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Phlorotannins in *Ecklonia cava* extract inhibit matrix metalloproteinase activity

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Abstract

Matrix metalloproteinase (MMP) inhibitors have been identified as potential therapeutic candidates for metastasis, arthritis, chronic inflammation and wrinkle formation. For the first time here we report a detailed study on the inhibitory effects of phlorotannins in brown algae, *Ecklonia cava* (EC) on MMP activities in cultured human cell lines. A novel gelatin digestion assay could visualize complete inhibition of bacterial collagenase-1 activity at 20 μg/ml of EC extract during preliminary screening studies. Sensitive fluorometric assay revealed that EC extract can specifically inhibit both MMP-2 and MMP-9 activities significantly ($P<0.001$) at 10 μg/ml. In addition, artificially induced activities of MMP-2 and MMP-9 in human dermal fibroblasts and HT1080 cells were inhibited by EC extract in a more or less similar manner to the positive control doxycycline. Even though the expression levels of MMPs differ from one cell type to the other, gelatin zymography clearly revealed that both MMP expression and activity in cells can be inhibited by EC extract. More interestingly, EC extract did not exert any cytotoxic effect even at 100 μg/ml anticipating its potential use as a safe MMP inhibitor.

Keywords: Matrix metalloproteinase; *Ecklonia cava*; Gelatin zymography; HT1080 cells; Human dermal fibroblasts

Introduction

MMPs are a family of secreted or transmembrane zinc-endopeptidases (Kohn et al., 1994) that are capable of digesting extracellular matrix (ECM), such as fibrillar and non-fibrillar collagens, fibronectin, laminin, elastin and basement membrane glycoproteins under physiological conditions (Birkedal-Hansen et al., 1993; Stetler-Stevenson et al., 1993; Chakrabarti and Patel, 2005). MMPs play an important role not only in physiologic degradation of ECM mediating tissue morohogenesis, tissue repair, and angiogenesis but also in pathologic conditions characterized by excessive degradation of ECM such as chronic inflammation, wrinkle formation, arthritis, osteoporosis, periodontal disease, tumor invasion and metastasis. Recently, it was reported that ultraviolet B-induced enhancement of gelatinase activity in the skin contributes to wrinkle formation through the destruction of basement membrane structure and dermal collagen, and thus, topical application of inhibitors of MMPs may be an effective way to overcome this problem (Inomata et al., 2003). In addition, direct evidence for the involvement of distinct MMPs in tumor growth and invasion has been revealed by studies with either MMP-2 or MMP-9 knockout mice having reduced melanoma tumor progression and angiogenesis (Itoh et al., 1998). Both MMP-2 and MMP-9 can degrade type IV collagen of basement membrane, the first barrier for cancer invasion. Expression of MMP-2 and MMP-9 is elevated in carcinomas and promote tumor progression in oral carcinoma (Ikebe et al., 1999), lung adenocarcinoma (Kodate et al., 1997), bladder (Papathoma et al., 2000) and ovarian carcinoma (Schmalfeldt et al., 2001). Therefore, inhibition of MMP activities in the extracellular space has been extensively studied as an approach to inhibit growth and invasion

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of neoplastic cells. At present, several MMP inhibitors are under clinical trials and it is expected that the use of these inhibitors would develop a new approach for the treatment of cancer in addition to traditional drugs. Most of these MMP inhibitors are synthetic peptides, chemically modified tetracyclines, bisphosphonates or compounds isolated from natural sources. However, most of these drugs are reported to exert side effects such as musculoskeletal pain in tendons and joints (Nelson et al., 2000).

To date, MMP inhibitors that are capable of applying to clinical trials have not been reported from marine resources. Therefore, we took effort to screen marine algal species possessing MMP inhibitory compounds. Based on our screening studies, the extracts of brown algae (Laminariaceae), *Ecklonia cava* (EC), present in the subtidal regions of Jeju Island in Korea had an excellent efficacy to inhibit MMP activities. In addition, extracts of several *Ecklonia* species have been reported to possess a number of other important biological activities, such as radical scavenging activity (Kang et al., 2004), antiplasmin inhibiting activity (Fukuyama et al., 1990), HIV-1 reverse transcriptase and protease inhibiting activity (Ahn et al., 2004) and tyrosinase inhibitory activity (Park et al., 2003). It is expected that high content of phlorotannins present in *Ecklonia* species is responsible for the above bioactivities. However, inhibitory effects of *Ecklonia* species on MMP activities have not been reported previously. Therefore, in the present work, we carried out a detailed study to investigate the inhibitory effects of EC extract on MMPs secreted from cultured human cell lines and compared with commercial MMPs.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), Trypsin–EDTA, penicillin/streptomycin/amphotericin (10,000 U/ml, 10,000 μg/ml, and 2500 μg/ml, respectively) and fetal bovine serum (FBS) were obtained from Gibco BRL, Life Technologies (USA). HT1080 cells were obtained from American Type of Culture Collection (Manassas, VA, USA). Human dermal fibroblasts (HDFs) were kindly donated by LG HG and CM Research Institute (Daejeon, Korea). Bacterial collagenase type I (234153), active MMP-2 (PF023) and active MMP-9 (PF024) were purchased from Calbiochem (Cambridge, MA, USA). FITC–Gelatin (CLN-100) was obtained from Collagen Technology Corporation (Tokyo, Japan). MTT reagent, gelatin, agarose, doxycycline, PMA (phorbol 12-myristate 13-acetate), phloroglucinol and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (USA).

Preparation of EC extract

The brown seaweed, EC extract was collected along Jeju Island coast of Korea during the period from October 2004 to March 2005. Fresh EC extract was washed three times with tap \water to remove salt, epiphytes and sand attached to the surface of the samples and stored at − 20 °C. The frozen samples were lyophilized and homogenized using a grinder before extraction. The dried EC powder (1 kg) was extracted with 95% EtOH (1:10 w/v) and evaporated in vacuo. The concentrated EC extract was freshly dissolved in DMSO before use.

Spectrophotometric determination of phlorotannin

Total phlorotannins content in the EC extract was determined according to a modified version of Folin-Ciocalteu method (Waterman and Mole, 1994), using phloroglucinol as the standard. Samples were diluted taking into account the measurable range of the spectrophotometer (e.g., a 0.005-ml aliquot of extract of soluble phenolics was mixed with 0.495 ml water). A 0.1-ml aliquot of the diluted sample was mixed in a test tube with 1.0 ml of 1N Folin-Ciocalteu reagent. The mixture was allowed to stand for 3 min following addition of 2.0 ml of 20% Na_2CO_3. Samples were incubated in the dark at room temperature for 45 min and centrifuged at 1600×*g* for 8 min. Optical density (OD) of the supernatant was measured at 730 nm using a GENios® microplate reader (Tecan Austria GmbH, Austria). Total phlorotannin content is calculated using the standard graph plotted and expressed as a percentage.

Cell culture

Cell lines were separately grown as monolayers in T-75 tissue culture flasks (Nunc, Denmark) at 5% CO_2 and 37 °C humidified atmosphere using appropriate media supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin–streptomycin. DMEM was used as the culture medium for HT1080 cells, human fibrosarcoma, and human dermal fibroblasts (HDFs) cultured primarily from human fetal skin. Cells were passaged three times a week by treating with trypsin–EDTA and used for experiments after five passages.

MTT assay

Cytotoxic levels of EC extract on HT1080 cells, and HDFs were measured using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as described by Hansen et al. (1989). The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of EC extract. After 48 h of incubation, cells were rewashed and 20 μl of MTT (5 mg/ml) was added and incubated for 4 h. Finally, DMSO (150 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using an GENios® microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan salt. Viability of cells was quantified as a percentage compared to the control (OD of treated cells − OD of blank/OD of control − OD of blank × 100) and dose–response curves were developed. The data were expressed as mean from at least three independent experiments and $P < 0.05$ was considered significant.

Gelatin digestion assay

Agarose solution (1%) was prepared in collagenase buffer (50 mM Tris–HCl, 10 mM $CaCl_2$, 0.15 M NaCl, 7.8 pH) with

0.15% porcine gelatin (Sigma Aldrich, Cat. G-2500) and allowed to solidify in wells of 6-well plate (3 ml/well) for 1 h at room temperature. Different concentrations of EC extract (1 μl) was incubated with 10 μl of bacterial collagenase-1 (0.1 mg/ml) in 89 μl of collagenase buffer for 1 h. The reaction products (10 μl) were loaded onto paper disks placed on gelatin–agarose gel and incubated for 18 h at 37 °C. The degree of gelatin digestion in agarose gel was visualized by Coomassie Blue staining after removal of the paper disks. Following destaining, the area of light translucent zone over blue background was determined to estimate gelatinase activity.

MMP activity assays by fluorescent gelatin

Enzyme activity assays were performed in 50 mmol/l Tris–HCl buffer, pH 7.5, 0.15 mol/l NaCl, 10 mmol/l $CaCl_2$, 0.02% NaN_3 (TNC buffer) containing 0.05% Brij 35 and 50 μmol/l $ZnSO_4$, as previously described (Netzel-Arnett et al., 1991). Various concentrations of EC extract were tested for their ability to digest a synthetic fluorogenic substrate (a general MMP substrate). Each concentration of EC extract was incubated with 1 μmol/l substrate at 37 °C for 20 h, and the reaction was terminated by the addition of 3% acetic acid. Fluorescence intensity was measured at 495 nm (excitation) and 520 nm (emission) with a GENios® fluorescence microplate reader (Tecan Austria GmbH, Austria).

Gelatin zymography

Activities of MMP-2 and MMP-9 were determined by zymography as described previously (Hrabec et al., 2002) in the presence or absence of EC extract. After cells were exposed to various concentrations of EC extract for 1 h prior to treatment of 10 ng/ml and 100 ng/ml PMA for HDFs and HT1080 cells, respectively, incubation was continued in FBS-free medium for 3 days. Conditioned medium containing 50 μg of total protein (or reaction products of various concentrations of EC extract with active MMP-2) was resuspended in a sample buffer (125 mM Tris–HCl, pH 6.8, 3% SDS, 40% glycerol, 0.02% bromophenol blue) without boiling and electrophoresed under non-reducing conditions on 10% polyacrylamide gels containing 1.5 mg/ml gelatin. After electrophoresis, the gels were washed twice with 50 mM Tris–HCl (pH 7.5) containing 2.5% Triton X-100 and incubated overnight at 37 °C in a developing buffer containing 10 mM $CaCl_2$, 50 mM Tris–HCl, and 150 mM NaCl. The gels were stained with 0.25% Coomassie Blue R-250 in 30% methanol and 10% acetic acid, and de-stained in the same solution without the Coomassie Blue dye. Gelatinolytic bands were observed as clear zones against the blue background and the intensity of the bands was estimated using Image Master Software, Amersham Pharmacia Biotech (Sweden).

Results

Phlorotannin content in EC extract

Phlorotannins are produced entirely by polymerization of phloroglucinol, which is a product of acetate–malonate pathway, also known as polyketide pathway. Following extraction with ethanol, EC extract consisted of 57.98±0.45% of phlorotannins at dry weight basis. This represented extraction of more than 90% of total pholorotannins from its starting material, dried EC (total phlorotannins in dried EC was 5.66±0.043% w/w).

Effect of EC extract on proliferation of human dermal fibroblasts and HT1080 cells

The purpose of the present study was to investigate the inhibitory effects of EC extract on MMP activities. Therefore, non-cytotoxic concentrations of EC extract dissolved in DMSO were determined by performing MTT assay on treated normal HDFs and HT1080 cells and used for MMP inhibition studies. Interestingly, as shown in Fig. 1, EC extract did not exert any cytotoxic effect on both normal and cancer cells even at the highest tested concentration (100 μg/ml). Therefore, concentrations of EC extract ranging from 10 to 100 μg/ml were selected for MMP activity assays.

Inhibitory effect of EC extract on bacterial collagenase-1 assessed by gelatin digestion assay

For visual investigation of the inhibitory effect of EC extract on MMPs, an indirect assay was developed using bacterial collagenase-1, as described above. Following incubation of bacterial collagenase-1 with different concentrations of EC extract, the remaining gelatinolytic activity was compared with initial enzyme activity represented by the control. As shown in Fig. 2A, the control group treated with reaction products of bacterial collagenase-1 and 0.1% of DMSO exhibited the highest gelatinolytic activity in the discrete zone, representing no enzyme inhibition. However, as shown in Fig. 2, gelatin digestion was clearly decreased following addition of 20 μg/ml well-known MMP inhibitor, doxycycline and area of the clear zone was reduced by 30%. Similarly, gelatinolytic activity was decreased following dose-dependent treatment of EC extract. A significant reduction in gelatin digestion was observed with 20 μg/ml or higher concentrations of EC extract, representing inhibition of more than 20% of bacterial collagenase-1 activity. In addition, low concentrations of EC extract (50 μg/ml) could inhibit the above activity at least 70% compared to the initial activity.

Fluorometric analysis of inhibitory effect of EC extract on bacterial collagenase-1

In order to elucidate the inhibitory effect of EC extract on bacterial collagenase-1, fluorescence-conjugated gelatin was used and compared with the positive control, doxycycline. In this study, fluorescence-conjugated gelatin was incubated with bacterial collagenase-1 for 18 h in the presence of two concentrations of EC extract and doxycycline, respectively. EC extract exhibited a significant inhibitory effect on bacterial collagenase-1 (Fig. 3), and that was more or less similar to the inhibitory effect of doxycycline at both concentrations, 100 μg/ml and 10 μg/ml. Treatment with 100 μg/ml of EC extract decreased the activity of bacterial collagenase-1 by 50%. Although the inhibitory effects of EC extract in the fluorometric assay were somewhat different from those of

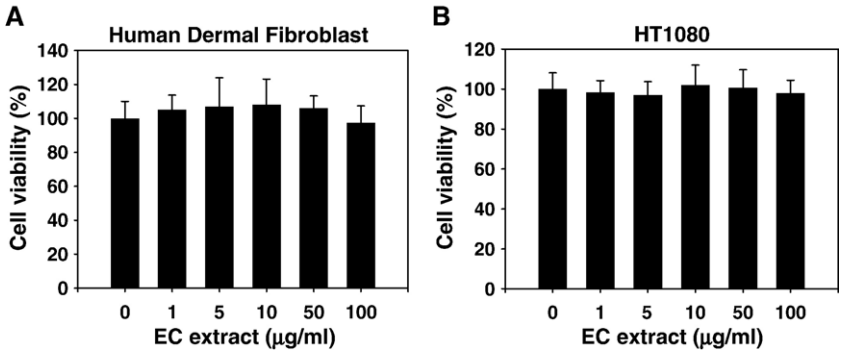

Fig. 1. Effect of EC extract on viability of human dermal fibroblasts (A) and HT1080 cells (B). Human dermal fibroblasts and HT1080 cells were treated with different concentrations of EC extract and cell viability was determined by MTT assay after 24 h. Data are given as means of values ± S.D. from three independent experiments.

gelatin digestion and azocoll assay, the trend of inhibitory effects were similar to those of doxycycline.

Fluorometric analysis of inhibitory effect of EC extract on MMP-2 and MMP-9 activities

Specifically, we examined whether EC extracts can inhibit MMP-2 and MMP-9 activities in addition to bacterial collagenase-1. Thus, fluorescence-conjugated gelatin was used as the substrate to determine the inhibitory effect of EC extract on MMP activities and compared with that of doxycycline. Fluorescence-conjugated gelatin was incubated with MMP for 18 h in the presence of two concentrations of EC extract and doxycycline, respectively. As shown in Fig. 4, EC extract exhibited significant inhibitory effects on MMP-2 and MMP-9 activity. Moreover, treatment with 10 μg/ml of EC extract decreased MMP-2 and MMP-9 activities by 80% and 85%, respectively. However, there was no significant difference between MMP-2 and MMP-9 inhibitory activities treated with EC extract and doxycycline. The inhibitory effect of EC extract on MMP-2 and MMP-9 activities was significantly different ($P<0.01$) from that on bacterial collagenase-1.

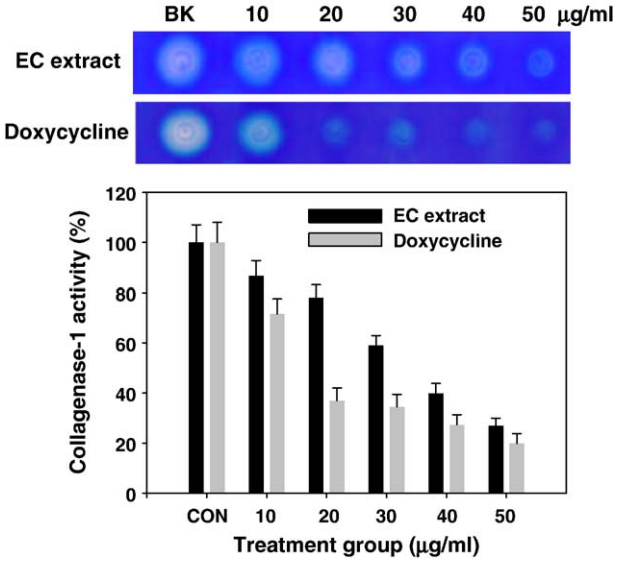

Fig. 2. Inhibitory effect of EC extract on bacterial collagenase-1 assessed by gelatin digestion assay. Bacterial collagenase-1 was reacted with 0.1% DMSO (A) as control, with doxycycline as positive control, and with EC extract at 10, 20, 30, 40 and 50 μg/ml; and then 10 μl of reaction products were loaded onto paper disks placed on agarose gel containing gelatin and incubated for 18 h. Enzyme activity of remaining bacterial collagenase-1 was calculated by densiometric determination of the gelatin digested clear zone visualized by Coomassie Blue staining.

Gelatin zymography of MMP-2 reacted with EC extract

In order to verify the inhibitory effect of EC extract on MMP-2 activity observed in fluorometric assay, gelatin zymography was performed. After 1 h, reaction products resulted between different concentrations of EC extract and MMP-2 were electrophoresed on gelatin gel, and MMP-2 activity was estimated by the area of clear bands following Coomassie Blue staining. As shown in Fig. 5, the gelatin gel was completely digested by the control group treated with MMP-2 alone. Addition of 100 μg/ml of doxycycline (positive control for MMP inhibition) could clearly inhibit the activity of MMP-2. EC extract exhibited a dose-dependent inhibitory effect on MMP-2 activity (20% at 10 μg/ml and 50% at 50 μg/ml), and complete inhibition was observed at concentrations above 500 μg/ml. This result was consistent with that of the gelatin digestion assay.

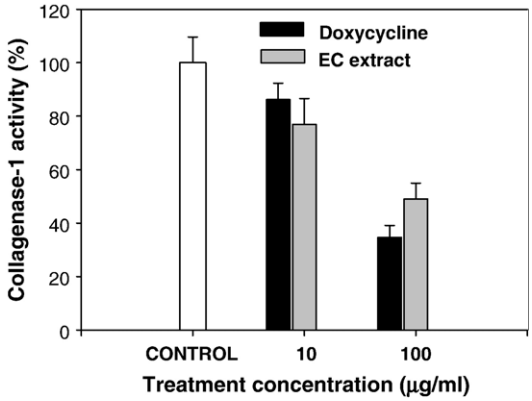

Fig. 3. Effect of EC extract on bacterial collagenase-1 activity tested using fluorometric assay. Bacterial collagenase-1 was reacted with 100 μg/ml and 10 μg/ml of EC for 18 h using doxycycline as the positive control. Fluorescence intensity, which represents bacterial collagenase-1 activity, was measured at 495/520 nm (excitation and emission). Error bars represent mean and S.D. from triplicate experiments after deduction of background values from raw values.

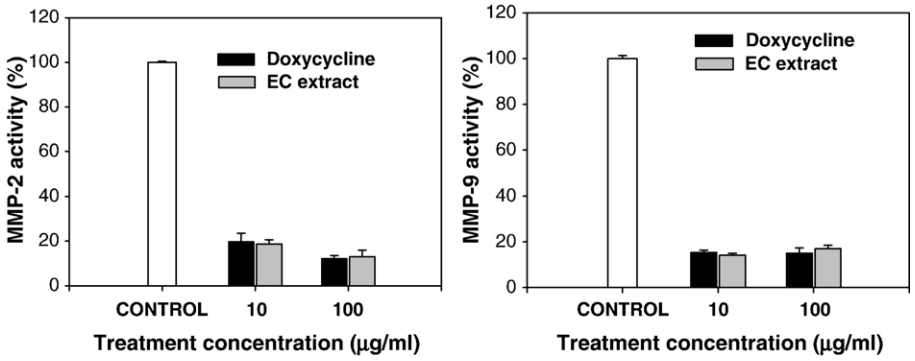

Fig. 4. Effect of EC extract on MMP-2 and MMP-9 activities assessed by fluorometric assay. MMPs were reacted with 100 and 10 μg/ml of EC extracts for 18 h using doxycycline as the positive control. Fluorescence intensities that represent MMP activities were measured at 495/520 nm (excitation and emission). Error bars represent mean and S.D. from triplicate experiments after deduction of background values from raw values.

Inhibitory effects of EC extract on activities of MMP-2 and MMP-9 from HT1080 cells

It has been reported that HT1080 cells secrete type IV collagenase, MMP-2 and MMP-9, which play a major role in cancer metastasis. Therefore, to clarify whether EC extracts can inhibit the gelatinolytic activity of MMPs secreted from HT1080 cells, PMA-treated conditioned medium was treated with various concentrations of EC extract for 1 and gelatin zymography was performed. As shown in Fig. 6, the expression level of MMP-9 was higher than that of MMP-2 in HT1080 cells and EC extract exhibited a dose-dependent inhibitory effect on both MMP-2 and MMP-9 activities. The inhibitory effect of EC extract on MMP-2 activity was greater than that of MMP-9 activity and complete inhibition of MMP-2 activity was observed at 500 μg/ml or higher concentrations. Furthermore, 50% inhibition of MMP-2 activity was observed at 50 μg/ml of EC extract. This result was in agreement with gelatin zymography performed using purified MMP-2. However, no significant inhibitory effect was observed on both MMP-2 and MMP-9 activities at 10 μg/ml of EC extract. In comparison with doxycycline, treatment with 100 μg/ml of EC extract exhibited a higher inhibitory effect on both MMP-2 and MMP-9 activities.

Inhibitory effects of EC extract on MMP activities from HDFs

To further investigate whether EC extract also inhibits MMPs secreted from HDFs cultured primarily from human fetal skin, gelatin zymography was carried out. For this, conditioned medium

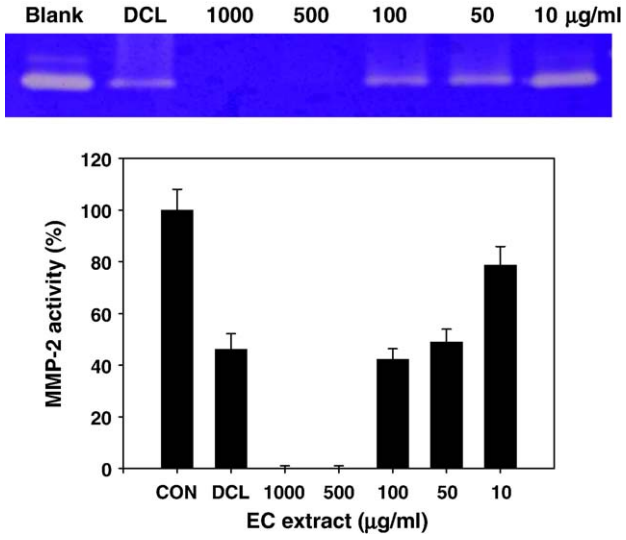

Fig. 5. Gelatin zymography of MMP-2 reacted with EC extract. MMP-2 was reacted with various concentrations of EC extracts and doxycycline (DCL, 100 μg/ml) as the positive control for 1 h and gelatin zymography was performed. Lower panel represents respective relative enzyme activities as percent of blank group.

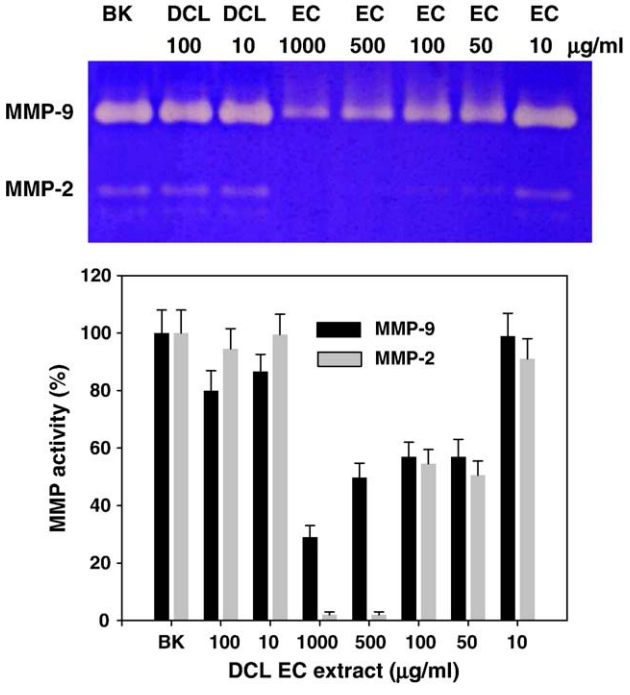

Fig. 6. Effects of EC extract on activities of MMP-2 and MMP-9 from HT1080 cells. Cells were treated with 10 ng/ml of PMA under serum-free conditions to induce MMP expressions. After 3 days of incubation, conditioned media were reacted with various concentrations of EC extract for 1 h, and then gelatin zymography was performed. Doxycycline (DCL) was used as the positive control. Lower panel represents respective relative enzyme activities as percent of blank group.

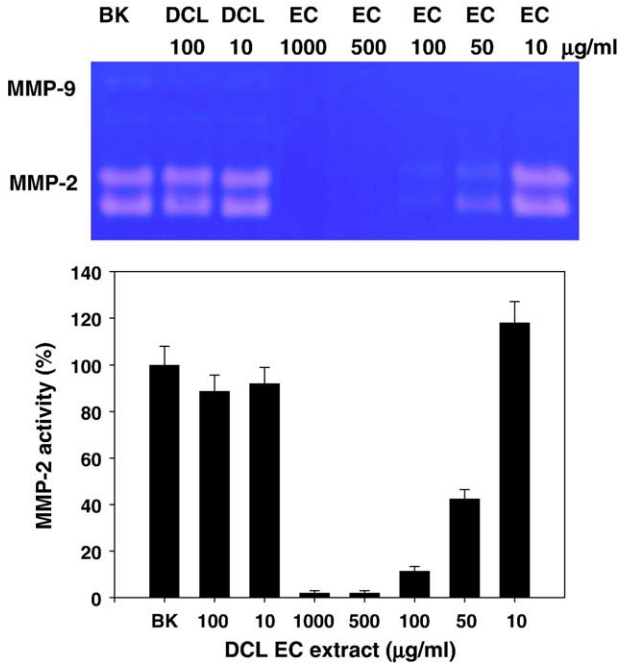

Fig. 7. Effects of EC extract on activities of MMP-2 and MMP-9 from HDFs. Cells were treated with 10 ng/ml of PMA under serum-free conditions to induce MMP expressions. After 3 days of incubation, conditioned media were reacted with various concentrations of EC extract for 1 h, and then gelatin zymography was performed. Doxycycline (DCL) was used as the positive control. Lower panel represents respective relative enzyme activities as percent of blank group.

of HDFs treated with PMA to induce MMPs expression was reacted with various concentrations of EC extract for 1 h. As shown in Fig. 7, in contrast to HT1080 cells, HDFs secreted mainly proMMP-2 and a little amount of proMMP-9. EC extract exhibited a dose-dependent inhibitory effect on MMP-2, but it was difficult to assess the inhibitory effect on MMP-9 activity. However, EC extract completely inhibited MMP-2 activity at 500 μg/ml similar to that observed for HT1080 cells. In addition, no MMP-2 inhibition was observed at 10 μg/ml similarly to the result of HT1080 cells, but exerted 70% inhibition at 50 μg/ml. Higher MMP-2 activity inhibition was observed with 100 μg/ml of EC extract compared to that of doxycycline at same concentration.

Inhibitory effects of EC extract on MMPs in HT1080 cells

Based on the above results, further studies were carried out to confirm direct MMP inhibitory activities of EC extract in cultured cells. Conditioned medium of HT1080 cells treated with EC extract was used for gelatin zymography. As shown in Fig. 8, treatment with PMA increased the expression level and activity of MMP-9 and MMP-2 compared with the blank group. EC extract exerted a higher inhibitory effect on the expression level and activity of MMP-2 than that of MMP-9. At 500 μg/ml of EC extract, a complete inhibition was observed for MMP-2 activity, but at the same concentration, MMP-9 activity was inhibited only by 60%. However, a significant inhibitory effect on both MMP-2 and MMP-9 activities was observed at 50 μg/ml or higher EC extract concentrations.

Discussion

In order to screen novel MMP inhibitors from marine natural resources, we collected 50 marine algal species from the southern coast of Korea and extracted them with ethanol and water, respectively. To screen the MMP inhibitory effects of these extracts, we developed a novel assay that confers several advantages such as reproducibility, reliability and economical efficiency. Using this novel assay, we investigated the inhibitory effects of all marine algal extracts on bacterial collagenase-1 and doxycycline was used as a positive control to compare these effects. Doxycycline is a synthetic tetracycline derivative that can inhibit MMP activity and potentially an effective therapeutic agent (Ashley, 1999). Longitudinal double-blind studies on humans with adult periodontitis have demonstrated that a sub-antimicrobial dose of doxycycline, previously reported to suppress collagenase activity in the periodontal pocket, is safe and effective and has recently been approved by the FDA as an adjunct to scaling and root planning (Golub et al., 1998). Furthermore, Periostat® (CollaGenex Pharmaceuticals Inc.), a tetracycline analog containing doxycycline, has been reported to inhibit collagenase activity but failed to act as an antibiotic (Golub et al., 1990). Our results indicated that EC, classified under the Laminaria family of brown algae, had the most excellent inhibitory effects on MMP activity among other tested algal species (screening data not shown). Previous studies on *Ecklonia* species have reported that high content of pholorotannins is responsible for some biological activities observed in these species. Therefore, to determine whether phlorotannins in EC extract can inhibit MMP or

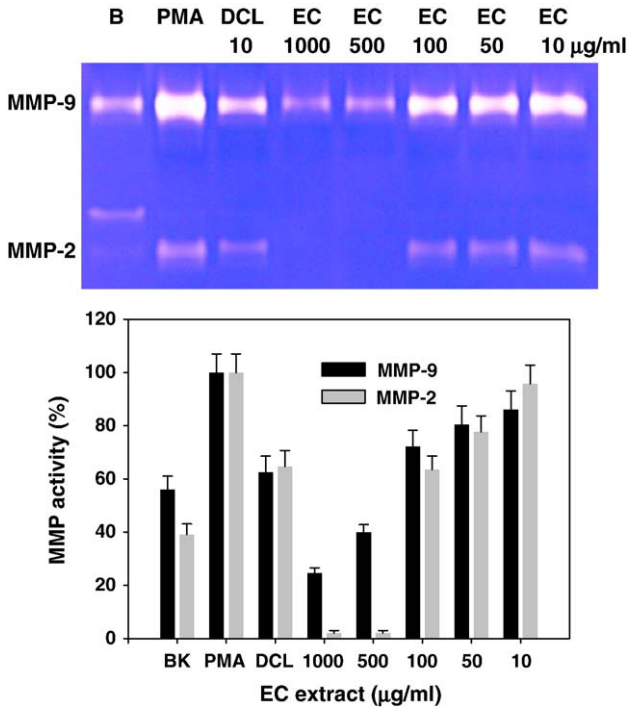

Fig. 8. Effects of EC extract on expression and activity of MMP-2 and MMP-9 in HT1080 cells. Cells stimulated with 10 ng/ml of PMA to induce MMP expression were treated with various concentrations of EC extract under serum-free conditions for 3 days. MMP activities in conditioned media were determined by zymography as described in the text. Lower panel represents respective relative enzyme activities as percent of blank group.

bacterial collagenase-1 activity, primary ethanol extract was re-extracted with different solvents and compared their phlorotannins content with respective enzyme inhibitory activities. Interestingly, there was a direct relationship between the content of phlorotannins and enzyme inhibition, suggesting the effect is due to phlorotanins. Very high content of phlorotannins in EC ethanol extract (58%) paved the path to study MMP inhibitory effect of phlorotanins in EC extract in the present study. EC is abundant in the subtidal regions of Jeju Island, Korea, and reported to contain eisenine, biotin and laminine (Hoppe, 1979). Recently, Kang et al. (2004) reported a radical scavenging effect of extracts from EC and their results suggested that phlorotannins from *E. cava* was responsible for this effect. Also, it is reported that some other brown alga contain higher amount of phlorotannins (Koivikko et al., 2005). Furthermore bieckol and dieckol, belonging to phlorotannins in EC extract, have exerted an inhibitory effect on HIV-1 RT and protease (Ahn et al., 2004). The greatest advantage of this novel assay was that it enabled visual observation of the inhibitory effect on MMP activity. However, this novel assay requires expensive MMPs to identify the specific inhibitory effects on MMPs. Therefore, during initial screening studies, much cheaper bacterial collagenase-1 which can digest gelatin was used instead of MMPs assuming any compound or an agent that inhibits the bacterial collagenase activity can inhibit gelatinases. Although the inhibitory effects of EC extract observed in the fluorometric assay (Beekman et al., 1996) were somewhat different from those of the novel assay, the trend of its inhibitory effects was similar to that of doxycycline. Also, some other studies were performed to investigate the inhibitory effects of EC extract on several kinds of human MMPs activities in detail. From the results of these studies, we found that there was no significant difference between the inhibitory effects of EC extract and doxycycline on MMP-2 and MMP-9 activities. However, EC extract exhibited a higher inhibitory effect on MMP-2 and MMP-9 activities than those on bacterial collagenase-1 activity. Therefore, we can suggest that the structure and function of human MMPs is different from those of bacterial collagenase-1. Further, recent studies have shown that gelatinases are responsible for the final degradation of fibrillar collagens after initial cleavage by collagenases (Egeblad and Werb, 2002), but on the other hand, bacterial collagenase-1 differs from mammalian collagenases that they attack many sites along the collagen helix (Seifter and Harper, 1971). However, both MMP-2 (Gelatinase-A) and MMP-9 (Gelatinase-B) can degrade type IV collagen of base membranes and are known to play a crucial role in cancer invasion such as oral carcinoma (Ikebe et al., 1999), lung adenocarcinoma (Kodate et al., 1997), bladder (Papathoma et al., 2000) and ovarian carcinoma (Schmalfeldt et al., 2001). In order to verify the inhibitory effects of EC extract on MMP-2 activity in fluorometric assay, gelatin zymography was conducted at various concentrations. EC extract exhibited a dose-dependent inhibitory effect on MMP-2 activity, and complete inhibition was observed at 500 μg/ml or higher concentration in agreement with the results of gelatin digestion assay. After we confirmed the inhibitory effects on MMPs activities, we further investigated whether EC extract has cytotoxicity on HDFs and HT1080 cells. EC extract did not exert any cytotoxic effect on both normal and cancer cells, and this was supported by the fact that EC has long been utilized as a traditional food in Korea. The inhibitory effects of EC extract on MMP activities were further confirmed clearly in conditioned media from HT1080 cells and HDFs. HT1080 cells mainly release proMMP-9 and a little amount of proMMP-2. In contrast, proMMP-2 is mainly expressed in HDFs. Previously, it has been reported that fibrosarcoma HT1080 cells secrete type IV collagenase, MMP-2 and MMP-9, and that these enzymes play a major role in cancer metastasis (Nagase et al., 1998; Yoon et al., 2001). In HT1080 cells, expression level of MMP-9 was higher than that of MMP-2 and EC extract exhibited a dose-dependent inhibitory effect on both MMP-2 and MMP-9 activities. Further, the inhibitory effect on MMP-2 activity was higher than that of MMP-9. In contrast to HT1080 cells, HDFs secreted mainly proMMP-2 and a little amount of proMMP-9. EC extract exhibited a higher dose-dependent inhibitory effect on MMP-2 activity compared to doxycycline. Moreover, based on the results shown above, we confirmed that MMP activity inhibition differs from direct treatment of the EC extract to cells. EC extract also exhibited a higher inhibitory effect on the expression level and activity of MMP-2 than that of MMP-9 in this study. In contrast to gelatinase activity of MMP-9, MMP-2 can degrade both gelatin and fibrillar collagen, which makes MMP-2 more important for the complete digestion of collagen during cellular invasion (Aimes and Quigley, 1995). Therefore, it can be presumed that the EC extract has an excellent efficacy in inhibiting invasion of cancer cells. In addition, development of MMP inhibitors has been considered to be a promising strategy for the prevention of wrinkle formation due to direct involvement of MMPs during aging (Ashcroft et al., 1997; Vayalil et al., 2004). Even though results of the present study exhibited in vitro effects of the primary algae extract on MMP activities, it provides the first experimental evidence that EC can inhibit MMP activities. We presume that these inhibitory effects on MMP activities are due to high content of phlorotannins present in EC extract. These findings are of considerable significance if future studies could demonstrate that a single active component purified from EC can inhibit MMP activity at a lower concentration and reduce the incidence of metastasis and skin wrinkle in vivo model.

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