Brief Report

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An aqueous extract from toad skin prevents gelatinase activities derived from fetal serum albumin and serum-free culture medium of human breast carcinoma MDA-MB-231 cells

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Summary **An aqueous extract from toad skin, cinobufacini, has been known to possess anticancer ability. The present study examined effect of toad skin extract on activity of gelatinases including matrix metalloproteinases-2 and -9 which play an important role in invasion of carcinoma cells. Gelatinase activities derived from fetal serum albumin and culture medium of human breast carcinoma cell line MDA-MB-231 were significantly prevented in the presence of toad skin extract. The inhibitory activity was found in water-soluble fraction of the extract prepared by the Bligh & Dyer method but not in $CHCl_3$-soluble lipid fraction. These results suggest that an aqueous extract from toad skin contains a water-soluble substance possessing a potent ability to prevent gelatinase activity. In conclusion, the water-soluble substance in toad skin extract cinobufacini may be able to regulate cancer cell migration accelerated by matrix metalloproteinases.**

Keywords: Toad skin extract cinobufacini, gelatinase, matrix metalloproteinase, cancer

1. Introduction

Carcinoma cells arise in epithelial tissues express extracellular matrix-degrading enzymes such as matrix metalloproteinases (MMPs) and invade into inner tissues while degrading basement membranes and stromal tissues (*1*). Of many types of MMPs, MMP-2 and -9, both of which possess gelatinase activity, are important to degrade basement membrane (*1,2*). Since the degradation of basement membrane is thought to be the initial step of metastasis, efforts to find and create compounds that can prevent gelatinase activity have proceeded (*3-6*).

An aqueous extract from the skin of toad *Bufo bufo* gargarizans Cantor, which is known as a source of the Chinese traditional medicine cinobufacini (*7*), has been focused in anticancer studies (*8,9*). Especially, a series of bufosteroids including bufalin, cinobufagin, and regibufogenin, which are ingredients of the toad skin extract and toad venom, has been found apoptosis-inducing ability against cancer cells *via* cell signaling pathways (*8,10-12*). Recently, the toad skin extract cinobufacini has been clinically applied to patients with cancer (*13-15*).

Our previous study indicated that the toad skin extract possessed not only cell toxicity such as apoptosis-inducing ability but also cancer cell migration-preventing ability *in vitro* (*16*). Considering that MMPs including gelatinases have an important role in cancer cell migration, it would be valuable to investigate whether the toad skin extract can inhibit gelatinase activity. Here, we describe that a type of water-soluble substance but not lipid such as bufosteroids in the toad skin extract could inhibit gelatinase activity.

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2. Materials and Methods

2.1. *Reagents*

Toad skin extract cinobufacini was kindly provided by Anhui Jinchan Biochemical Co., Ltd., Anhui, China. Bufosteroids such as bufalin and cinobufagin were obtained from Cosmo Bio, Tokyo, Japan and another bufosteroid regibufogenin was from Wako Pure Chemical Industries, Osaka, Japan. Fetal serum albumin (FCS) was obtained from GE Healthcare Life Sciences, HyClone Laboratories, Logan, UT, USA. Galardin (GM6001; *N*-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide) was obtained from Sigma-Aldrich Japan, Tokyo, Japan. All the chemicals used were of analytical grade.

2.2. *Cell culture*

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 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) supplemented with penicillin-streptomycin, fungizone (Invitrogen), and 2 mM glutamine at 37°C in a 5% CO_2 atmosphere as described previously (*17*). Before use, the cells were cultured in medium containing 0.1% bovine serum albumin instead of FCS for 24 h at 37°C. The culture medium was centrifuged and the supernatant was subjected to experiments as a source of gelatinase.

2.3. *Gelatin zymography*

MMP species in FCS and culture supernatant of MDA-MB-231 cells were detected using gelatin zymography (*18*). Samples were mixed with the same volume of sample buffer (125 mM Tris-HCl, pH 6.8, containing 4% SDS, 10% glycerol, and 0.01% bromophenol blue). Fifteen µL each of the sample was applied to SDS-polyacrylamide gel electrophoresis (4% gel) in the presence of 10% gelatin (Nacalai Tesque, Kyoto, Japan) in the separating gel at 4°C. After the electrophoresis, the gel was treated with 1 mM 4-aminophenylmercuric acetate (Sigma-Aldrich Japan) for 20 h at 4°C to convert proenzymes such as proMMP-2 and proMMP-9 to active forms (*19,20*). The gel was washed with a renature buffer (50 mM Tris-HCl, pH 7.5, containing 2.5% Triton X-100, 0.15 M NaCl) for 15 min 3 times and then gently shaked in a developing buffer (50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, 0.7 mM $CaCl_2$, 1 mM $ZnCl_2$) for 70 h at 37°C to allow gelatinase degrade the gelatin *in situ*. The gel was stained with 0.25% Coomassie brilliant blue and destained with 10% acetic acid. Intensity of enzyme reaction bands appeared in the destained gel was quantified by an image analysis using a gel imaging system Printgraph with CS Analyzer software (Atto, Tokyo, Japan).

2.4. *Colorimetric assay of gelatinase activity*

Colorimetric assay of gelatinase activity was performed using a thiopeptolide substrate (Ac-Pro-Leu-Gly-SCH[$CH_2CH(CH_3)_2$]-CO-Leu-Gly-OC_2H_5; Funakoshi, Tokyo, Japan) (*21,22*). FCS was used as a source of gelatinase. Assay was performed using a 96-well microassay plate. Reaction mixture contained 50 µL of 50 mM Hepes-NaOH, pH 7.5, containing 10 mM $CaCl_2$, 0.05% Briji-35, and 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; Wako Pure Chemical Industries), 20 µL of FCS, and 20 µL of sample solution such as toad skin extract, galardin, or bufosteroid or the relevant solvent as control. Enzyme reaction was started by adding 10 µL of 1 mM thiopeptolide substrate dissolved in dimethyl sulfoxide and the mixture was incubated at 37°C. 2-Nitro-5-thiobenzoic acid produced by a reaction of DTNB and sulfhydryl group of cleaved thiopeptolide substrate was continuously detected at 415 nm for 6 h at 1 h intervals using a microplate reader (Model iMark; Bio-Rad Laboratories, Hercules, CA, USA).

2.5. *Fluorometric assay of gelatinase activity*

Fluorometric assay of gelatinase activity was performed using gelatin-fluorescein isothiocyanate (FITC) (Cosmo Bio) as a substrate (*23*). Gelatin-FITC was dissolved in 10 mM acetic acid to a concentration of 1 mg/mL. The developing buffer for gelatin zymography described above was used as a reaction buffer. Reaction mixture was prepared in a shading tube, which contained 70 µL of reaction buffer, 60 µL of MDA-MB-231 cell culture supernatant as a source of gelatinase, and 20 µL of sample solution of interest or the relevant solvent as control. Enzyme reaction was started by adding 50 µL of gelatin-FITC solution and the mixture was incubated for 24 h at 37°C. The reaction was stopped by adding 200 µL of 22%(w/v) trichloroacetic acid and then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant (300 µL) was mixed with 100 µL of pH adjusting solution, a mixture of 1.5 M Tris-HCl, pH 7.5, and 3.14 M NaOH in the ratio of 11: 21 (v/v). FITC released by the enzyme reaction was measured at 520 nm with excitation at 495 nm using Fluorometer RF-1500 (Shimadzu, Kyoto, Japan).

2.6. *Fractionation of toad skin extract by the Bligh & Dyer method*

Fractionation of water-soluble and $CHCl_3$-soluble components in toad skin extract was performed according to the Bligh & Dyer method (*24*). To 0.9 part of toad skin extract, 1 part each of methanol (MeOH)

and chloroform ($CHCl_3$) were added and vortexed for 10 min followed by a centrifugation for 10 min at 3,000 rpm. The upper layer was moved into another sample vial. The lower layer was extracted again after adding a previously prepared upper layer of $CHCl_3$/ MeOH/ water (1:1:0.9, v/v) and the lower layer was recovered after centrifugation. In contrast, the upper layer of the first extraction was secondary extracted after adding a previously prepared lower layer of $CHCl_3$/ MeOH/ water (1: 1: 0.9, v/v) and the upper layer was recovered after centrifugation. Each layer was dried out *in vacuo* using VaporMix (EYELA, Tokyo, Japan) after removing $CHCl_3$ under N_2 gas. The residues obtained from the upper and lower layer were dissolved in distilled water and MeOH, respectively, of the same volume of toad skin extract sample used.

2.7. *Data analysis*

Data were analyzed by Student's *t* test with a StatMate III software (ATMS, Tokyo, Japan) and a *p* value less than 0.05 was considered significant.

3. Results and Discussion

Gelatinase species in FCS and serum-free culture medium of MDA-MB-231 cells were detected by gelatin zymography. As shown in Figure 1A, a different profile of enzyme reaction bands was shown between FCS and MDA-MB-231 cell culture medium. The bands a-d at positions of molecular weights 92, 80, 72, and 67 kDa, respectively, were deduced as proMMP-9, MMP-9, proMMP-2, and MMP-2, respectively (*20*). Quantification of the enzyme reaction bands by the image analysis showed that the major gelatinase species contained in FCS and MDA-MB-231 cell culture medium are MMP-2 and MMP-9, respectively (Figures 1B and 1C).

Gelatinase activity in FCS in which MMP-2 was a major MMP component (Figure 1B) was assayed using colorimetric assay in the presence or absence of diluents of toad skin extract. As shown in Figure 2A, the enzyme activity was prevented depending on the dilution rate of toad skin extract. In contrast, as shown in Figure 2B, gelatinase activity was not prevented by bufosteroids including bufalin, cinobufagin, and regibufogenin (1 μM each), all of which are known as antitumor ingredients in toad skin extract (*10,25*), while the activity was effectively prevented by 1 μM galardin, a peptide-mimic gelatinase inhibitor (*26*). These results suggest that toad skin extract contains some component which can inhibit gelatinase activity but it is presumably different from bufosteroids.

Next, culture supernatant of MDA-MB-231 cells was used as a gelatinase source in which MMP-9 was a major MMP component (Figure 1C). When gelatinase activity was estimated using FITC-gelatin as a substrate in the presence of 100-fold diluent of toad skin extract, the activity was reduced to 32.9 ± 9.3% compared with untreated control (data not shown). Similarly, in the presence of 10 μM galardin, the residual gelatinase activity was 26.2 ± 4.6% (data not shown).

The enzyme reaction was assayed in the presence of water-soluble or $CHCl_3$-soluble fractions of toad skin extract that were prepared by the Bligh & Dyer method

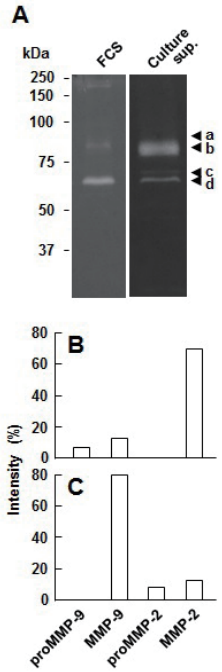

Figure 1. Detection of gelatinases in FCS and culture supernatant of MDA-MB-231 cells using gelatin zymography. A, Gelatin zymography. Arrow heads a-d are deduced to be proMMP-9 (92 kDa), MMP-9 (80 kDa), proMMP-2 (72 kDa), and MMP-2 (67 kDa), respectively. **B** and **C**, Relative intensity of active bands of various MMP species contained in FCS (**B**) and culture supernatant of MDA-MB-231 cells (**C**).

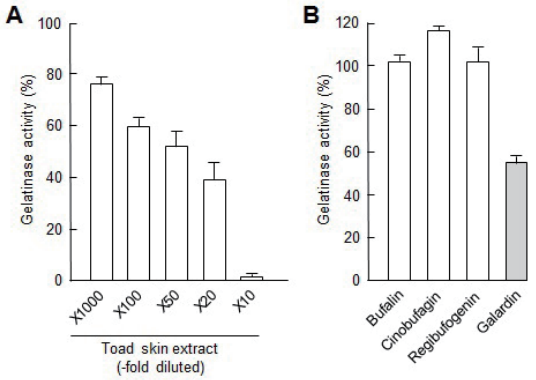

Figure 2. Inhibition of gelatinase activity in FCS by toad skin extract but not by bufosteroids. A, Gelatinase activity was assayed in the presence of various degree of dilution of toad skin extract using colorimetric assay as described in Materials and Methods. **B**, Gelatinase activity was assayed in the presence of 3 types of bufosteroids indicated in the figure or galardin (1 μM each). Data represent percentages compared with untreated control (means ± SD, $n = 4$).

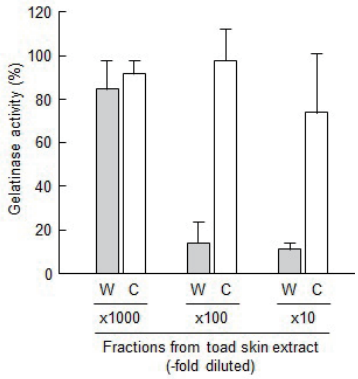

Figure 3. Inhibition of gelatinase activity in culture supernatant of MDA-MB-231 cells by water-soluble fraction but not by $CHCl_3$-soluble fraction of toad skin extract. Toad skin extract was separated to water-soluble (W) and $CHCl_3$-soluble (C) fractions by the Bligh & Dyer method. Gelatinase activity was assayed in the presence of the fractions diluted as indicated using fluorometric assay as described in Materials and Methods. Data represent percentages compared with untreated control (means ± SD, $n = 3$).

as described in Materials and Methods. As shown in Figure 3, gelatinase activity was significantly reduced in the presence of water soluble fraction depending on the dilution rate of the fraction. In contrast, significant reduction was not found in the presence of $CHCl_3$-soluble fraction. This suggests that inhibitory ability of toad skin extract against gelatinases is due to some water-soluble substance.

Major ingredients getting attention in the toad skin extract have been bufosteroids due to their apoptosis-inducing ability to cancer cells (*8,10*). In the present study, bufosteroids such as bufalin, cinobufagin, and regibufogenin at the concentration of 1 μM did not show inhibitory ability against gelatinases (Figure 2B). In addition, $CHCl_3$-soluble lipid fraction prepared by the Bligh & Dyer method did not have apparent ability to prevent gelatinase, although the bufosteroids could be contained in this lipid-rich fraction. Actually, analysis of cell toxicity of water-soluble and $CHCl_3$-soluble fractions using a sulforhodamine B method (*27*) suggested that $CHCl_3$-soluble fraction showed a remarkable toxicity to MDA-MB-231 cells, but water-soluble fraction not (unpublished data).

The present data suggests that toad skin extract have some water-soluble ingredient possessing inhibitory ability to gelatinases. Gelatinases are known to act in the degradation of basement membranes at the time of cancer cell migration into stromal tissues (*1,2*). We have previously found that toad skin extract can suppress the migration of MDA-MB-231 cells not only into a model stromal tissue constituted by type I collagen gels (*16*) but also into a basement membrane model tissue constituted by type IV collagen gels (unpublished data). Therefore, the water-soluble substance in toad skin extract may influence cancer cell migration *via* preventing activity of gelatinases such as MMP-2 and -9.

A lot of compounds that can inhibit MMP activity or expression have been reported, in which some are synthetic compounds and the others are from naturally-occurring substances. Many of synthetic compounds, which mainly categorized into a zinc-binding group and a peptide-mimic group, act as competitive inhibitors against MMP activity (*3,28-31*). In contrast, many of naturally-occurring substances that can control MMP activity have a diversity in the structure including steroids, flavonoids, rotenoids, and terpenoids, and suppress expression of MMP gene by a regulation of signal transduction in cells (*32-36*). Although structural property and action mechanism to prevent gelatinase activity of the substance in toad skin extract have remained unknown, the substance, which is water-soluble and derived from natural product, might have novelty.

In conclusion, our present study suggests that an aqueous extract from toad skin possesses a potent ability to prevent activity of gelatinases such as MMP-2 and -9. Further experiments on the causative substance would improve usability of the toad skin extract cinobufacini to regulate cancer cell migration accelerated by the MMPs.

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