Inhibition of Activator Protein-1 Binding Activity and Phosphatidylinositol 3-Kinase Pathway by Nobiletin, a Polyphenol 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

Takashi Sato, Leona Koike, Yoshihi Miyata, Michiko Hirata, Yoshihiro Mimaki, Yutaka Sashida, Masamichi Yano, and Akira Ito

Department of Biochemistry [T. S., L. K., Y. M., M. H., A. I.] and Medical Plant Science [Y. M., Y. S.], Tokyo University of Pharmacy and Life Science, School of Pharmacy, Tokyo 192-0392; Bio-Oriented Technology Research Advancement Institution (BRAIN), Tokyo 105-0001 [M. H.]; and Department of Citriculture, Okayama University, National Institute of Fruit Tree Science, Shizuka 424-0292 [M. Y.], Japan

ABSTRACT

Medicinal plants contain pharmacological substances including flavonoids, and their extracts have been therapeutically administered for cancer therapy in vivo and in vitro. We investigated the efficacy of a polyphenol flavonoid, nobiletin, from Citrus depressa on tumor invasion in vitro. Nobiletin inhibited the tumor-invasive activity of human fibrosarcoma HT-1080 cells in the Matrigel model, whereas a similar inhibition was observed upon exogenously adding tissue inhibitors of metalloproteinases (TIMPs)-1 and -2. The gene expression and production of promatrix metalloproteinase 9 (proMMP-9)/progelatinase B and proMMP-1/interstitial procollagenase were specifically suppressed by nobiletin in 12-O-tetradecanoylphorbol 13-acetate-stimulated HT-1080 cells. In contrast, the gene expression and production of TIMP-1, but not TIMP-2, were enhanced by nobiletin. We also demonstrated that nobiletin suppressed the 12-O-tetradecanoylphorbol 13-acetate-induced binding activity of activator protein-1. Furthermore, a phosphatidylinositol 3-kinase inhibitor, LY-294022, was found to mimic the different actions of nobiletin on the production of proMMP-9 and TIMP-1. These results suggest that nobiletin inhibits tumor cell invasive activity not only by suppressing the expression of MMPs but also augmenting TIMP-1 production in tumor cells, and that the nobiletin-mediated inhibition of activator protein-1 binding activity is at least partly involved in the suppression of MMP expression.

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 enzymic activity of MMPs is involved in the divergent regulation of proMMP-1 and TIMP-1. These results suggest that nobiletin is an attractive candidate with an effect that prevents tumor invasion, not only by suppressing MMP production but also by augmenting TIMP-1 production.

MATERIALS AND METHODS

Cell Culture and Treatment. Human fibrosarcoma HT-1080 cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in Eagle’s MEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD) and nonessential amino acids (Life Technologies, Inc., Rockville, MD). The harvested culture medium was stored at −20°C until use.

Received 7/20/01; accepted 12/14/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by a grant from private universities provided by The Promotion and Mutual Aid Corporation for Private Schools of Japan and in part by Takeda Science Foundation.

To whom requests for reprints should be addressed, at Department of Biochemistry, Tokyo University of Pharmacy and Life Science, School of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Phone: 81-426-76-5728; Fax: 81-426-76-5734; E-mail: satotak@ps.toyaku.ac.jp.

The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane type-MMP; TIMP, tissue inhibitor of metalloproteinases; PI3K, phosphatidylinositol 3-kinase; TPA, 12-O-tetradecanoylphorbol 13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AP-1, activator protein-1; NF-κB, nuclear factor-κB.
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.
Three independent experiments were highly reproducible, and typical data are shown.

Fig. 2. Effect of nobiletin on the production of proMMPs and TIMP-1 in HT-1080 cells. Confluent HT-1080 cells were treated with TPA (10 nM) and nobiletin (8–64 μM) for 24 h, and then the harvested culture medium was subjected to Western blot analysis for proMMP-1 [A], proMMP-9 [B], and TIMP-1 [C] as described in “Materials and Methods.” Three independent experiments were highly reproducible, and typical data are shown.

Fig. 3. Effect of nobiletin on the gene expression of proMMPs and TIMP-1 in HT-1080 cells. Confluent HT-1080 cells were treated with TPA (10 nM) and/or nobiletin (64 μM) for 24 h, and then the isolated mRNA (2 μg) was subjected to Northern blot analysis for proMMP-1 [A], proMMP-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 MMP-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 TPA-mediated augmentation of AP-1 binding activity was abolished by nobiletin in HT-1080 cells. However, the binding activity of NF-κB was not altered by nobiletin in TPA-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 proMMP-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 proMMP-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 proMMP-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., we found that it suppresses the production of proMMP-1, proMMP-3, and proMMP-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, Kandaswami 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).

ACKNOWLEDGMENTS

We thank Dr. H. Nagase for providing recombinant human TIMP-1 and TIMP-2, sheep anti-(human proMMP-1), anti-(human proMMP-9), and anti-(human TIMP-1) antibodies and human TIMP-1 cDNA probe.

REFERENCES


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

Takashi Sato, Leona Koike, Yoshiki Miyata, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/4/1025

Cited articles
This article cites 44 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/4/1025.full#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/4/1025.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/62/4/1025.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.