Broccoli and watercress suppress matrix metalloproteinase-9 activity and invasiveness of human MDA-MB-231 breast cancer cells

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Abstract

A high dietary intake of cruciferous vegetables has been associated with a reduction in numerous human pathologies particularly cancer. In the current study, we examined the inhibitory effects of broccoli (Brassica oleracea var. italica) and watercress (Rorippa nasturtium aquaticum) extracts on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cancer cell invasion and matrix metalloproteinase-9 activity using human MDA-MB-231 breast cancer cells. Aberrant overexpression of matrix metalloproteinases, including metalloproteinase-9, is associated with increased invasive potential in cancer cell lines. Our results demonstrate that extracts of broccoli and Rorippa suppressed TPA-induced MMP-9 activity and invasiveness in a concentration dependant manner as determined by zymographic analysis. Furthermore, fractionation of individual extracts followed by liquid chromatography mass spectroscopy analysis (LC-MS) revealed that the inhibitory effects of each vegetable were associated with the presence of 4-methysulfinylbutyl (sulforaphane) and 7-methylsulphinylheptyl isothiocyanates. Taken together, our data indicate that isothiocyanates derived form broccoli and Rorippa inhibit metalloproteinase 9 activities and also suppress the invasive potential of human MDA-MB-231 breast cancer cells in vitro. The inhibitory effects observed in the current study may contribute to the suppression of carcinogenesis by diets high in cruciferous vegetables.

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Introduction

The process of metastasis consists of a series of complex cascades involving cancer cell migration, adhesion, and invasion. Indeed, cell invasion, the process of translocation of neoplastic cells across extracellular matrix barriers, is recognized as an essential biological event required for tumor metastasis (Basset et al., 1997; Bogenrieder and Herlyn, 2003). Although the mechanism(s) for the translocation of tumor cells across matrix barriers are not fully understood, it would appear that the secretion of proteolytic enzymes is essential. The metalloproteinases (MMP) represent a family of proteolytic enzymes that can degrade extracellular matrix (ECM) components including collagen, fibronectin, and laminin (Nabeshima et al., 2002). The MMP family is represented by several sub-groups each recognized for the type of substrates they degrade these include the gelatinases, collagenases, stromelysins, stromelysin-like MMPs, matrilysins, and MMPs (Kerkela and Saarialho-Kere, 2003). With regards to cell invasion both matrix metalloproteinases 2 (MMP-2) and matrix metalloproteinases 9 (MMP-9) are known to be essential (Bernardo and Fridman, 2003; Westermarck and Kahari, 1999). Indeed, elevated levels and activities of both MMP-2 and MMP-9 are found in cancerous tissues and tumor cells.

Due to the significant role that MMPs play in cancer as well as additional human pathologies, considerable interest...
has focused on identifying natural and synthetic compounds that can inhibit MMP activities. To date, these have included screening extracts derived from the traditional Korean prescription drug Daesungki-Tang, the Chinese medicinal herb *Euonymus alatus*, as well as polyphenolic compounds isolated from green tea. The results obtained from these studies indicate that extracts and compounds of plant origin are potent sources of principles with inhibitory effects against MMP activities (Cha et al., 2003; Chung et al., 2004; Corps et al., 2004; Demeule et al., 2000; Garbisa et al., 2001; Kaegi, 1998; Ha et al., 2004; Lin et al., 1998; Mohan et al., 2000); however, the mechanism(s) for this action remains largely unknown.

Considering the reported association of high cruciferous vegetable consumption and reduced incidence of cancer, little evidence exists showing the possible effects of cruciferous vegetables on tumor invasion. An early report by Scholar et al. (1989), demonstrated that a diet high in crucifers (cabbage and collards) decreased the number of pulmonary metastases after animals were intravenously injected with mammary tumor cells. However, the potential mechanism(s) and contributing active principles were not determined. Therefore, in the current investigation, we studied the inhibitory effects of extracts of broccoli and *Rorippa* on the invasion potential of human MDA-MB-231 breast cancer cells and the activity of MMP-9. These data could contribute to the cancer-inhibitory effects of diets high in cruciferous vegetables.

**Materials and methods**

**Cell culture**

The human MDA-MB-231 breast cancer cell line was purchased from ATCC. Cells were cultured in monolayers at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U/ml penicillin.

**Cell viability testing.** Cell viability was determined as previously described (Hansen et al., 1989). In brief, MDA-MB-231 cells were seeded at a density of 1 x 10⁴ cells/well in a 96-well microtitre plate and incubated for 24 h. Following cell adhesion the cells were washed twice in PBS and fresh media (DMEM FBS free) were replaced containing the designated concentrations of vegetable extract. Plates were incubated for a further 24 h prior to the determination of cell viability, as measured by the calorimetric change using a plate reader at 595 nm (Bio-Rad, model 3550, Biorad, Hercules, CA, USA).

Activity of lactate dehydrogenase (LDH) in the medium was measured using an Abbott VP Biochemical Analyser with the test kit (Abbott Laboratories, Irving, TX, USA). The total LDH activity was determined by ultrasonication and assessed by expressing as percentage LDH leakage (LDH in medium/total LDH activity ×100).

**Plant material and liquid chromatography mass spectroscopy (LC-MS) analysis.** Individual cruciferous vegetables, broccoli (*Brassica oleracea var. italica*) and watercress (*Rorippa nasturtium aquaticum*), were collected over a 3-month period from local supermarkets. Both species were placed on dry ice and freeze dried immediately to preserve freshness. All individual vegetable samples were then pooled, this being conducted to eliminate variation. Extracts were made as previously described (Mithen et al., 2003; Rose et al., 2000). In brief, 100 mg of freeze-dried tissue was weighed into a 50-ml polypropylene tube, hydrated with 2.0 ml of deionized water, and homogenized for 15 s (Ultraturrax homogenizer) and left at room temperature for 1 h with occasional vortexing. Boiling 70% methanol (3.0 ml) was added to the mix and incubated for a further 15 min at 70 °C. The mixture was cooled to room temperature, and centrifuged at 3000 rpm for 5 min. After centrifugation 1 ml aliquots were removed and vacuum condensed to 200 μl volumes. The resultant concentrates were filtered through sterile non-pyrogenic filters (0.2 μm; Millipore) and stored at −70 °C prior to testing. Extracts gave an equivalent concentration of 100 mg ml⁻¹ for each sample. All extracts were analyzed for their respective ITC composition using a Finnigan-LCQ LC-MS system.

**Gelatin zymography**

Gelatin zymography was performed essentially as reported earlier with modifications (Albini et al., 2003; Ha et al., 2004; Huang et al., 2004). Cells were seeded onto six-
well plates in DMEM with 10% FBS and cultured to 80% confluence. Cells were subsequently washed in PBS twice and cultured in serum-free medium for an additional 24 h. Following serum starvation the cells were then treated with TPA (80 nM) and/or vegetable extract (0.1–1 mg/ml) for 24 h. The conditioned media for each designated treatment was then collected and standardized to cell number (5 × 10^5 cells), mixed with non-reducing sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% (v/v) glycerol, 0.01% bromophenol blue), and subjected to electrophoresis on a 10% SDS–PAGE gel containing 0.1% (w/v) gelatin (Sigma). The resulting gels were washed twice for 60 min in wash buffer (10 mM Tris (pH 8.0) containing 2.5% (v/v) Triton X-100) to remove residual SDS and incubated for a further 16 h at 37 °C in developing buffer (50 mM Tris–HCl, pH 7.5, 0.2 M NaCl, 10 mM CaCl₂, and 1 mM ZnCl₂). Gels were stained using 0.5% Coomassie blue R-250 in 5% (v/v) methanol and 10% (v/v) acetic acid for 1 h and destained in 10% (v/v) methanol, 5% (v/v) acetic acid until the bands could be visualized. The presence of MMP-9 was indicated as an opaque band, the density of which was quantified using a Kodak Scientific Imaging system (Kodak, CT, USA).

**Cell invasion assay**

The ability of cells to invade through the BD Matrigel invasion chambers were conducted as previously described (Baba et al., 2000). In brief, cells (2 × 10^5/ml) were inoculated into the culture inserts and pretreated with different concentrations of broccoli and watercress extracts (0.1–1 mg/ml) for 1 h prior to stimulation with 80 nM TPA. After 12 h incubation, the lower surfaces of the membrane were fixed with 100% methanol and stained

![Fig. 2. Inhibitory effect of broccoli and Rorripa extracts against MMP-9 activity. (A) Conditioned media derived from TPA-treated MDA-MB-231 cells exposed to increased concentrations of vegetable extract. Conditioned media were normalized to cell number prior to loading on a 10% SDS–polyacrylamide gel co-polymerized with 0.1% gelatin. Following electrophoresis, gelatin zymography was performed and the presence of MMP-9 determined as an opaque unstained band. (B) Densitometric intensity of the MMP-9 band quantified using a Kodak Scientific Imaging system. All experiments were repeated 3 times on separate days using freshly prepared reagents.](image-url)
with Giemsa solution. Cells that had invaded to the lower surface of the membranes were counted using a high-power field microscope.

**Statistical analysis**

Data are presented as means ± SD and analyzed by Student’s *t* test.

**Results**

**Cruciferous vegetable extract cytotoxicity on MDA-MB-231 cells**

The cytotoxicity of each vegetable extract towards MDA-MB-231 breast cancer cells was evaluated using the MTT and LDH leakage assays. The results showed that no appreciable loss in cell viability was observed in cells incubated with increased concentrations of broccoli or *Rorippa* extract (0, 0.1, 0.2, 0.5, and 1 mg/ml) (Fig. 1).

**Effect of broccoli and Rorippa extracts on MMP-9 activities**

To examine the inhibitory effect of cruciferous vegetable extracts against MMP-9 enzymatic activity, cultured conditioned media of MDA-MB-231 cells were subjected to zymographic analysis. In the presence of increased concentrations of broccoli or *Rorippa* extract (0, 0.1, 0.2, 0.5, and 1 mg/ml), MMP-9 activity was shown to be reduced in a concentration-dependent manner (Figs. 2A–B).

4-Methylsulfinylbutyl and 7-methylsulfinylheptyl isothiocyanates contribute to the inhibitory effects of broccoli and Rorippa on MMP-9

Identification of candidate MMP-9 inhibitor(s), in extracts of broccoli and *Rorippa*, was determined by LC-
MS analysis. Because both species are members of the family Cruciferae we focused our attention towards isothiocyanate (ITCs) constituents. ITCs have previously been shown to have numerous biological effects in mammalian cells, including phase I enzyme inhibition and phase II enzymatic induction properties. The major ITC products found in the vegetable extracts were the non-volatile ITCs, 4-methylsulfinylbutyl (broccoli; 4-MSB ITC), and 7-methylsulfinylheptyl isothiocyanates (Rorippa; 7-MShep ITC). In addition, we also found 4-methylsulfinylbutyl nitrile (4-MSB nitrile) in the broccoli extract (Fig. 7-MShep ITC). The presence of each individual ITC was confirmed by LC-MS; the ITCs having a [M + H+] ion and a major component with a peak consistent with the fragmentation patterns of synthetic standards, sulforaphane, and 8-methylsulfinyloctyl ITCs. Moreover, the relative amount of the [M + H+] and [M + H+]2 were consistent with the predicted isotopic occurrence of 13C, 15N, and 33S. Thus, in each extract we had identified putative candidate compounds that may inhibit MMP-9 induction 4-MSB nitrile, 4-MSB ITC, and 7-MShep ITC.

We next fractionated the broccoli and Rorippa extracts into 6 individual fractions. The candidate compounds 4-MSB ITC and 7-MShep ITC were separated into fraction 4, while 4-MSB nitrile was present in fraction 3. Cells were incubated for 24 h in the presence of each fraction at 1 mg/ml equivalents and cell viability determined. None of the fractions tested were cytotoxic in either of the assays used (Fig. 3B). Next we analyzed each vegetable fraction (1–6) to determine the inhibitor effects of towards MMP-9 activity using the zymography assay. Using the conditioned media obtained from the MDA-MB-231 cells treated with each fraction (1 mg/ml) we found that only fraction 4 exhibited any strong inhibitory effect against MMP-9, as shown in Figs. 3C–D. None of the other components showed any inhibition action towards MMP-9 activity. Therefore, ITCs present within broccoli and Rorippa can be regarded as the main active components with respect to the inhibition of MMP-9 activity.

**Suppressive effect of broccoli and Rorippa extracts on TPA-induced invasion of MDA-MB-231 cells**

To determine whether inhibition of MMP-9 activity could prevent the invasive properties of MDA-MB-231 cells. Using transwell plates we measured the cell invasion property following TPA stimulation. As shown in Figs. 4A and B, crude extracts of broccoli and Rorippa and their respective ITC fractions could inhibited the invasiveness of TPA-stimulated MDA-MB-231 cells through the Matrigel-coated membrane in a concentration-dependent manner. We can rule out the possibility that such inhibition is due to cytotoxicity, as the cell viability was unaffected by the vegetable extracts, as determined using the MTT assay and LDH leakage assay. These results show that the broccoli and Rorippa extracts could inhibit MDA-MB-231 cells invasion, a breast cancer cell line with a high invasive capacity.

**Discussion**

The process of invasion requires the active degradation of environmental barriers including components of the basement membrane (BM) and extracellular matrix (ECM). Essential in this step are several proteolytic enzymes including the MMPs. To date, MMP-9 (gelatinase B) and -2 (gelatinase A) have received considerable attention due to their ability to degrades type IV collagen, this being a major structural component of the BM and ECM (Nabeshima et al., 2002). Moreover, increased expression and activities of MMP-9 and -2 are often found in tumor tissues and malignant cells (Woessner, 1991) and are known to be crucial in the invasion process (Aimes and Quigley, 1995; Woessner, 1991). Indeed, elevated protein levels and activities of MMP-9 and -2 in invasive breast cancers are associated with poor survival rates in patients. It is therefore not surprising that current research seeks to identify mechanism(s) to inhibit or reduce the invasive process.

Natural products including green tea polyphenols, isothiocyanates (ITC), resveratrol, limonene, and Allium-derived organosulfur compounds can all inhibit chemically induced tumor formation in rodent models (Park and Pezzuto, 2002; Surh, 2003). Attributed to the anticancer effects of these dietary agents is their ability to inhibit cancer cell proliferation, invasion, and metastatic potential. For example, with regards to invasion, green tea polyphenols are strong inhibitors of the gelatinolytic activity of MMP-9 and the secretion of MMP-2 (Annabi et al., 2002). Surprisingly, little is known of the role of cruciferous vegetable species or their phytochemical constituents in the prevention and or inhibition of cancer cell invasion. Recent epidemiological evidence has shown that a diet high in cruciferous vegetables can reduce the incidence of breast cancer risk in females (Ambrosone et al., 2004; Fowke et al., 2003). These data are further supported by the findings from experimental rodent models in which the principal bioactive components of cruciferous vegetables, the ITCs, can induce phase II detoxification enzymes, induce cell cycle arrest, and promote of apoptosis in breast cancer cells in vitro (Jackson and Singletary, 2004; Tseng et al., 2004; Zhang et al., 1992). Sulforaphane, derived from broccoli, has also been demonstrated to inhibit chemically induced breast cancer in female rats (Fahey et al., 1994). Moreover, early work by Wattenberg (1981), showed that benzyl isothiocyanate could inhibit 7, 12-dimethylbenz(a)anthracene-induced neoplasia of the breast of Sprague–Dawley rats. To date, no studies have addressed whether phytochem-
icals derived from cruciferous vegetables are inhibitory against cancer cell invasion in vitro. Therefore, in the current investigation we evaluated extracts of broccoli, *B. oleracea* var. *italica*, and watercress, *R. nasturtium aquaticum*, both major dietary sources of ITCs, against the invasive potential of a human breast cancer cell line. We show that extracts of broccoli and *Rorripa* can inhibit MMP-9 activities and the invasive potential of the human breast cancer cell line MDA-MB-231. Analysis of individual plant extracts using LC-MS revealed that two major ITC were present, these being 4-methylsulfinylbutyl and 7-methylsulfinylheptyl ITCs, respectively. This finding corresponded with previous reports (Mithen et al., 2003; Rose et al., 2000). Fractionation of each plant extract showed that the anti-invasive properties of broccoli and *Rorripa* were mediated by the ITC constituents. Previous investigations have shown that ITCs can inhibit the NF-KB signaling pathway in murine Raw 264.7 macrophages, human pancreatic cancer BxPC-3, and human colon HT29 cells (Heiss et al., 2001; Jeong et al., 2004; Murakami et al., 2003; Srivastava and Singh, 2004). Moreover, recent evidence has also demonstrated that the ITC sulforaphane can inhibit AP-1 DNA binding in human keratinocyte exposed to UV-B irradiation (Zhu et al., 2004). Considering the role of NF-KB and AP-1 signaling pathways in the expression of pro-inflammatory genes, including MMPs, perhaps detailed characterization of the AP-1 and NF-KB pathways in the anti-invasive properties of other cruciferous vegetable species and ITCs is warranted.

In conclusion, the findings herein suggest that broccoli and *Rorripa* have anti-invasive and anti-metalloproteinase activities, and that the phytochemical constituents, the ITCs, are a new class of invasion inhibitors. The results provide...
further insight as to how cruciferous vegetable consumption perhaps contributes to human chemoprevention.

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