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Anti-metastatic activity of an apple polyphenol crude fraction against human Ha ras-transformed metastatic mouse tumor (r/m HM-SFME-1) cells

Research Article

Kazuo Ryoyama*, Yoshitaka Shimotai, Taichi Higurashi, Tomomi Kokufuta, Yumi Kidachi, Hideaki Yamaguchi#, and Ichiro Hatayama§

Department of Bioscience and Biotechnology, Faculty of Engineering;
#Graduate School of Environmental Sciences, Aomori University, Aomori 030-0943, Japan
§Aomori Prefectural Institute of Public Health and Environment, Aomori 030-8566, Japan

***Correspondence:** Kazuo Ryoyama; Tel: +81 177 38 2004; Fax: +81 177 38 2030; e-mail: ryoyama@aomori-u.ac.jp

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Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; NO, nitric oxide; VEGF, vascular endothelial growth factor

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Summary

Oral administration of a 0.5% crude fraction of an apple polyphenol significantly inhibited the spontaneous lung metastasis of r/m HM-SFME-1 tumor cells, but did not significantly inhibit tumor growth at the site of transplantation. This fraction dose-dependently inhibited the *in vitro* invasion and migration of the tumor, and inhibited slightly the MMP-9 production and IFN- plus LPS-augmented VEGF gene expression of the tumor, although non-augmented VEGF gene expression was stimulated in a dose-dependent fashion by the fraction. In addition, the polyphenol fraction inhibited the MMP-9 production of the fibroblast cell line NIH3T3, but not that of the macrophage cell line J774.1, and inhibited the VEGF gene expression of both stromal cell types. NO production by J774.1 cells was also inhibited significantly. These findings indicate that the anti-metastatic activity of the crude polyphenol fraction occurs *via* the inhibition of both tumor and stromal cell activities. It is noteworthy that the anti-metastatic activity of the polyphenol fraction occurs in the absence of any direct inhibition of tumor growth.

I. Introduction

Increasing interest in the health benefits of plants (tea, grapes, etc.) that are rich in polyphenols has led to the inclusion of plant extracts in dietary supplements and functional foods (Kelloff et al, 2000). Animal studies have shown that a polyphenol-rich diet is associated with a lower incidence of cancer, and epidemiological evidence, although inconclusive, suggests that consuming food and beverages that are rich in polyphenols may reduce the risk of some cancers in humans (Kelloff et al, 2000; Lin, 2002; Mouria et al, 2002).

Polyphenols are distributed widely in the plant kingdom, and are structurally diverse (Freidman and Jugens, 2000). They are plentiful in certain plants but not in others (Paganqa et al, 1999; Leontowicz et al, 2002; Mouria et al, 2002). For example, tannic acid is not found in tea but is found in apples (Graham, 1992; Kanda et al, 1998). The polyphenol content and composition are affected by various factors, such as the plant variety, growth conditions, and manufacturing processes (Graham, 1992; Astill et al, 2001; van der Sluis et al, 2001). In this respect, green tea is rich in catechins, whereas black tea is rich in theaflavins (Graham, 1992).

The most studied of these compounds are the tea polyphenols, particularly catechins, and there are many reports regarding the antitumor activities of epigallocatechin-3-gallate (Blanko et al 2003; Gupta et al, 2003). Other polyphenols, such as curcumin, rutin, quercetin, and trans-reveratrol, have also been reported to be chemopreventive through their anti-proliferative, anti-metastatic, and/or anti-invasive properties (Menon et al, 1995; Maeda-Yamamoto et al, 1999; Menon et al, 1999; Caltagirone et al, 2000; Mouria et al, 2002).

Immature apples have been reported to contain large amounts of several types of polyphenol, which include

chlorogenic acid, catechin, epicatechin, rutin, and condensed tannins (Kanda et al, 1998). Although the biological activities of these apple-derived compounds, especially as they pertain to the control of tumor progression, remain to be determined, a crude polyphenol fraction has been reported to inhibit histamine release from RBL-2H3 cells and rat mast cells (Kanda et al, 1998).

Therefore, we examined the effects of a crude fraction of the apple polyphenol on the growth, metastasis, and invasion of tumor cells both *in vivo* and *in vitro*.

II. Materials and methods

A. Reagents

The apple polyphenol crude fraction (5% in solution) was kindly supplied by Nikka Whisky Distilling Co. Ltd. (Chiba, Japan). Dulbecco's modified Eagle's medium mixture F-12 Ham (DME/F-12), RPMI-1640, insulin, transferrin, and gelatin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin and LPS (*E. coli* 055:B5) were obtained from DIFCO Laboratories (Detroit, MI, USA). Fetal bovine serum (FBS), bovine fibronectin, gelatinase zymography standards (human MMP-2 and -9), recombinant mouse IFN- , Perfect Protein™ Markers, ISOGEN®, RNA PCR kit, AmpliTaq Gold with Gene Amp 10× PCR Gold Buffer, agarose-LE, and the DIG Oligonucleotide Tailing Kit were purchased from JRH Biosciences (Lenaxa, KS, USA), Biomedical Technologies Inc. (Stoughton, MA, USA), Chemicon International Inc. (Temecula, CA, USA), Novagen Inc. (Madison, WI, USA), Genzyme Corp. (Cambridge, MA, USA), Nippon Gene (Tokyo, Japan), Takara Biochemicals, Inc. (Tokyo, Japan), Applied Biosystems (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), and Roche Diagnostics Co. Ltd. (Tokyo, Japan), respectively. All of the other reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

B. Cell lines and culture conditions

The r/m HM-SFME-1 cells were maintained in a humidified 5-7% CO_2 atmosphere at 37°C under serum-free culture conditions, as described previously (Matano et al, 1995), and passaged every four days. The culture medium (F/D medium) was DME/F-12 that was supplemented with sodium bicarbonate (1.2 g/l), sodium selenite (10 nM), and gentamicin sulfate (10 µg/ml). The NIH3T3 cells were maintained under similar conditions, with the following modifications: EGF (50 ng/ml) was added to the culture medium, and the culture dishes and plates were pre-coated with type I collagen (0.3 mg/6-cm-diameter dish; coated twice). The J774.1 cells were cultured in RPMI 1640 that contained 5% FBS.

C. Mouse strains and in vivo antitumor experiments

Female BALB/c mice were obtained from Charles River (Japan) Inc. (Kanagawa, Japan) or from our own colony, and used in the experiments at 7-10 weeks of age. The r/m HM-SFME-1 cells (2×10^5) were injected subcutaneously into the right footpad of each mouse. Every other week, some of the injected mice were sacrificed and their lungs were removed. The mice had free access to drinking water that contained the apple polyphenol crude fraction from two weeks before tumor implantation to the end of the experiment.

D. Evaluation of r/m HM-SFME-1 cell metastasis in the lungs

The degree of r/m HM-SFME-1 cell metastasis in the lungs was estimated by quantifying the levels of the human c-Ha-ras 1 gene in the cells, as described previously (Matano et al, 1995), with minor modifications. In brief, the DNA samples were amplified for 35 cycles of 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 1min. The 72ºC incubation period was extended to 10 min in the last cycle. The PCR products were electrophoresed in a 3% agarose-LE gel, and transferred to a positively charged nylon membrane (Roche Diagnostics). The transferred PCR products were hybridized with probes that had been labeled with the DIG Oligonucleotide Tailing Kit. The membrane was washed and exposed in the Luminescence Image Analyzer (Fujifilm LAS-1000; Fuji Photo Film Co. Ltd., Tokyo, Japan), and the intensities of the PCR products were measured. The 123-bp PCR product of the human c-Ha-ras 1 gene was used as a probe for this gene, since the total region rather than partial regions of the gene was required to detect the PCR products. In the case of the human c-Ha-ras 1 gene, the upstream (sense) and downstream (antisense) primers were 5'-ATgACggAATATAAgCTggT-3' and 5'-CgCTAggCTCACCTCTATA-3', which correspond to nucleotide positions 1670-1689 and 1773-1792, respectively. The standard used for estimation of the metastasized tumor cell numbers was derived from the DNA samples from normal lungs that contained 10^2 to 10^6 r/m HM-SFME-1 cells.

E. Chemoinvasion and migration assays

The invasiveness of r/m HM-SFME-1 cells was assayed using 8.0-µm pore size polyvinylpyrrolidone-free polycarbonate filter chambers (Chemotaxicell®, Kurabo Industries, Ltd. Osaka, Japan). The filter had been previously coated on the reverse side with fibronectin (10 µg/filter) and dried at 37ºC for 2 days. Gelatin (25 µg/filter) was applied to the front side of the filter, which was then dried at 37ºC for 1 day. The gelatin coating was applied twice, and fibronectin (10 µg/filter) was applied subsequently. The cell suspension (100 µl of 1×10^6 cells/ml) in F/D medium containing 5% FBS and 100 µl apple polyphenol solution (0-50 µg/ml) was plated onto the Chemotaxicells, which were placed in 24-well microplates, and 0.6 ml of F/D medium containing 5% FBS and the apple polyphenol solution (0-50 µg/ml) was immediately added to the outer well of the microplates. The chambers were incubated at 37ºC. The culture medium was removed after 3 days, and the cells on the gelatin-coated (front) side of the filter were removed by wiping with a cotton swab. The cells on the fibronectin-coated (reverse) side of the filter were collected by rinsing with phosphate-buffered saline that contained 0.1% trypsin, and were counted with a Coulter counter (model Z_BI; Coulter Electronics Inc., Hialeah, FL, USA).

The migration assay for the r/m HM-SFME-1 cells was similar to the invasion assay, with the following modifications: fibronectin and gelatin were not applied to the reverse and front sides of the filters in the Chemotaxicell, and fibronectin (3 µg in 0.6 ml) was added to the outer well of the 24-well microplate.

F. Gelatin zymography

After a 2 h pre-incubation of the cells (10^6) in 6-cm-diameter culture dishes that contained 2 ml of culture medium, 10 µl of the apple polyphenol crude fraction (0.1-10 mg/ml) was added, and the dishes were incubated for 24 h at 37ºC. Cell-free culture supernatants were prepared. The culture supernatants of the r/m HM-SFME-1 cells, but not those of the NIH3T3 and J774.1 cells, were concentrated 30-fold using an Ultrafree-CL concentrator (Amicon, UFC4LCC25; Millipore Corp., Bedford,

MA, USA). Aliquots of the culture supernatants were electrophoresed in a 10% polyacrylamide gel that contained sodium dodecyl sulfate and 0.1% gelatin. After electrophoresis, the gel was washed with 2.5% Triton-X100 and incubated at 37ºC for 20 h in 100 mM Tris-HCl (pH 8.0) that contained 5 mM $CaCl_2$, 0.005% polyoxyethylene lauryl, and 0.001% sodium azide. The gel was then stained with Quick-CBB® that contained 1% Coomassie brilliant blue R. Perfect™ Protein Markers were used as the molecular mass standards, and gelatinase zymography standards for human MMP-2 and MMP-9 were used as the positive controls for MMP-2 and MMP-9, respectively.

G. RT-PCR

The cDNA was prepared as described previously (Ryoyama et al, 2003). Aliquots of the cDNA samples were subjected to PCR using AmpliTaq Gold with the Gene Amp 10× PCR Gold Buffer that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 µM of each primer (sense and antisense), 0.2 mM of each dNTP, and 25 units/ml Taq polymerase.

The primers for mouse MMP-2, MMP-9, TIMP-1, and VEGF were synthesized according to the GenBank sequences. The primers for MMP-2 were derived from the sequence with accession no. M84324: forward primer, 5'-ggCCATgCCATggggCTg-3' (nucleotides 1259-1276) and reverse primer, 5'-CCAgTCTgATTTgATgCTTC-3' (nucleotides 2001-2020). The MMP-9 primers were derived from the sequence with accession no. D12712: forward primer, 5'-gggCAACTCggCAggAgAgC-3' (nucleotides 1044-1063) and reverse primer, 5'-CCAggTgACgggCTgCTTgT-3' (nucleotides 1513-1532). The TIMP-1 primers were derived from the sequence with accession no. X04684: forward primer, 5'-CTgTgCCCCACCCCACCCAC-3' (nucleotides 184-203) and reverse primer, 5'-AAggCTTCAggTCATCgggC-3' (nucleotides 716-735). The VEGF primers were derived from the sequence with accession no. NM009505: forward primer, 5'-CACgACAgAAggAgAgCAgAAgTC-3' (nucleotides 79-119) and reverse primer, 5'-gCCATCATCgTCACCgTTgA-3' (nucleotides 742-761). The mouse beta-actin gene was used as the internal control with the forward primer, 5'-gTgggCCgCTCTAggCACCAA-3' (nucleotides 25-45) and the reverse primer, 5'-CTCTTTgATgTCACgCACgATTTC-3' (nucleotides 541-564).

The PCR was initiated at 95ºC for 10 min, and then performed for 30 cycles of 94ºC for 45 s, 60ºC (65ºC for VEGF) for 45 s, and 72ºC for 45 s. The reaction was terminated by heating to 70ºC for 10 min, followed by chilling on ice. The PCR products were electrophoresed, and transferred to a nylon membrane, as described above. The expected PCR products for the mouse MMP-2, MMP-9, TIMP-1, and beta actin gene were 762 bp, 489 bp, 552 bp, and 540 bp, respectively.

H. Determination of NO levels

The levels of NO in the culture supernatants were determined with the Griess reagent, as described previously (Ryoyama et al, 1993).

I. Data presentation and statistical analysis

All of the experiments were repeated two to five times, with similar results. The statistical significance of the differences between the groups was determined as described previously (Ryoyama et al, 2003).

III. Results and Discussion

A. Effect of the apple polyphenol crude fraction on the spontaneous metastasis of r/m HM-SFME-1 cells in the lung

The effects of the crude fraction of the apple polyphenol on spontaneous metastasis of r/m HM-SFME-1 cells in the lung were assayed according to the method of Matano et al. (Matano et al, 1995) with minor modifications. At 39 days after tumor implantation, the number of tumor cells in the lungs was estimated to be between 1.5×10^4 and 3.0×10^6 cells/lung (**Figure 1A**). Oral administration of the apple polyphenol fraction significantly inhibited tumor metastasis; the estimated number of tumor cells after treatment was between 1.0×10^2 and 3.9×10^5 cells/lung. A lower dose of the fraction (0.05%) also tended to inhibit lung metastasis, although this level of inhibition was not statistically significant (data not shown). In addition, since the mice drank 4.4 ± 0.5 ml/day of fluid, each mouse had an approximate intake of 22 ± 2.5 mg/day of the apple polyphenol crude fraction.

Figure 1B shows the effects of the crude fraction on the subcutaneous growth of the r/m HM-SFME-1 cells. Cell growth appeared 14 days after implantation, and the size of the tumor-transplanted footpads increased thereafter in a time-dependent manner. In the mice that were fed the apple polyphenol fraction, the increases in footpad thickness were smaller than those of the control, but were not significant between the controls and treated mice for each timepoint. In addition, within the concentration range of 0.5-500 µg/ml, the crude fraction did not inhibit the *in vitro* growth of the r/m HM-SFME-1 cells (data not shown).

B. Effects of the apple polyphenol crude fraction on the in vitro invasion and migration of r/m HM-SFME-1 cells

Since the apple polyphenol fraction scarcely affected the growth of r/m HM-SFME-1 cells, its effect on the *in vitro* invasion and migration of these cells was examined. It has been reported that r/m HM-SFME-1 cells are highly metastatic in the lungs of mice (Matano et al, 1995). Indeed, r/m HM-SFME-1 cells could be detected in the lungs seven days after the injection of 10^5 to10^6 cells into the footpad (unpublished data). This result suggests that these cells are highly invasive in the *in vitro* invasion model. Thus, we attempted to clarify the invasive activities of the r/m HM-SFME-1 cells. A preliminary experiment showed that 10% to 50% of the input cells had invaded three days after incubation. Since the rates of invasion varied from experiment to experiment, the effects of the apple polyphenol crude fraction on invasion are expressed as percentages of the mean number of invading cells in the control group for each experiment. **Figure 2A** shows the effect of the crude fraction on r/m HM-SFME-1 invasion. The fraction dose-dependently inhibited cell invasion, and the level of inhibition at 50 µg/ml crude fraction was significant, at 40-50% of the control level of invasion. This inhibition was not due to cytotoxicity, since the fraction was not cytotoxic at these concentrations (data

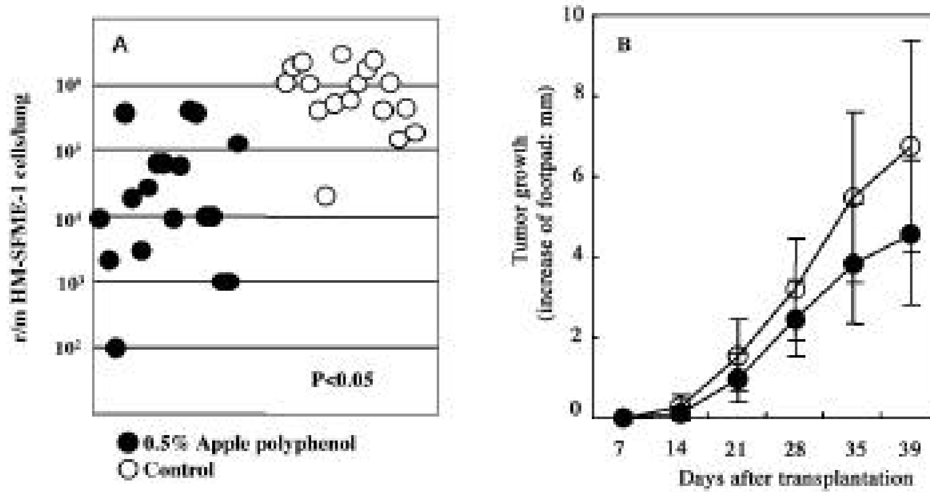

Figure 1. Effects of the crude fraction of the apple polyphenol on spontaneous lung metastasis and the *in situ* growth of r/m HM-SFME-1 cells.

The r/m HM-SFME-1cells (2×10^5) were injected subcutaneously into the right footpad of each BALB/c mouse. The crude fraction (0.5%) of the apple polyphenol was provided freely to the mice in the drinking water, from two weeks before tumor implantation to the end of each experiment. At 39 days after tumor implantation, the numbers of lung tumor cells that had metastasized spontaneously were estimated (A). The method of estimating the numbers of tumor cells is described in the *Materials and Methods* section. Footpad thickness, which was taken as an indicator of tumor growth, was measured with a caliper (B). In (B), there were no statistically significant differences between the controls and treated mice for each timepoint.

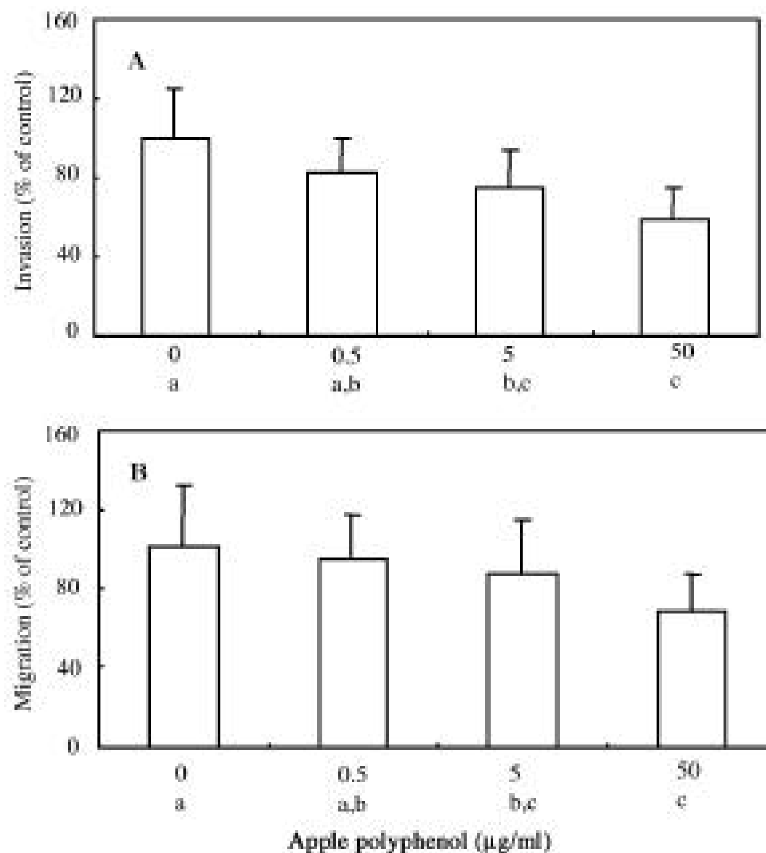

Figure 2. Effects of the crude fraction of the apple polyphenol on *in vitro* invasion (A) and *in vitro* migration (B) of the r/m HM-SFME-1 cells.

The r/m HM-SFME-1 cells (10^5/Chemotaxicell®) were incubated for three days with various concentrations of the crude fraction. Invasion and migration of the tumor cells were determined as described in the *Materials and Methods* section. The results are represented as percentages of the control values: [invaded (migrated) cells with treatment / mean invaded (migrated) cells without treatment]. The values shown are means ± SD of 5-7 independent experiments (each consisting of six Chemotaxicells). The letter below each treatment indicates the results of the statistical analysis (P<0.05).

not shown).

Since the inhibition of invasion by the apple polyphenol crude fraction might be due to the inhibition of cell migration *in vitro*, we examined the effect of the fraction on the migration activity of the r/m HM-SFME-1 cells. Initially, the migrating activities of the cells were assayed, and found to be 1% to 6% of the input cells after the three-day incubation. Since the rates of migration varied from experiment to experiment, the effects of the crude fraction on migration are expressed as described above for the invasion assays. **Figure 2B** shows the effect of the apple polyphenol crude fraction on the migration of r/m HM-SFME-1 cells. The fraction inhibited migration in a dose-dependent manner, and the level of inhibition at 50 µg/ml of the crude fraction was significant, at about 30% of the control level of migration.

C. Effects of the apple polyphenol crude fraction on the activities and gene expression of MMP, and on NO production

Our preliminary experiments showed that r/m HM-SFME-1 cells produced MMP-9 at low levels, but did not produce MMP-2, and that LPS augmented MMP-9 gene expression. Moreover, Wang et al. (Wang et al, 2002) have reported that fibroblasts promote breast cancer cell invasion by stimulating MMP-9 synthesis. Therefore, we examined the effects of the apple polyphenol crude fraction on MMP-9 activity and mRNA expression in r/m HM-SFME-1 cells, in the presence and absence of LPS and in the conditioned medium from a culture of the fibroblast cell line NIH3T3. **Figure 3A** shows that the fraction scarcely affected the MMP-9 activities of the tumor cells. The MMP-9 activity in the presence of the conditioned medium was not tested because the conditioned medium itself had potent MMP-9 activity.

Both LPS and the conditioned medium augmented MMP-9 gene expression, which was inhibited in a dose-dependent fashion by the fraction (**Figure 3B**), whereas MMP-9 gene expression in the absence of stimulation was not affected by the fraction. Furthermore, TIMP-1 gene expression in the presence of the conditioned medium was apparently inhibited at 50 µg/ml (**Figure 3B**).

Since tumor growth sites appear to consist of both tumor cells and inflammatory cells, increases in footpad thickness reflect not only tumor growth but also inflammation. Many reports have indicated a close correlation between tumor progression and *in situ* inflammation; tumor invasion and metastasis appear to be affected by soluble factors and free radicals that are produced locally by host tissue cells (fibroblasts, endothelial cells, macrophages) (Lala and Chakraborty, 2001; Coussens and Werb, 2002; Wang et al, 2002). Furthermore, in the majority of human and experimental tumors, NO appears to stimulate tumor growth and metastasis by enhancing the invasive, angiogenic, and migratory capacities of the tumor cells (Lala and Chakraborty, 2001). In fact, the spontaneous metastasis of r/m HM-SFME-1 in the lung is blocked by inhibitors of NO production (manuscript in preparation). The apple polyphenol crude fraction inhibited slightly but not significantly the increase in thickness of the footpads into which r/m HM-SFME-1 cells had been implanted (**Figure 1B**), and did not inhibit *in vitro* tumor growth (data not shown). Furthermore, the fraction inhibited the augmentation of MMP-9 gene expression caused by the addition of fibroblast-conditioned medium (**Figure 3B**). These results suggest that the apple polyphenol crude fraction inhibits inflammation.

Therefore, we examined the effects of the crude fraction on the MMP-9 and MMP-2 activities of NIH3T3 cells and on the MMP-9 activity of J774.1 cells. **Figure 3C** shows that the crude fraction dose-dependently inhibited the MMP-9 activity of NIH3T3 cells, and also tended to inhibit MMP-2 activity. However, the fraction did not inhibit the MMP-9 activity of the J774.1 cells (data not shown). The production of NO by J774.1 cells, which was induced by treatment with IFN- and LPS, was inhibited slightly but significantly by 50 µg/ml of the crude fraction (data not shown).

Plasminogen activation systems have been implicated in extracellular matrix degradation (Sidenius and Blasi, 2003). One of these systems, the urokinase-type plasminogen activator (uPA), has been clearly implicated in cancer progression, particularly invasion and metastasis (Andreasen et al, 2000; Rabbani and Mazar, 2001). Therefore, we examined the effect of the apple polyphenol crude fraction on uPA production by the r/m HM-SFME-1 cells. The addition of 0.5-50 µg/ml of the fraction did not affect uPA production (data not shown). On the other hand, the crude fraction augmented significantly the uPA activity of J774.1 cells, whereas that of the NIH 3T3 cells was scarcely affected (data not shown).

These results show that the stromal cell responses of tumor tissues to apple polyphenols may affect tumor growth in either a positive or a negative fashion. It remains to be seen which constituents of the apple polyphenol are responsible for the individual responses.

D. Effect of the apple polyphenol crude fraction on VEGF gene expression

Neovascularization is widely accepted as being a crucial step in tumor progression (Folkman, 1995). Tumor cells and various host cells, such as macrophages, fibroblasts, and epithelial cells, secrete various angiogenic factors, the most important of these being vascular endothelial growth factor (VEGF). Thus, we examined the effect of the crude fraction on VEGF gene expression. Our preliminary experiments showed that r/m HM-SFME-1, NIH3T3, and J774.1 cells express VEGF 188 (666 bp) and VEGF 120 (462 bp). **Figure 4A** shows that 0.5-50 µg/ml of the crude fraction dose-dependently augmented VEGF 188 and VEGF 120 gene expression in r/m HM-SFME-1 cells. Moreover, treatment with INF- plus LPS augmented the expression of both forms of VEGF; this augmentation was slightly increased at 0.5 µg/ml and inhibited at 50 µg/ml of the crude fraction (**Figure 4A**). The expression levels of the two forms of VEGF in the NIH3T3 cells were inhibited by 50 µg/ml of the crude fraction (**Figure 4B**). On the other hand, the expression

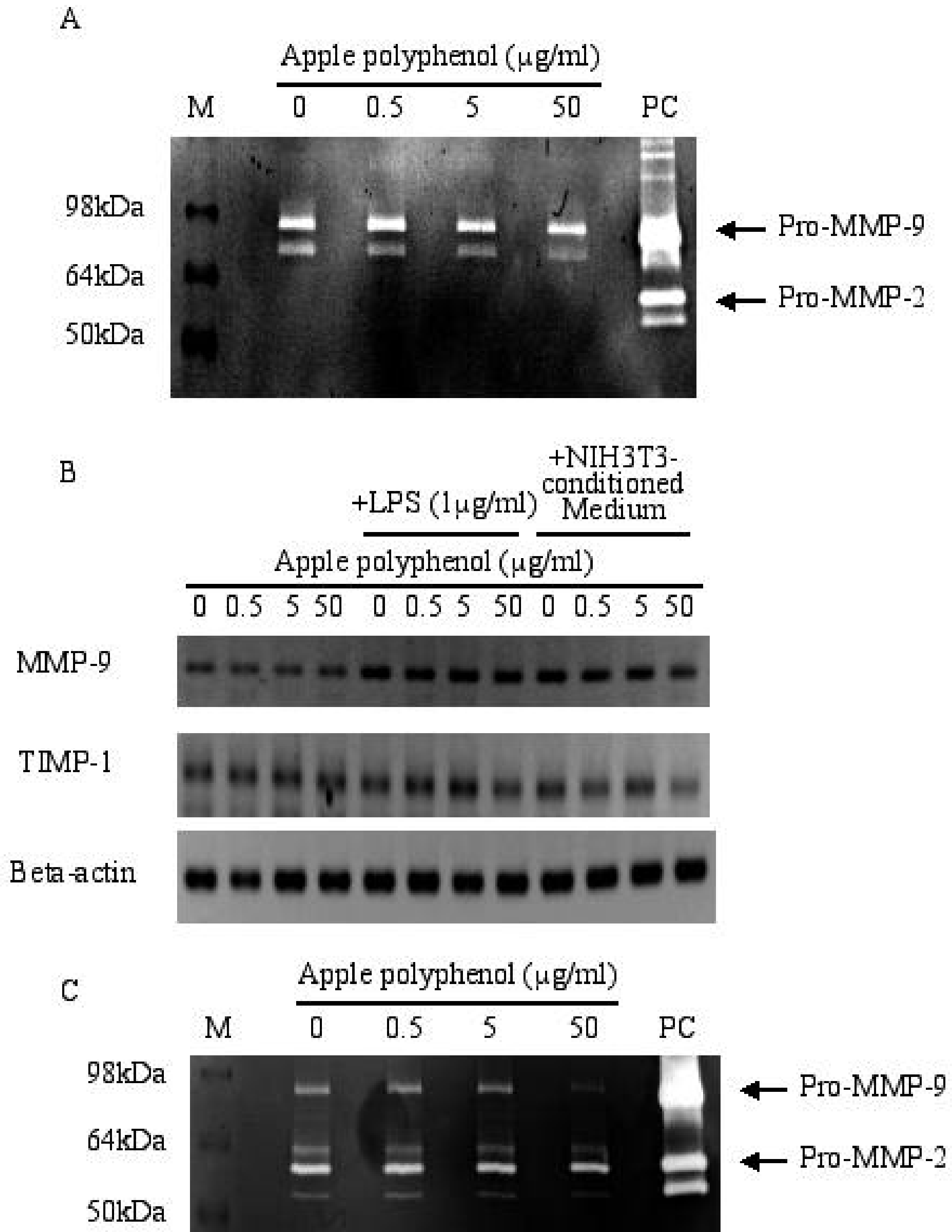

Figure 3. Effects of the crude fraction of the apple polyphenol on the activity and gene expression of MMP-9 in r/m HM-SFME-1 (A, B) and NIH3T3 (C) cells.

The cells (10^6) were treated for 24 h with various concentrations of the crude fraction. The culture supernatants and cells were processed to determine the MMP-9 activity and the expression of the MMP-9 and TIMP-1 genes, respectively, as described in the *Materials and Methods* section.

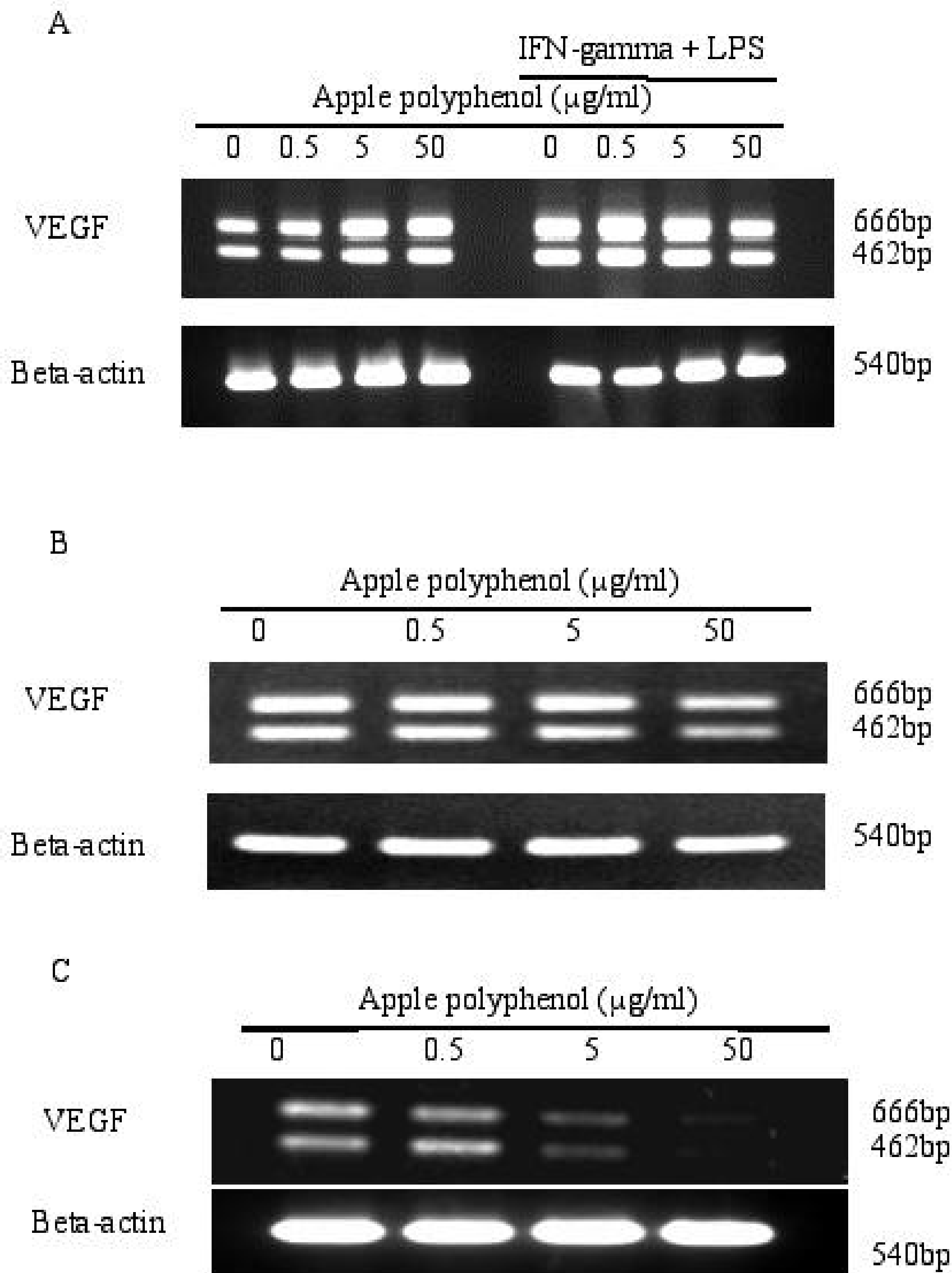

Figure 4. Effects of the crude fraction of the apple polyphenol on VEGF gene expression in r/mHM-SFME-1 (A), NIH3T3 (B) and J774.1 (C) cells.

The cells (10^6) were treated for 24 h with various concentrations of the crude fraction, and were then processed to determine VEGF gene expression, as described in the *Materials and Methods* section. VEGF 188 and 120 correspond to 666 bp and 462 bp, respectively.

levels of both VEGFs in J774.1 cells were much weaker than in r/mHM-SFME-1 cells, and treatment with IFN- plus LPS augmented their expression (data not shown). As shown in **Figure 4C**, the crude fraction dose-dependently inhibited the augmented expression, and the expression of both VEGF forms was abrogated by 50 μg/ml of the crude fraction. These results suggest that the apple polyphenolinhibits neovasculalization, resulting in the inhibition of tumor metastasis.

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Dr. Kazuo Ryoyama