 3 h at 37°C in a 5% CO_2 atmosphere. After incubation, the gel surface was rinsed twice with 250 μL of phosphate-buffered saline and then DMEM containing 0.1% BSA to remove unbound cells. The gel was subsequently incubated for 15 h at 37°C in a 5% CO_2 atmosphere to allow the cells to migrate into the gel.

After removing the medium on the gel surface, the gel was then mounted using an embedding compound (Tissue-Tek O.C.T. Compound; Sakura Finetechnical, Tokyo, Japan) and frozen at –80°C. The frozen gel was sliced perpendicularly to the gel surface with a cryostat at a 50 μm thickness and the section was placed on a glass slide.

Frozen sections of reconstituted type I collagen gel into which cells were allowed to migrate were stained by the azan staining method as described previously (*12*). The sections stained were observed under a microscope (×200; BX-51, Olympus, Tokyo, Japan).

2.5. *Data analysis*

Migration distance of each cell from the gel surface was measured using at least 5 photographs of microscopic visual fields (× 200) or at least 150 cells. The Mann–Whitney *U* test was conducted with StatMate III software (ATMS, Tokyo, Japan) and a p value less than 0.05 was considered significant.

3. Results and Discussion

Figures 1A and 1B show typical microphotographs of frozen sections of reconstituted collagen gels after incubating cells in the absence or presence of toad skin extract, respectively. Histochemical observation of MDA-MB-231 cells in type I collagen gels showed that some cells remained on the gel surface and the others migrated into the gel with a wide range of distribution from the gel surface (Figure 1A). In contrast, at a glance, the number of cells observed in a microscopic field was decreased in the presence of toad skin extract (Figure 1B). The decrease in the number of cells depended on the concentration of toad skin extract when undiluted and ×100-diluted extract was added in the cell suspensions (Table 1).

Toad skin extract has been known to have potent anticancer activity (*2-4,13,14*). Some reports have suggested that apoptosis of cancer cell lines was induced by bufosteroids, unique steroid compounds contained in toad skin extract and toad venom, such as bufalin and cinobufagin (*15-19*). Decrease in MDA-MB-231 cell numbers observed in our experiments may

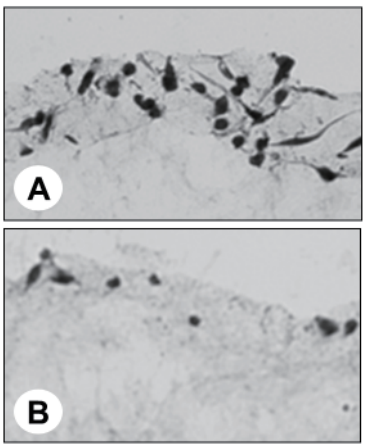

Figure 1. Typical histochemical observations of type I collagen gels where cells were allowed to migrate into the gels. MDA-MB-231 cells were allowed to migrate into type I collagen gels in the absence **(A)** or presence **(B)** of toad skin extract. The frozen sections were stained with azan and observed under a microscope. Original magnification: ×200.

Table 1. Decrease in the numbers of cells observed in microscopic fields by toad skin extract treatment

Treatments	% (means ± SD)[a]
Control	100
Toad skin extract	
×100-Diluted	35.5 ± 7.6
Undiluted	19.8 ± 5.3

[a] MDA-MB-231 cells treated with or without toad skin extract diluents were allowed to migrate into type I collagen gels. The frozen sections were stained with azan as described in Materials and Methods. Cell numbers observed in microscopic fields were counted and compared with untreated control.

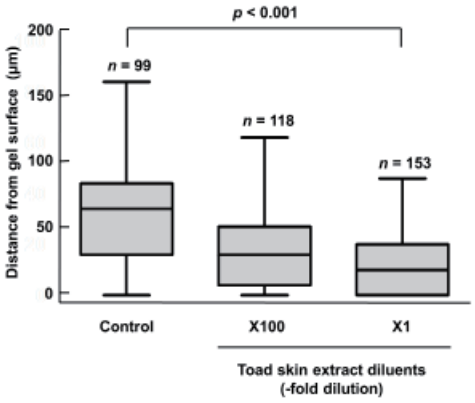

Figure 2. Effect of toad skin extract on cancer cell migration into type I collagen gels. MDA-MB-231 cells were treated with toad skin extract diluents as indicated in the figure and allowed to migrate into type I collagen gels with or without toad skin extract diluents. The frozen sections were stained with azan and then the distance from the gel surface was measured for each cell in microscopic fields.

be due to apoptosis of the cells in the presence of toad skin extract.

Figure 2 shows the distribution of the distance that cells migrated from the gel surface for untreated control cells and cells treated with various concentrations of toad skin extract. Some cells migrated a relatively long distance in the presence of toad skin extract but the median distance that cells migrated from the gel surface was significantly shorter depending on the concentration of toad skin extract (Figure 2).

Cancer cell migration and invasion are promoted by a degradation of matrix collagen by metalloproteinases (*20-22*). In our previous paper, we reported suppression of MDA-MB-231 cell migration into type I collagen gels in the presence of galardin which is known as an MMP inhibitor (*12*). Our present data suggest that toad skin extract may contain unknown compounds possessing MMP inhibitor activity. Actually, a preliminary study using metalloproteinase activity of FCS and a synthetic peptide analog as a substrate showed that the enzyme activity was inhibited by diluents of toad skin extract but not by bufosteroids such as bufalin, cinobufagin, and regibufogenin (unpublished data). This suggests that unknown compounds other than bufosteroids might participate in inhibiting metalloproteinase activity and result in suppressing cancer cell migration in the collagen gels. Further investigation to clarify the nature of the compounds is needed.

4. Conclusion

Toad skin extract cinobufatini study has been focused on anticancer activity, especially apoptosis-inducing activity by bufosteroids. Recently, cinobufatini has been clinically applied to patients with cancer (*23-25*). The present study suggests that the toad skin extract has an additional anticancer activity because it prevents cancer cell migration in model stromal tissues. Multifaceted effects such as apoptosis-inducing and migration-preventing activities should improve the usefulness of cinobufatini as an anticancer medicine.

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