Inhibition of Activator Protein-1 Binding Activity and Phosphatidylinositol 3-Kinase Pathway by Nobiletin, a Polymethoxy Flavonoid, Results in Augmentation of Tissue Inhibitor of Metalloproteinases-1 Production and Suppression of Production of Matrix Metalloproteinases-1 and -9 in Human Fibrosarcoma HT-1080 Cells

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INTRODUCTION

The metastatic progression of malignant tumors requires the proteolytic degradation of ECM components in basement membrane and stroma tissues, and MMPs play important roles in the degradation of ECM (1–3). Different sets of MMPs, such as gelatinase A (72-kDa type IV collagenase)/MMP-2, gelatinase B (92-kDa type IV collagenase)/MMP-9, interstitial collagenase/MMP-1, stromelysin-1/MMP-3, and MT-MMPs act in concert in the breakdown of ECM components during tumor invasion (1, 4–8). The enzymatic activity of MMPs is inhibited by TIMPs; TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (9), and they inhibit the invasion and metastasis of malignant tumor cells in vivo and in vitro (10–14). Thus, it is likely that the suppression of MMP expression or the augmentation of TIMP expression efficiently exerts an effect of interference on tumor invasiveness.

Flavonoids possess pharmacological properties for preventing tumor progression by inhibiting cell proliferation and tumor promotion (15–21). Recently, we reported that a polymethoxy flavonoid, nobiletin, which is contained as a major component in juice from Citrus depressa, inhibits the invasion and metastasis of malignant tumor cells (9), and MT-MMPs act in concert in the breakdown of ECM components (1–3). Different sets of MMPs, such as gelatinase A (72-kDa type IV collagenase)/MMP-2, gelatinase B (92-kDa type IV collagenase)/MMP-9, interstitial collagenase/MMP-1, stromelysin-1/MMP-3, and MT-MMPs act in concert in the breakdown of ECM components during tumor invasion (1, 4–8). The enzymatic activity of MMPs is inhibited by TIMPs; TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (9), and they inhibit the invasion and metastasis of malignant tumor cells in vivo and in vitro (10–14). Thus, it is likely that the suppression of MMP expression or the augmentation of TIMP expression efficiently exerts an effect of interference on tumor invasiveness.

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on the lower side were fixed and then stained with hematoxylin (Wako Pure Chemical Co., Osaka, Japan). The number of cells in 10 randomly chosen areas/membrane was visually counted under a light microscope at 200-fold magnification.

**Western Blot Analysis.** After being treated with reagents, the harvested culture medium was subjected to SDS-PAGE with 10% (w/v) acrylamide gel (for proMMPs) and 12.5% (w/v) acrylamide gel (for TIMP-1). The proteins separated in the gel were electrotransferred onto a nitrocellulose membrane, and the membrane was reacted with sheep anti-human proMMP-1, anti-human proMMP-9, or anti-human TIMP-1 antibody (generous gifts of Dr. H. Nagase), which was then complexed with horseradish peroxidase-conjugated goat anti-sheep IgG IgG. Immunoreactive proMMP-1, proMMP-9, and TIMP-1 were visualized with enhanced chemiluminescence-Western-blotting detection reagents (Amersham Pharmacia Biotech, Tokyo, Japan). The relative amounts of the proteins were quantified by densitometric scanning using the Image Analyzer LAS-1000 plus (Fuji Film Co., Tokyo, Japan).

**Northern Blot Analysis.** Cytoplasmic RNA (20 μg) or mRNA (2 μg) isolated from the reagent-treated cells was electrophoresed on a 1.0% (w/v) formaldehyde-denatured agarose gel and then transferred on a nylon membrane. The membrane was hybridized with a 32P-labeled human cDNA probe for proMMP-1, proMMP-9 (25), TIMP-1 (generous gifts of Dr. H. Nagase), TIMP-2 (24), MT1-MMP, or GAPDH cDNA (Clontech Laboratories, Inc., Palo Alto, CA). The relative amounts of steady-state mRNA were quantified by densitometric scanning using the Image Analyzer LAS-1000 plus (Fuji Film) and calculated after correction for GAPDH mRNA. The cDNAs of proMMP-1 and MT1-MMP were amplified by reverse transcription-PCR using their specific primers and mRNA derived from TPA-treated human uterine cervical fibroblasts (26) and HT-1080 cells, respectively, and then ligated into pGEM-T Easy vector (Promega Corp., Madison, WI), followed by confirming their cDNA sequences as described previously (24). The human proMMP-1 primers were 5'-GGTTGATGAAGTCAGCCGGAA-3' (antisense, 742–759 bp; Ref. 27), 5'-GGTGATGAAGCAGCCCAG-3' (sense, 254–270 bp) and 5'-GGAACCTGACTCACCCCCAT-3' (antisense, 959–979 bp; Ref. 28).

**Gelatin Zymography.** The harvested culture medium (10 μl) was subjected to SDS-PAGE with 10% (w/v) acrylamide gel containing gelatin (0.6 mg/ml; Difco Laboratories, Detroit, MI). The gel was washed with washing buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM CaCl2, 1 μM ZnCl2, and 0.1% (v/v) Triton X-100) to remove SDS and then incubated at 37°C in incubation buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM CaCl2, and 1 μM ZnCl2). Thereafter, the gel was stained with Coomassie Brilliant Blue R-250, and gelatinolytic activity was detected as unstained bands on a blue background.

**Nuclear Extracts and Gel Shift Assay.** The cells were washed with Ca2+ - and Mg2+ -free PBS once and then harvested with lysis buffer A [10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 0.25% (v/v) NP40] supplemented with 1 mM dithiothreitol and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 5 μM pepstatin, 10 μM leupeptin, and 1 mM sodium vanadate). After centrifugation at 4°C, the precipitate containing nuclear proteins was resuspended in lysis buffer B [50 mM HEPES-KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl2, and 2% (v/v) glycerol] supplemented with 1 mM dithiothreitol and the protease inhibitors and then subjected to Gel Shift Assay Systems (Promega) with AP-1 and NF-κB consensus oligonucleotides according to the manufacturer’s instructions. Briefly, nuclear extract (30 μg) was preincubated at room temperature for 10 min in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% (v/v) glycerol, 0.05 mg/ml poly(deoxyinosinic-deoxycytidylic acid) in the presence or the absence of a 100-fold excess of unlabelled oligonucleotide for AP-1 (5′-CCGTGTAGATGTCGGCGGA-3′) or NF-κB (5′-AGTGAGGCGATTCCACGCGG-3′). The reaction mixture was incubated with 32P-labeled oligonucleotides of AP-1 or NF-κB for 20 min at room temperature and then electrophoresed on 6% (w/v) nondenaturing polyacrylamide gel. The gel was rinsed with TBE buffer (89 mM Tris, 89 mM boric acid, and 1 mM EDTA), dried, and exposed to X-ray film at ~80°C.

**Statistical Analysis.** Data were analyzed by Student’s t test; P < 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

**Nobiletin Inhibits in Vitro Tumor Invasion.** We examined the effect of nobiletin on the invasiveness of HT-1080 cells using Matrigel-coated insert chambers. As shown in Fig. 1, nobiletin inhibited the invasive activity of HT-1080 cells in a dose-dependent manner (52% inhibition at 64 μM). In addition, the number of invading cells decreased by exogenously adding human recombinant TIMP-1 and TIMP-2 (41 and 52% inhibition, respectively), indicating that the invasive activity of HT-1080 cells was dependent on MMP activity. We further confirmed that the inhibition of tumor cell invasiveness was not attributable simply to the cytotoxicity of nobiletin (data not shown), as was confirmed by examining living cells using the Alamer Blue assay (22), and that nobiletin did not interfere with the adhesion of HT-1080 cells to ECM components such as type IV collagen and fibronectin (data not shown). These results, therefore, suggest that nobiletin exerts an anti-invasive action by a mechanism in which the proteolytic activity of MMP may be inhibited in HT-1080 cells.

**Nobiletin Suppresses the Production of MMP-1 and MMP-9 and Augments the Production of TIMP-1.** To clarify the anti-invasive action mechanism of nobiletin observed in the in vitro invasion model with Matrigel, we examined the effect of nobiletin on the production of MMPs and TIMPs in HT-1080 cells. As shown in Fig. 2B, the TPA-induced production of proMMP-9 was suppressed by nobiletin in a dose-dependent manner; 70% inhibition was observed at 64 μM. The nobiletin-mediated suppression was attributable to a decrease in the steady-state level of proMMP-9 mRNA (50% inhibition; Fig. 3B). The TPA-induced production and gene expression of proMMP-1 was also inhibited by nobiletin in a dose-dependent manner, but the suppressive effect (30% inhibition at 64 μM at both the protein and gene levels) was less than that for proMMP-9 (Figs. 2A and 3A). However, nobiletin did not influence the expression of proMMP-1 and proMMP-9 in unstimulated HT-1080 cells (Fig. 3). In addition, HT-1080 cells constitutively expressed proMMP-2, MT1-MMP, and TIMP-2, and nobiletin did not alter their expression and the activation of proMMP-2 in HT-1080 cells treated with or without TPA (Fig. 4). On the other hand, the TPA-induced production of TIMP-1 was transcriptionally enhanced by nobiletin in a dose-dependent manner; TIMP-1 production and gene expression were increased 2.8- and 1.3-fold, respectively, at 64 μM (Figs. 2C and 3C). Therefore, these results suggest that nobiletin exerts different effects on the expression of MMPs and TIMPs in HT-1080 cells.

![Fig. 1. Nobiletin suppresses tumor cell invasion in vitro. HT-1080 cells were treated with nobiletin (8–64 μM), recombinant human TIMP-1 (TIMP-1; 100 ng/ml) or recombinant human TIMP-2 (TM2; 100 ng/ml) for 24 h in the Matrigel invasion model as described in "Materials and Methods." The number of invading cells on the lower side in 10 randomly chosen areas/membrane was visually counted under a light microscope at 200-fold magnification. Three independent experiments were highly reproducible, and typical data are shown. * and **, significantly different from untreated cells (Cont; P < 0.05 and 0.01, respectively).](image_url)
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NF-κB

whether nobiletin could influence the binding activity of AP-1 and expression of MMPs and up-regulates that of TIMP-1, we examined for pro-MMP-1 (A), pro-MMP-9 (B), TIMP-1 (C), and GAPDH (D) mRNA as described in "Materials and Methods." Three independent experiments were highly reproducible, and typical data are shown.

Nobiletin Inhibits the Binding Activity of AP-1 but not of NF-κB. The expression of MPP-1, MMP-9, and TIMP-1 is regulated by several transcriptional factors such as AP-1 and NF-κB (2). Therefore, to evaluate the mechanism whereby nobiletin down-regulates the expression of MMPs and up-regulates that of TIMP-1, we examined whether nobiletin could influence the binding activity of AP-1 and NF-κB to their predicted promoter sequences. As shown in Fig. 5, the corresponding bands (69 and 90% inhibition, respectively; Fig. 5B) were not altered by nobiletin in HT-1080 cells. However, the binding activity of NF-κB was not altered by nobiletin in STP-1 stimulated HT-1080 cells. This suggests that the inhibition of AP-1 binding activity by nobiletin is at least partly involved in the suppression of the production of MMP-1 and MMP-9 in HT-1080 cells.

PI3K Inhibitor Mimics the Different Effect of Nobiletin on MMP-9 and TIMP-1 Production. To further examine whether nobiletin interferes in the intracellular signal pathway(s), we investigated the effect of kinase inhibitors on MMP-9 and TIMP-1 production to mimic the actions of nobiletin in HT-1080 cells. As shown in Fig. 6A, a PI3K inhibitor, LY-294002, was found to mimic the divergent action of nobiletin; it inhibited the production of pro-MMP-9 and augmented that of TIMP-1 in a dose-dependent manner (58% inhibition and a 2.8-fold increase, respectively, at 10 μM). We also demonstrated that a mitogen-activated protein/extracellular signal-regulated kinase inhibitor, PD98059, inhibited both the production of pro-MMP-9 and that of TIMP-1 (69 and 90% inhibition, respectively; Fig. 6B). However, a potent inhibitor of p38 kinase, SB202190, did not influence the expression of pro-MMP-9 and TIMP-1 in TPA-stimulated HT-1080 cells (Fig. 6C). Therefore, these results suggest that nobiletin may interfere at least in the PI3K pathway, which is involved in the divergent control of MMP and TIMP-1 expression.

DISCUSSION

Medicinal plants are well known to contain therapeutic components such as flavonoids, which possess various pharmacological effects in vivo and in vitro (15–21). Recently, we characterized a novel chondroprotective action of nobiletin, which is a polymethoxy flavonoid from C. depressa, i.e., found that it suppresses the production of pro-MMP-1, pro-MMP-3, and pro-MMP-9 in rabbit articular chondrocytes and synoviocytes (22). In the present study, we demonstrated that nobiletin inhibited the invasion of HT-1080 cells in a Matrigel invasion model, and this inhibition may depend on a decrease in MMP activity. In addition, the action of nobiletin in preventing tumor cell invasion was observed in peritoneal dissemination of human gastric carcinoma in severe combined immunodeficient mice in vivo (29). Moreover, Kangaswami et al. (15) reported that citrus flavonoids including nobiletin have antiproliferative effects on human squamous carcinoma cells. Taken together, these results suggest that nobiletin has both antiproliferative and anti-invasive effects and is an attractive candidate for cancer therapy.

The augmented expression of MMPs such as MMP-1, MMP-2, MMP-3, and MMP-9 has been implicated in the metastatic phenotype of many cancers in vivo and in vitro (1, 2, 4–7), and the suppression of expression and activity of the MMPs is considered to contribute to...
the prevention of tumor progression and spreading. We demonstrated that nobiletin suppressed the gene expression and production of proMMP-9 and proMMP-1 in TPA-stimulated HT-1080 cells, whereas its effect on the expression of proMMP-2 and MT1-MMP was negligible. Baldyuk et al. (30) reported that the in vitro invasion of human breast carcinoma MDA-MB-231 is dependent on the augmentation of MMP-9 expression induced by cell contact with Matrigel, assuming that the in vitro invasive activity of HT-1080 cells is attributable to an MMP-9-dependent penetration by interacting with Matrigel. Moreover, we demonstrated that a similar suppression of MMP-9 expression was observed in peritoneal dissemination of human gastric carcinoma in severe combined immunodeficient mice in vivo (29). On the other hand, it is of interest that nobiletin transcriptionally enhanced the expression of TIMP-1 in TPA-stimulated HT-1080 cells. The augmentation of TIMP-1 expression results in the inhibition of tumor invasion in vivo and in vitro by decreasing the overall MMP activity (10–12). Thus, these observations suggest a possible mechanism in that the anti-invasive action of nobiletin may be attributable not only to the down-regulation of MMP-9 production but also the up-regulation of TIMP-1 production.

Prominent flavonoids, quercetin and genistein, have been reported to exert an antitumorigenic effect on malignant tumors (17–20). In addition, genistein has been reported to suppress the expression of MT1-MMP and MMP-9 in human breast carcinoma cells (19, 31). Recently, Huang et al. (20) reported that quercetin suppresses the epidermal growth factor-induced production of MMP-2 and MMP-9 in human squamous carcinoma A431 cells. These results are similar to our findings, but the suppressive actions of nobiletin seem to be distinguishable from the effects of genistein and quercetin. In the present study, we found that nobiletin suppressed the expression of proMMP-9 more than that of proMMP-1 but did not inhibit the expression of proMMP-2 and MT1-MMP. Similar results were obtained in A431 cells (data not shown). Thus, it is likely that nobiletin may predominantly act as a potent suppressor of MMP-9 expression in tumor cells.

The expression of MMPs and TIMPs is divergently regulated by the transcriptional activation of their genes, the promoter sequences of which contain putative binding sites for AP-1, NF-κB, and PEA3 (2). The promoter activity of the MMP-9 gene is induced by AP-1 and/or NF-κB (32, 33), whereas AP-1 and/or PEA3 are required for stimulation of the transcription of MMP-1 and MMP-3 (34, 35). The gel shift assay in this study showed that nobiletin inhibited the binding activity of AP-1 but not NF-κB, suggesting that nobiletin suppresses the transcription of the MMP-1 and MMP-9 genes by interfering with AP-1 binding activity. Furthermore, Murakami et al. (21) reported that nobiletin blocks lipopolysaccharide and IFN-γ-induced inducible nitric oxide synthase expression in mouse macrophage RAW264.7 cells. Because the expression of inducible nitric oxide synthase is dependent on NF-κB activation (36, 37), our evidence that nobiletin did not inhibit the binding activity of NF-κB in HT-1080 cells suggests that nobiletin may have some cell type specificity.

Similar to MMP-1 and MMP-9, TIMP-1 and TIMP-2 have AP-1 binding sites in their promoters, and the transcription of TIMP-1 is AP-1 dependent (38, 39). Therefore, it is suggested that the different responses of TIMP-1 and TIMP-2 expression to nobiletin may be attributable to the different contributions of AP-1 to the transcriptional activation (40). Furthermore, regardless of the suppression of AP-1 activity by nobiletin, the TPA-induced gene expression and production of TIMP-1 were enhanced in nobiletin-treated HT-1080 cells. The divergent regulation of transcriptional activity is likely to be controlled by an interplay among various transcriptional factors or upstream signal cascades including mitogen-activated protein kinases (41–44). We therefore speculate that the nobiletin-mediated augmentation of TIMP-1 expression may result from counterbalancing the depression of AP-1 activity by activating other crucial transcriptional factors.

It has been reported that genistein is a well-known tyrosine kinase inhibitor that blocks signal transduction pathways mediated by mitogen-activated protein kinase (45) and 1-phosphatidylinositol 4-phosphate 5-kinase (17) in various cell species. In addition, quercetin inhibits protein kinase C and/or tyrosine kinase in human HL-60 leukemia cells (18) and phosphatidylinositol kinase in human breast carcinoma cells MDA-MB-435 (16). Therefore, we speculate that nobiletin may modify the protein kinase-mediated intracellular signal pathway(s). In this regard, we demonstrated that LY-294002 mimicked the different effects of nobiletin on the regulation of proMMP-9 and TIMP-1 production in TPA-stimulated HT-1080 cells. However, PD98059 suppressed both the expression of proMMP-9 and that of TIMP-1, whereas SB202190 did not alter the production of either one. Thus, we suggest a possible mechanism in that nobiletin may interfere at least in the PI3K-mediated signal pathway(s) to induce the divergent control of MMP and TIMP-1 production.

In conclusion, we demonstrated that nobiletin inhibited an in vitro invasion of HT-1080 cells in the Matrigel model and transcriptionally down-regulated the expression of MMP-1 and MMP-9 but up-regulated that of TIMP-1, suggesting that nobiletin prevents tumor invasion not only by suppressing the production of MMPs but also by augmenting TIMP-1 production in tumor cells. These divergent actions of nobiletin are very likely to result from interference in the PI3K-mediated signal pathway(s).

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REFERENCES


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