

Extracts of *Aster* species Inhibit Invasive Phenotype and Motility of H-ras MCF10A Human Breast Epithelial Cells Possibly via Downregulation of MMP-2 and MMP-9

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Abstract – Cancer metastasis represents the most important cause of cancer death and antitumor agents that may inhibit this process have been extensively pursued. Invasion and metastasis of malignantly transformed cells involve degradation of the extracellular matrix (ECM) components by matrix metalloproteinases (MMP), especially MMP-2 and -9. We previously showed that H-ras-induced invasive phenotype may involve MMP-2, rather than MMP-9, in MCF10A cells. In the present study, we investigated the chemopreventive effect of *Aster*, a widely used culinary vegetable in Korea. We screened twelve extracts from three *Aster* species (*Aster scaber*, *Aster oharai* and *Aster glehni*) for the inhibitory effect on MMP activities of H-ras MCF10A human breast epithelial cells. All of the extracts tested in this study efficiently inhibited the gelatinolytic activities of MMP-2 and MMP-9. A more prominent inhibition was observed in MMP-2 activity compared to MMP-9. Out of twelve extracts, eight extracts showed >90% inhibition of MMP-2 activity in H-ras MCF10A cells while only one extract showed >90% inhibition of MMP-9 activity. We selected three extracts (AO-3, AG-3 and AS-EA) for further studies since they exerted a marked inhibition in the ratio of MMP-2 to MMP-9. Treatment with AO-3, AG-3 and AS-EA in H-ras MCF10A cells caused a significant inhibition of invasive phenotype and migration, proving a chemopreventive potential of these extracts. Taken together, our results demonstrate that extracts of *Aster* effectively inhibit invasion and migration of highly malignant human breast cells, possibly via downregulation of MMP-2 and MMP-9.

Key words □ *Aster*, matrix metalloproteinase, invasion, migration

1. Introduction

Tumor invasion and metastasis are aggressive processes during which tumor cells, as well as normal endothelial cells, invade, proliferated, and migrate. Thus, the control of matrix proteolysis has long been proposed as a rational therapeutic antitumor strategy (Liotta *et al.*, 1991, Brown, 1999, Stetler-Stevenson, 1999, Werb, 1999, Yip, 1999). An essential part of the metastatic process includes degradation of the basement membrane and the stromal extracellular matrix (ECM), which allows cells to migrate into neighboring tissue. Invasion of malignantly transformed cells is dependent on their capacity to degrade basement membranes and ECM in an effective controlled manner (Stetler-Stevenson *et al.*, 1993). Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases

collectively capable of degrading essentially all ECM components, and they apparently play a key role in tumor cell invasion (Stetler-Stevenson *et al.*, 1993, Kahari and Saarialho-Kere, 1997). Mounting evidence suggests a role for members of the MMP family on tumor invasion and metastasis formation, especially, MMP-2 (72 kDa type IV collagenase, gelatinase A) and MMP-9 (92 kDa type IV collagenase, gelatinase B), which degrade type IV collagen, the major structural collagen of the basement membrane (Liotta *et al.*, 1991, Stetler-Stevenson, 1990, Tryggvason *et al.*, 1993). Numerous studies show a correlation between the levels of MMP-2 and/or MMP-9 and the invasive phenotypes of cancer cells (Ura *et al.*, 1989). We previously showed that H-ras-induced invasive phenotype in MCF10A human breast epithelial cells involved MMP-2 rather than MMP-9 (Moon *et al.*, 2000).

Aster scaber T. (Asteraceae) is a widely used culinary vegetable in Korea (Lee, 1989). *Aster* species has also

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been used in traditional Chinese medicine for treatment of bruises, snakebite, headache, and dizziness (Kim *et al.*, 1997). Recently studies revealed that quinic acid derivatives from *Aster scaber* exhibited diverse biological activities, especially antiviral (Gesa *et al.*, 1999, Mahmood *et al.*, 1997). and antihepatotoxic activities. In addition, neuroprotective and neurotrophic effects of quinic acids from *Aster scaber* was reported (Hur *et al.*, 2001). Less studies have been performed for the biological activities of other *Aster* species, *Aster oharai* and *Aster glehni*.

In the present study, we attempted to study *in vitro* chemopreventive effects of extracts of three *Aster* species (*Aster scaber*, *Aster oharai* and *Aster glehni*) in H-ras-transformed MCF10A human breast epithelial cells. Here, we report that extracts of *Aster* inhibits invasive phenotype and migration in parallel with a downregulation of MMP-2 and MMP-9, suggesting a possible application to prevent and treat cancer.

2. Materials and Methods

Cell lines and culture condition

Establishment of H-ras MCF10A cells was previously described (Moon *et al.*, 2000). Cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, 20 ng/ml EGF, 0.1 µg/ml cholera enterotoxin, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin and 0.5 µg/ml fungizone. Cells were cultured in a humidified 5% CO₂ incubator at 37°C.

Extraction and Isolation of Plant Material

Aster oharai and *Aster glehni* were collected on Ullung island, Kyongsangbuk-Do, Korea in July 1999. *Aster scaber* was collected in ChukRyung Mt., Kyungi-Do, Korea in September 1999. The partially dried and chopped aerial parts of *Aster oharai* (500 g) was extracted with MeOH three times at room temperature. The resultant MeOH extract (AO-1) was subjected to successive solvent partitioning to give *n*-hexane (AO-2), EtOAc (AO-3) and *n*-BuOH (AO-4) fractions. *Aster glehni* (500 g) was extracted with MeOH three times at room temperature. The resultant MeOH extract (AG-1) was subjected to successive solvent partitioning to give *n*-hexane (AG-2), EtOAc (AG-3) and *n*-BuOH (AG-4) fractions. *Aster scaber* (500 g) was extracted with MeOH three times at room temperature. The each resultant MeOH extract (AS-

MeOH Ex.) was subjected to successive solvent partitioning to give methylene chloride (AS-MC), EtOAc (AS-EA) and *n*-BuOH (AS-BU) fractions. Twelve extracts (AO-1, AO-2, AO-3, AO-4, AG-1, AG-2, AG-3, AG-4, AS-MeOH Ex., AS-MC, AS-EA and AS-BU) were resuspended in DMSO to make final concentrations of 1, 5 and 10 mg/ml.

Gelatin zymography

Cells cultured in serum-free DMEM/F12 medium were treated with the *Aster* extracts for 24 hr. Conditioned medium was collected and centrifuged at 3,000 rpm for 10 min to remove cell debris. The protein concentration was measured using BCA protein assay reagents (Pierce, IL). Equal amounts of conditioned media were mixed with 2× laemmli non-reducing sample buffer, incubated for 15 min at room temperature, and then electrophoresed on 10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 three times for 30 min, rinsed for 15 min with 50 mM Tris-HCl buffer (pH 7.6) containing 5 mM CaCl₂, 0.02% Brij-35, 0.2% sodium azide, and incubated overnight at 37°C. The gels were stained with 0.5% Coomassie Brilliant Blue R-250 solution containing 10% acetic acid and 20% methanol for 30 min and destained with 10% acetic acid solution. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background. Relative band intensities of MMPs were determined by quantitation of each band with an Image Analyzer (Vilber Lourmet, France).

In vitro invasion assay

In vitro invasion assay was performed using 24-well Transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA) as previously described (Kim *et al.*, 2000). The lower side of the filter was coated with type I collagen, and the upper side was coated with Matrigel (Collaborative Research, Lexington, KY). Lower compartment was filled with serum-free media containing 0.1% BSA. Cells were placed in the upper part of the Transwell plate, incubated for 17 hr, fixed with methanol and stained with hematoxylin for 10 min followed briefly by eosin. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at ×400. Thirteen fields were counted for each filter and each sample was assayed in triplicate.

Wound Migration Assay

Cells were pretreated with mitomycin C (25 µg/ml) for 30 min before injury line was made. The injury line was made with a tip with 2 mm in width on the cells plated in culture dishes at 90% confluency. After rinsed with PBS, cells were allowed to migrate in complete media and photographs were taken (×40) at indicated time points.

3. Results and Discussion

To determine the effect of *Aster* extracts on MMP-2 and/or MMP-9 expression, H-ras MCF10A cells were

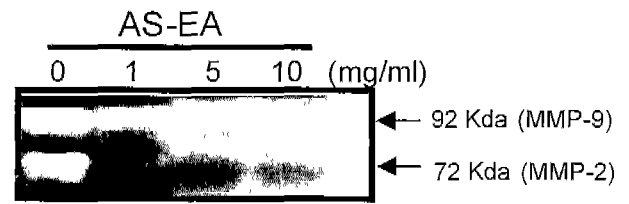


Fig. 1. AS-EA downregulated MMP-2 and MMP-9 in a dose dependent manner. A total of 5×10^5 H-ras MCF10A cells were treated with 1 mg/ml, 5 mg/ml and 10 mg/ml AS-EA extract for 24 h in serum-free media. Conditioned media were collected and subsequently analyzed for secretion of MMP-2 (72kDa) and MMP-9 (92 kDa) by gelatin zymogram analysis.

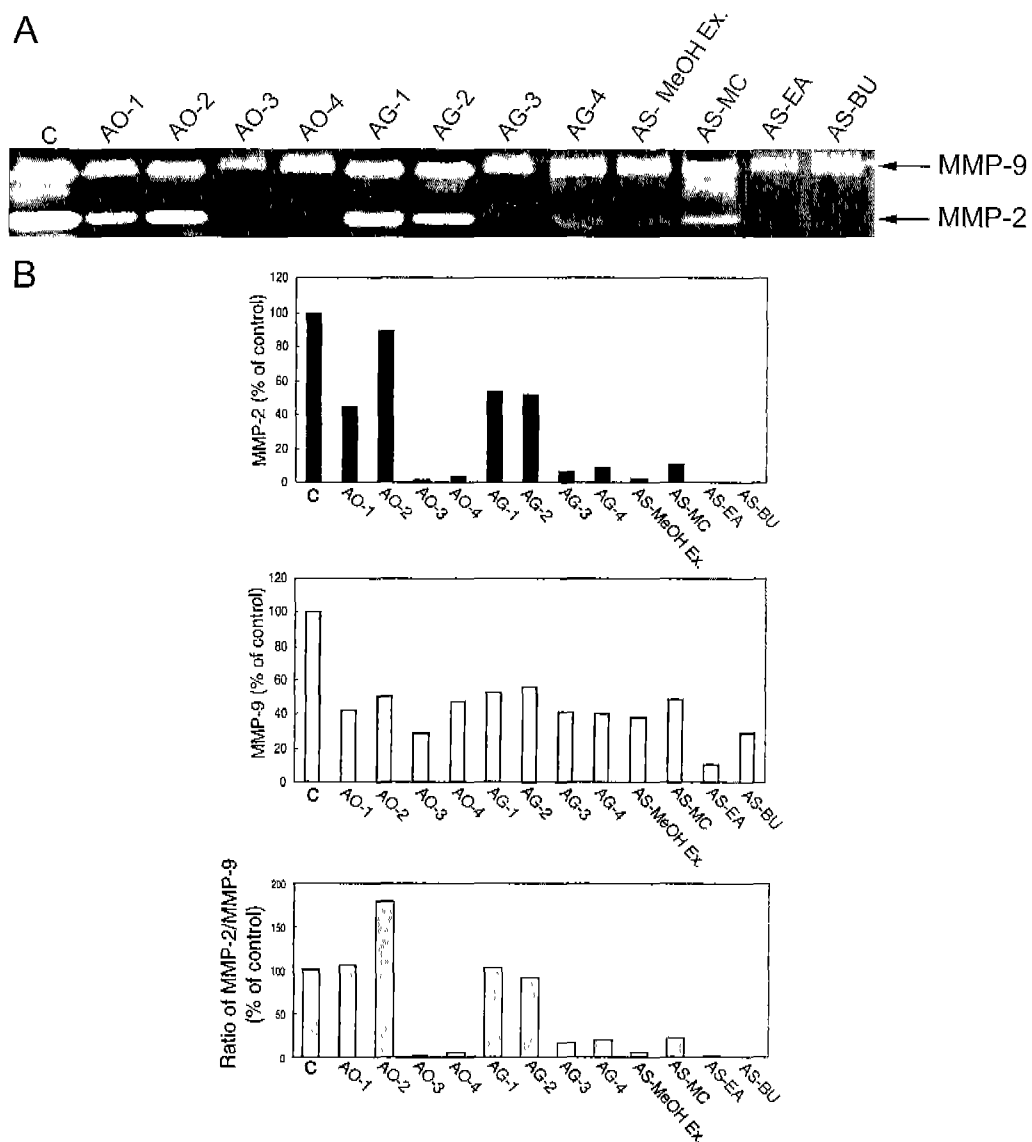


Fig. 2. *Aster* extracts downregulated MMP-2 and MMP-9. *A*, H-ras MCF10A cells were treated with 5mg/ml *Aster* extracts (AO-1, AO-2, AO-3, AO-4, AG-1, AG-2, AG-3, AG-4, AS-MeOH Ex., AS-MC, AS-EA and AS-BU) for 24h in serum-free media. Conditioned media were subsequently analyzed for secretion of MMP-2 (72kDa) and MMP-9 (92kDa) by gelatin zymogram analysis *B*, Relative band intensities were measured for quantification and plotted.

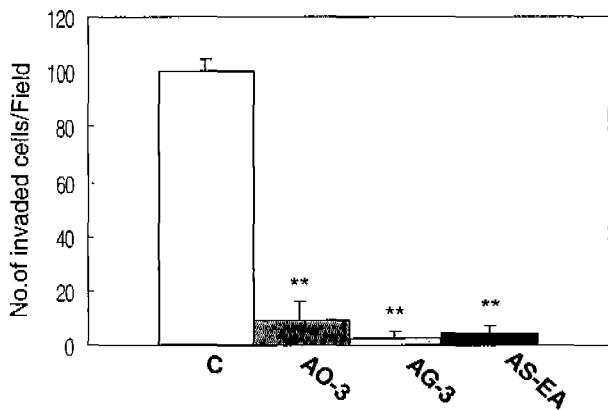


Fig. 3. Aster extracts inhibited invasive phenotype. H-ras MCF10A cells were incubated with 5 mg/ml Aster extracts (AO-3, AG-3 and AS-EA) in a Matrigel-coated transwell chamber for 17h. The number of invaded cells per field was counted ($\times 400$) in thirteen fields. The results represent means \pm S.E. of triplicates. **Statistically different from control at $p < 0.01$.

treated with various concentrations (1, 5 and 10 g/ml) of AS-EA for 24 hr in serum-free medium. Gelatin-based zymographic assay showed that treatment of Aster extracts resulted in a marked decrease of a gelatinolytic activity in the harvested conditioned medium, which comigrated with a 72,000 molecular weight marker in a dose-dependent manner (Fig. 1). The gelatinolytic activity of MMP-9 (92,000 molecular weight) was not substantially reduced upon treatment of AS-EA compared to MMP-2. Secreted levels of MMP-2 was almost completely suppressed by treatment with 5 mg/ml of AS-EA, indicating that this concentration would be appropriate for investigating the effect of Aster extracts on MMP-2 and/or MMP-9.

We then screened twelve extracts from Aster for the inhibitory effect on MMP activities of H-ras MCF10A

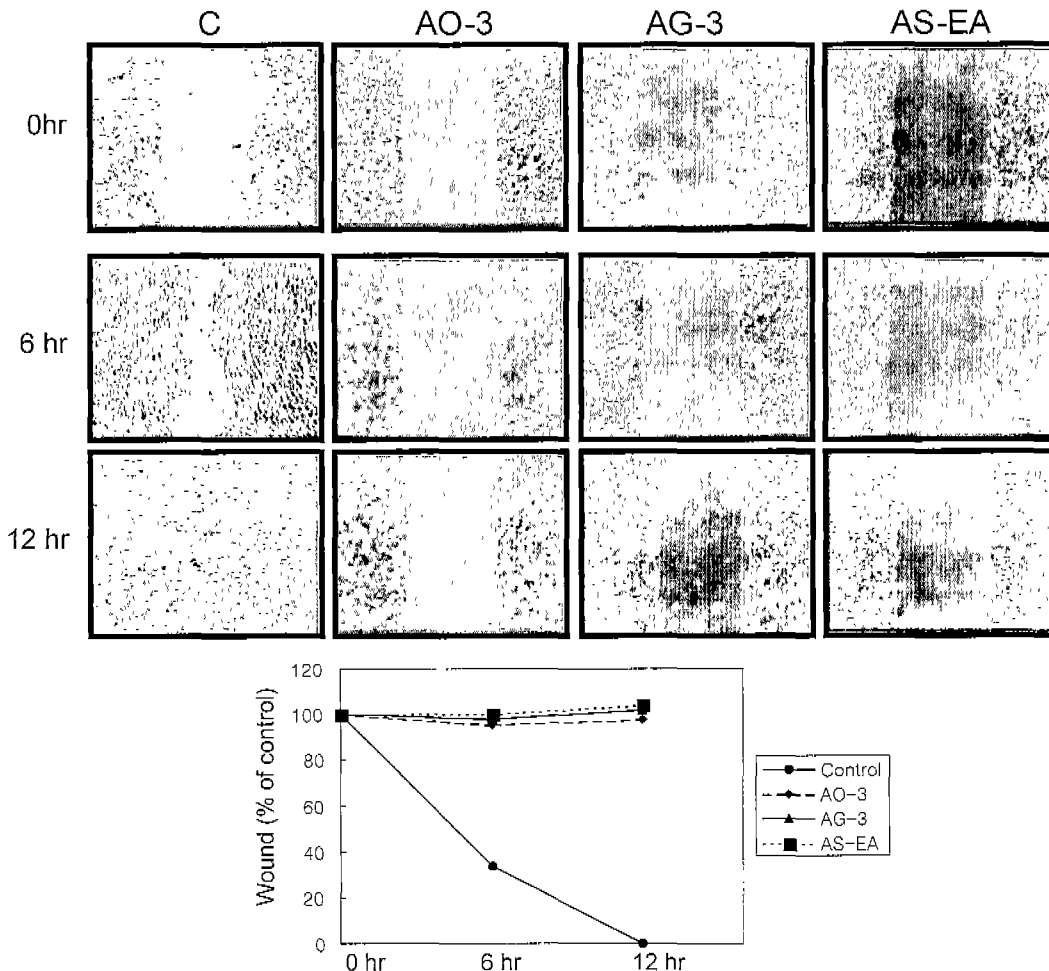


Fig. 4. Aster extracts inhibited cell migration. H-ras MCF10A cells were treated with 5mg/ml Aster extracts (AO-3, AG-3 and AS-EA) for 24 h in complete media. The cells were preincubated with 25 μ g/ml mitomycin C for 30 min. Injury line was made on the cells plated in culture dishes at 90% confluency with a tip of 2 mm in width. Cellular migration was observed with light microscope ($\times 40$) at indicated time points. Width of injury line (wound) was measured and plotted.

cells. The cells were treated with 5 mg/ml of each extracts for 24 hr in serum-free medium and gelatin zymographic assay was performed. As shown in Fig. 2A, all of the extracts tested in this study efficiently inhibited the gelatinolytic activity of MMP-2. Eight extracts, AO-3, AO-4, AG-3, AG-4, AS-MeOH Ex., AS-MC, AS-EA and AS-BU, showed >90% inhibition of MMP-2 activity in H-ras MCF10A cells. MMP-9 activity was also inhibited by treatment with the *Aster* extracts, in a lesser degree compared to MMP-2: only one extract (AS-EA) showed >90% inhibition of MMP-9 activity. Relative band intensities of MMPs were determined by quantitation of each band and the levels of MMP-2 and MMP-9 and the ratio of MMP-2 to MMP-9 were plotted (Fig. 2B). We selected one extract from each *Aster* species, AO-3 from *Aster oharai*, AG-3 from *Aster glehn* and AS-EA from *Aster scaber*; for further studies since they exerted an efficient downregulation of MMPs and decrease in the ratio of MMP-2 to MMP-9.

In vitro invasion assay was performed on highly invasive H-ras MCF10A cells to investigate the effect of *Aster* extracts on invasive phenotype of the malignant breast cells. As shown in Fig. 3, treatment with 5 mg/ml of AO-3, AG-3 and AS-EA for 17 hr markedly inhibited H-ras MCF10A cell invasion. AO-3, AG-3 and AS-EA reduced the number of invaded cells through a reconstituted basement membrane by 91%, 98% and 96%, respectively.

Since migrative capacity is a prerequisite for cell invasion through the basement membrane, we examined the effect of *Aster* extracts on cell migration by *in vitro* wound migration assay. As shown in Fig. 4, migration of H-ras MCF10A cells was inhibited by treatment with treatment with 5 mg/ml of AO-3, AG-3 and AS-EA. To confirm that the healing of wound is solely due to a migrative property and not due to a proliferative effect, cells were pretreated with mitomycin C (25 µg/ml), a cell cycle blocker at the S phase (Jozaki *et al.*, 1990), for 30 min before the injury line was made. The results show that the *Aster* extracts effectively inhibit cell migration regardless of cell cycle transition, suggesting that the anti-invasive effect of the extracts may involve inhibition of migrative property of the cells.

Many studies have focused on the anticarcinogenic, antimutagenic or chemopreventive activities of phytochemicals, particularly those included in the human diet

(Ferguson, 1994; Stavric, 1994). *Aster* has been shown to exert biological activities including antiviral, antihepatotoxic, neuroprotective and neurotrophic activities in various experimental systems (Gesa *et al.*, 1999, Mahmood *et al.*, 1997, Hur *et al.*, 2001). Our study was conducted to investigate the chemopreventive potential of *Aster* extracts that efficiently inhibit invasion and migration of malignant cancer cells. In this study, we show that *Aster* extracts exert inhibitory effect on invasiveness and migration of H-ras MCF10A cells in which MMP-2 and -9 downregulation is possibly closely associated. Taken together, the present study suggests that the *Aster* extracts, especially AO-3, AG-3 and AS-EA, inhibit invasive phenotype and migration of H-ras MCF10A cells through MMP-2 and -9 dependent mechanism which may contribute to the chemopreventive potential of these extracts.

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