Isoginkgetin inhibits tumor cell invasion by regulating phosphatidylinositol 3-kinase/Akt–dependent matrix metalloproteinase-9 expression

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Abstract

Matrix metalloproteinase (MMP)-9 plays a key role in tumor invasion. Inhibitors of MMP-9 were screened from *Metasequoia glyptostroboides* (Dawn redwood) and one potent inhibitor, isoginkgetin, a biflavonoid, was identified. Noncytotoxic levels of isoginkgetin decreased MMP-9 production profoundly, but up-regulated the level of tissue inhibitor of metalloproteinase (TIMP)-1, an inhibitor of MMP-9, in HT1080 human fibrosarcoma cells. The major mechanism of Ras-dependent MMP-9 production in HT1080 cells was phosphatidylinositol 3-kinase (PI3K)/Akt/nuclear factor-κB (NF-κB) activation. Expression of dominant-active H-Ras and p85 (a subunit of PI3K) increased MMP-9 activity, whereas dominant-negative forms of these molecules decreased the level of MMP-9. H-Ras did not increase MMP-9 in the presence of a PI3K inhibitor, LY294002, and a NF-κB inhibitor, SN50. Further studies showed that isoginkgetin regulated MMP-9 production via PI3K/Akt/NF-κB pathway, as evidenced by the findings that isoginkgetin inhibited activities of both Akt and NF-κB. PI3K/Akt is a well-known key pathway for cell invasion, and isoginkgetin inhibited HT1080 tumor cell invasion substantially. Isoginkgetin was also quite effective in inhibiting the activities of Akt and MMP-9 in MDA-MB-231 breast carcinomas and B16F10 melanoma. Moreover, isoginkgetin treatment resulted in marked decrease in invasion of these cells. In summary, PI3K/Akt is a major pathway for MMP-9 expression and isoginkgetin markedly decreased MMP-9 expression and invasion through inhibition of this pathway. This suggests that isoginkgetin could be a potential candidate as a therapeutic agent against tumor invasion. [Mol Cancer Ther 2006;5(11):2666–75]

Introduction

Throughout the cancer progression, the microecology of the local host tissue is a consistently active participant in the evolving tumor. Invasion occurs at the tumor-host interface, where the tumor and stromal cells exchange enzymes and cytokines that modulate the local extracellular matrix and stimulate cell migration (1). For tumor invasion and migration, the tumor cells and host coordinately regulate matrix degradation, cell-cell attachments, and cell-matrix attachment. The rate-limiting step of the invasion is the breakdown of connective tissue barriers, extracellular matrix, which comprise collagens, proteoglycan, elastin, laminin, and fibronectin (2). The main groups of proteolytic enzymes involved in the extracellular matrix degradation are matrix metalloproteinases (MMP) and zinc-dependent proteinases. MMPs are synthesized as preproenzymes and most are secreted from the cells as proenzymes. Among previously reported human MMPs, MMP-9 (gelatinase B/M_r 92,000 type IV collagenase) is thought to be a key enzyme for degrading type IV collagen, which is a major component of the basement membrane (2, 3). MMP-9 is abundantly expressed in various malignant tumors and contributes to invasion and metastasis (4, 5). Therefore, inhibition of MMP-9 activity could affect tumorigenesis in many ways, including the inhibition of invasion, metastasis, and angiogenesis (6).

Both naturally occurring and synthetic MMP inhibitors have been identified and characterized. The most studied physiologic inhibitors of MMPs are tissue inhibitor of metalloproteinases (TIMP). TIMPs inhibit the MMPs in a 1:1 stoichiometric fashion. Among several TIMPs, TIMP-1 binds the hemopexin domain of MMP-9. Through the inhibition of MMPs, TIMPs can block the degradation of the extracellular matrix, tumor invasion, and metastasis (7, 8). In addition, they inhibit the release of angiogenic factors from the matrix, block endothelial cell migration (9), and inhibit the formation of endothelial tube *in vitro* (10). Most synthetic MMP inhibitors target the catalytic site of the MMPs and act by chelating the catalytically essential zinc ion. In clinical trials, however, many direct MMP inhibitors have not been successful in treating cancer. Therefore, therapeutic inhibition of MMPs may be achieved by other indirect means, such as targeting the signal transduction pathways that regulate MMPs expression. In this direction, we screened MMP-9 inhibitors from

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Metasequoia glyptostroboides (Dawn redwood, family: Taxodiaceae). Fruits, vegetables, common beverages, and several herbs and plants with diversified pharmacologic properties have been shown to be rich sources of microchemicals with cancer preventive and other therapeutic effects in humans (11). Among these, naturally occurring flavonoids and isoflavones, such as genistein, epigallocatechin 3-gallate, silymarin, etc., have received increasing attention in recent years (12).

The key signaling pathway that regulate tumor cell invasion is phosphatidylinositol 3-kinase (PI3K)/Akt pathway (13). PI3K/Akt pathway can be activated by Ras and growth factors/growth factor receptors. About 30% of tumors contain constitutively active mutant Ras and most tumor cells express excessive growth factors. In addition, growth factor receptors can be autoactivated even without growth factors in tumors. Some tumors also have somatic mutation and biallelic inactivation of PTEN tumor suppressor, the negative regulator of PI3K/Akt pathway (14). Therefore, PI3K/Akt pathway is constitutively active in most tumors. In addition to its role in tumor cell invasion, this pathway also regulates many cellular processes implicated in tumorigenesis, cell size/growth, proliferation, survival, glucose metabolism, genome stability, metastasis, and angiogenesis (13). In this study, we screened inhibitors of MMP-9 and found one biflavonoid, isoginkgetin. Further mechanistic studies revealed that isoginkgetin potently inhibited MMP-9 expression and tumor cell invasion through the inhibition of Ras/PI3K/Akt/nuclear factor-κB (NF-κB) pathway.

Materials and Methods

Screening, Extraction, and Purification of MMP-9 Inhibitors

The leaves of *M. glyptostroboides* (Dawn redwood, family: Taxodiaceae) were washed with water and dried in the dark. The components of leaves were extracted with various solutions, and active fraction was further fractionated using the methods as shown in Fig. 1. Among several fractions, ethyl acetate fraction was the most active for inhibiting MMP-9 activity. This fraction was further fractionated by TLC (Merck, Darmstadt, Germany), silica gel column chromatography (230–400 mesh, Merck), Sephadex LH-20 column chromatography (Amersham Pharmacia Biotech, Piscataway, NJ), and HPLC (YMC Co., Kyoto, Japan). With each step of these purifications, cells were treated with fractions for 24 hours and conditioned medium was collected for MMP-9 assay. MMP-9 activity was measured using zymography and fluorogenic MMP-9 substrates, and active fraction was collected and subsequently fractionated. The most potent MMP-9 inhibitor was selected and analyzed by nuclear magnetic resonance and mass spectrophotometry. It turned out to be isoginkgetin. The purity of isoginkgetin was >95%. For further studies, isoginkgetin was provided by Dr. Hyun Po Kim (Kangwon National University, Gangwon-do, Republic of Korea).

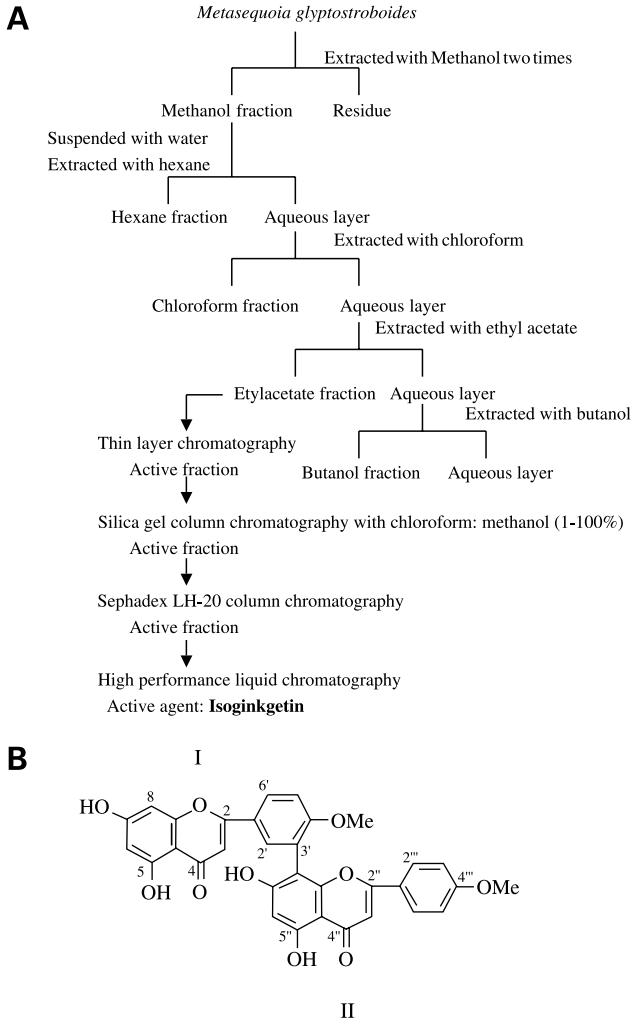

Figure 1. Screening of MMP-9 inhibitors. **A**, inhibitors of MMP-9 were screened from *M. glyptostroboides*. At each step, cells were treated with compounds for 24 h and conditioned medium was collected. MMP-9 activity was measured using zymography and fluorogenic MMP-9 substrate. **B**, structure of isoginkgetin.

Cells and Reagents

HT1080 fibrosarcoma cells and MDA-MB-231 breast carcinoma cells were maintained in DMEM supplemented with 10 mmol/L HEPES, 50 mg/L gentamicin, and 10% heat inactivated fetal bovine serum. B16F10 melanoma cells were grown in RPMI 1640 supplemented with 10 mmol/L HEPES, 50 mg/L gentamicin, and 10% heat inactivated fetal bovine serum. SP600125, SB203580, FPTI III, PD98059, LY294002, and MMP-9 inhibitor were purchased from Calbiochem (San Diego, CA). Gelatin was obtained from Sigma (St. Louis, MO) and Matrigel was purchased from Becton Dickinson (Bedford, MA).

Zymography

All experiments, including zymography, were done in the absence of serum. Enzymatic activity of MMP-9 was assayed by gelatin zymography. Samples were

electrophoresed on a gelatin containing 10% SDS-polyacrylamide gel. After electrophoresis, the gel was washed twice with washing buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 2.5% Triton X-100], followed by a brief rinsing in washing buffer without Triton X-100. The gel was incubated with incubation buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L $CaCl_2$, 0.02% NaN_3, and 1 μmol/L $ZnCl_2$] at 37°C. After incubation, the gel was stained and destained. In this gel, a clear zone of gelatin digestion indicates the presence of MMP-9.

MMP-9 Activity Assay

MMP-9 activity was measured using fluorogenic MMP-9 substrate [*N*-(2,4-dinitrophenyl)-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg amide; Sigma]. Briefly, conditioned medium was mixed with substrate and incubated at 37°C. Fluorescence was measured by using a fluorometer, according to the instruction from the manufacturer.

Cell Death Assays

Cells were washed once with medium, once with PBS, once with Annexin V–FITC buffer [10 mmol/L HEPES-NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$], and then incubated for 15 minutes at room temperature with 5 μg/mL Annexin V–FITC (Biosource International, Camarillo, CA). After washing once with Annexin V buffer, the samples were resuspended in the same buffer. Immediately before analysis, 5 μg/mL propidium iodide (Biosource International) were added to distinguish apoptotic cells from necrotic cells, and the cells were analyzed using a flow cytometer.

Invasion Assays

The lower and upper parts of Transwell (Corning Costar, Acton, MA) were coated with 10 μL of type I collagen (0.5 mg/mL) and 20 μL of 1:2 mixture of Matrigel/DMEM, respectively. Cells (5×10^4 per chamber) were plated in the upper chamber. The lower chambers contained 0.1 mg/mL bovine serum albumin. The inserts were incubated for 18 hours at 37°C. The cells that had invaded the lower surface of the membrane were fixed with methanol and stained with H&E. The number of cells that had invaded was quantified by counting 10 random fields using ×20 objective of light microscope.

Plasmids

MMP-9 promoter region (−670 to +3) was PCR amplified and inserted upstream of the pGL3 luciferase basic vector (Promega, Madison, WI). NF-κB reporter plasmids were purchased from Clontech (Palo Alto, CA). pGL3-TIMP-1 promoter-reporter and pECE-MyrAkt (myristoylated Akt) were provided by Dr. Ian M. Clark (University of East Anglia, Norwich, United Kingdom) and Dr. Thomas F. Franke (Columbia University, New York, NY), respectively. H-Ras constructs were gifts from Dr. Kun-Liang Guan (University of Michigan, Ann Arbor, MI).

Luciferase Assay

Cells were cotransfected with an indicated construct, a luciferase reporter plasmid, and a control renilla luciferase construct using LipofectAMINE 2000. After 36 hours, luciferase activity was measured according to the instruction from the manufacturer (Promega) using a luminometer.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was purified from cultured cells using TRI reagent (Molecular Research Center). For Northern blot analysis, 15 μg RNA were electrophoresed on 1% agarose gels containing 37% formaldehyde, and transferred to Hybond-N membranes (Amersham Pharmacia Biotech) by capillary transfer. Membrane was fixed using an optimized UV cross-linking procedure. Prehybridization and hybridization were done at 68°C in ExpressHyb hybridization solution (Clontech). cDNA probes for MMP-9, TIMP-1, and glyceraldehyde-3-phosphate dehydrogenase were labeled with [^{32}P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) by using a random primer kit (TakaRa, Shiga, Japan). The blot was then washed twice with 2× SSC [300 mmol/L NaCl, 30 mmol/L sodium citrate (pH 7.0)] containing 0.05% SDS at 25°C and with 0.1× SSC containing 0.1% SDS at 55°C in order, and autoradiographed at −70°C.

Akt Kinase Assay

Cells were transfected with hemagglutinin-tagged Akt plasmids and lysed in a buffer solution containing 20 mmol/L Tris-HCl (pH 7.5), 12 mmol/L β-glycerophosphate, 150 mmol/L NaCl, 5 mmol/L EGTA, 10 mmol/L NaF, 1% Triton X-100, 0.5% deoxycholate, 3 mmol/L DTT, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1.5% aprotinin. Cell extracts were centrifuged, and 200 μg supernatants were immunoprecipitated with hemagglutinin antibody and protein A-agarose. The beads were washed three times with a solution containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EGTA, 2 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride. Akt activities were assayed in a reaction mixture consisting of 1 μg histone H2B (*in vitro* substrate for Akt), 2 μg PKI, 5 μmol/L ATP, and 5 μCi [γ-^{32}P]ATP in a buffer [20 mmol/L HEPES (pH 7.2), 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, 1 mmol/L DTT, and 0.2 mmol/L EGTA] at 30°C for 20 minutes. Protein kinase assays were terminated by the addition of SDS sample buffer, and the samples were subjected to SDS-PAGE. Phosphorylated proteins were visualized by autoradiography.

Immunoblot Analysis

Cells were extracted in a buffer [20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L EGTA, 10% glycerol, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 1 mg/mL pepstatin], and samples were resuspended in reducing buffer [5×: 60 mmol/L Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mmol/L 2-mercaptoethanol, and 0.1% bromophenol blue]. These were boiled for 5 minutes and electrophoresed by SDS-PAGE. Proteins were then transferred to Hybond-ECL (Amersham Biosciences, Piscataway, NJ). Blots were blocked with a TBST [25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.05% Tween 20] containing 5% nonfat dried milk or 5% bovine serum

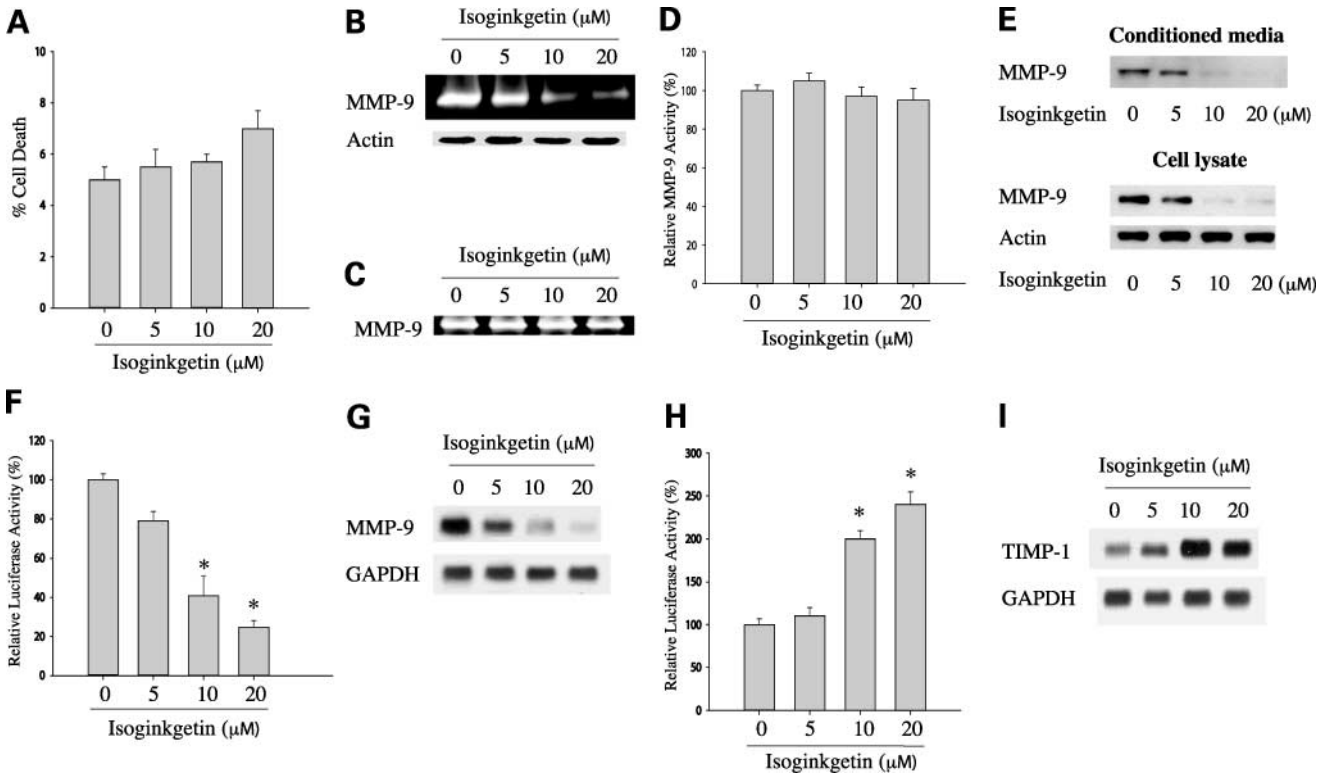

Figure 2. Isoginkgetin inhibits expression of MMP-9, whereas it increases TIMP-1 expression. **A,** cells were treated with the indicated concentration of isoginkgetin for 24 h. Cell death was assessed using Annexin-FITC as described in Materials and Methods. *Columns,* mean of three independent experiments; *bars,* SD. **B,** cells were treated with the indicated concentration of isoginkgetin for 24 h and conditioned medium was collected. MMP-9 activity was measured by gelatin zymography. Actin was detected in total cell lysates by immunoblot analysis to normalize protein loadings. **C** to **D,** cells were incubated in serum-free medium for 24 h and conditioned medium was collected. Isoginkgetin was incubated with conditioned medium for 3 h at 37°C. MMP-9 activity was measured using gelatin zymography (**C**) and fluorogenic MMP-9 substrate (**D**). **E,** cells were treated with the indicated concentration of isoginkgetin for 24 h, and conditioned medium was collected and concentrated. Cells were lysed with lysis buffer. MMP-9 in conditioned medium and in cells was identified by immunoblot analysis using anti-MMP-9 antibody. **F,** cells were cotransfected with a MMP-9 promoter – containing reporter construct and a control renilla luciferase reporter vector. After 24 h, cells were treated with the indicated concentration of isoginkgetin for another 24 h in the absence of serum. Cells were lysed and luciferase activity was measured. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test. **G,** cells were treated with isoginkgetin for 24 h, and mRNA level of MMP-9 was detected by Northern blot analysis. **H,** cells were cotransfected with a TIMP-1 promoter – containing reporter construct and a control renilla luciferase reporter vector. After 24 h, cells were treated with the indicated concentration of isoginkgetin for another 24 h. Cells were lysed and luciferase activity was measured. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*$P < 0.01$) using Student's *t* test. **I,** cells were treated with isoginkgetin for 24 h, and the mRNA level of TIMP-1 was detected by Northern blot analysis.

albumin and probed with primary antibodies for overnight and secondary antibodies coupled to peroxidase for 1 hour. Blots were developed using the enhanced chemiluminescence system (Amersham Biosciences).

Results

Isoginkgetin Inhibits MMP-9 Activities

Given the important roles of MMP-9 activity in tumor cell invasion, MMP-9 inhibitors were screened from the leaves of *M. glyptostroboides.* Throughout the screening, cells were treated with each fraction for 24 hours and conditioned medium was collected. MMP-9 activity assay was done using fluorogenic MMP-9 substrate and zymography (Fig. 1A). The most potent inhibitor of MMP-9 was selected and it was identified as isoginkgetin (Fig. 1B). For further studies, isoginkgetin was obtained from Dr. Hyun Po Kim.

We first examined the cytotoxicity of isoginkgetin. HT1080 fibrosarcoma cells exhibited no significant increase in death rate by the treatment with 5 to 20 μmol/L of isoginkgetin for 24 hours (Fig. 2A). To confirm the effect of isoginkgetin on MMP-9 activity, cells were treated with isoginkgetin for 24 hours and gelatin zymography was done. HT1080 cells secreted high levels of MMP-2 and MMP-9. MMP-9 bands were confirmed by size markers (data not shown). As shown in Fig. 2B, isoginkgetin decreased MMP-9 activity. The β-actin immunoblot of cells showed that equal numbers of cells were present during the conditioning of the medium. To assess the possibility that isoginkgetin may interact with and inhibit MMP-9 directly, conditioned medium was incubated with isoginkgetin, and MMP-9 activity was determined by zymography (Fig. 2C) and fluorogenic substrates of MMP-9 (Fig. 2D).

As shown in Fig. 2C and D, activity of MMP-9 in conditioned medium was not changed by isoginkgetin.

Isoginkgetin Decreases MMP-9 Expression but Increases TIMP-1 Expression

The data shown in Fig. 2B to D suggest that isoginkgetin may inhibit MMP-9 activity by regulating MMP-9 expression. To evaluate this possibility, cells were treated with isoginkgetin, and conditioned medium was collected and concentrated for MMP-9 immunoblot analysis. As shown in Fig. 2E, isoginkgetin decreased MMP-9 level. Isoginkgetin treatment also decreased cellular MMP-9 level as determined by immunoblot analysis of cell lysate (Fig. 2E). We next examined the changes of MMP-9 transcription. For this, cells were transfected with MMP-9 promoter containing reporter constructs and treated with isoginkgetin. As shown in Fig. 2F, isoginkgetin profoundly inhibited MMP-9 promoter activity. Given the fact that promoter activity reflects transcriptional activity, the mRNA level of MMP-9 was detected by Northern blot analysis. Indeed, isoginkgetin decreased MMP-9 mRNA (Fig. 2G). We next examined the effect of isoginkgetin on the promoter activity and mRNA level of TIMP-1, an inhibitor of MMP-9, using TIMP-1 promoter containing reporter constructs and Northern blot analysis, respectively. In contrast to MMP-9, both TIMP-1 promoter activity (Fig. 2H) and mRNA level (Fig. 2I) were increased by isoginkgetin.

Ras/PI3K/Akt/NF-κB Is the Major Pathway for MMP-9 Expression in HT1080 Cells

The finding in Fig. 2 led us to the hypothesis that isoginkgetin may regulate signaling molecules that control MMP-9 expression and eventually total activity. To identify which signal pathways are responsible for MMP-9 activity, HT1080 cells were treated with various signaling molecule inhibitors and zymography was done. The inhibitory

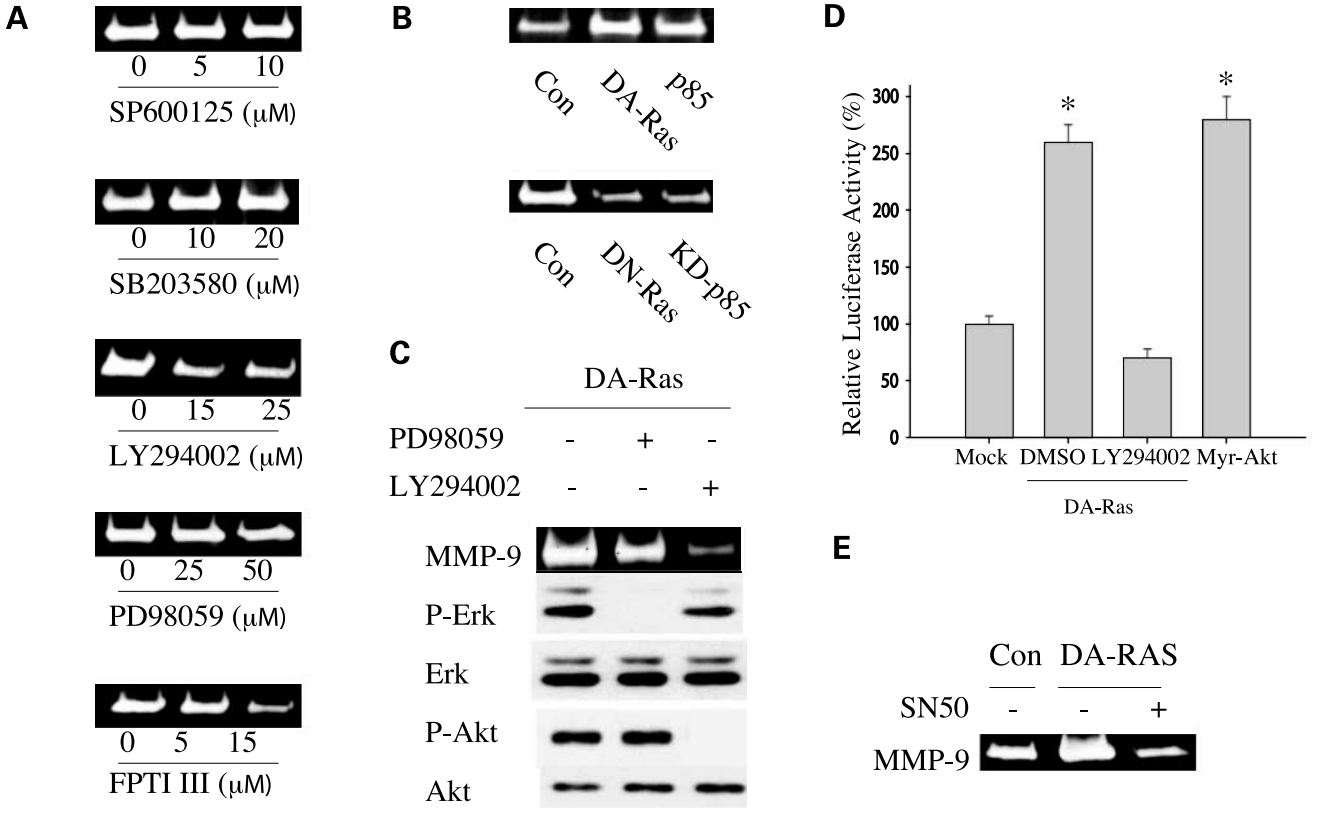

Figure 3. PI3K/Akt/NF-κB is the major pathway for MMP-9 expression in HT1080 cells. **A,** cells were treated with the indicated concentration of SP600125, SB203580, LY294002, PD98059, and FPTI III. After 24 h, conditioned medium was collected and zymography was done. **B,** cells were transfected with control, dominant-active H-Ras, p85, dominant-negative H-Ras, or dominant-negative p85. After 24 h, cells were washed and incubated in serum-free medium for 24 h. MMP-9 activity was measured by zymography. **C,** cells were treated with either 30 μmol/L PD98059 or 25 μmol/L LY294002 for 24 h. Conditioned medium was collected and gelatin zymography was done. Phospho-ERK, ERK, phospho-Akt, and Akt levels were detected in total cell lysates by immunoblot analysis. **D,** cells were cotransfected with a luciferase construct driven by NF-κB–binding sites, a control renilla luciferase reporter vector, and either dominant-active H-Ras or myr-Akt. After 24 h, cells were washed and incubated in serum-free medium for another 24 h. For LY294002 treatment, washed cells were incubated for 12 h in serum-free medium and treated with 25 μmol/L LY294002 for another 12 h. Cells were lysed and luciferase activity was measured. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test. **E,** cells were transfected with either control or dominant-active H-Ras. After 24 h, cells were treated with a NF-κB–specific inhibitor (SN50) or control peptide (SN50M) for another 24 h. Conditioned medium was collected and gelatin zymography was done.

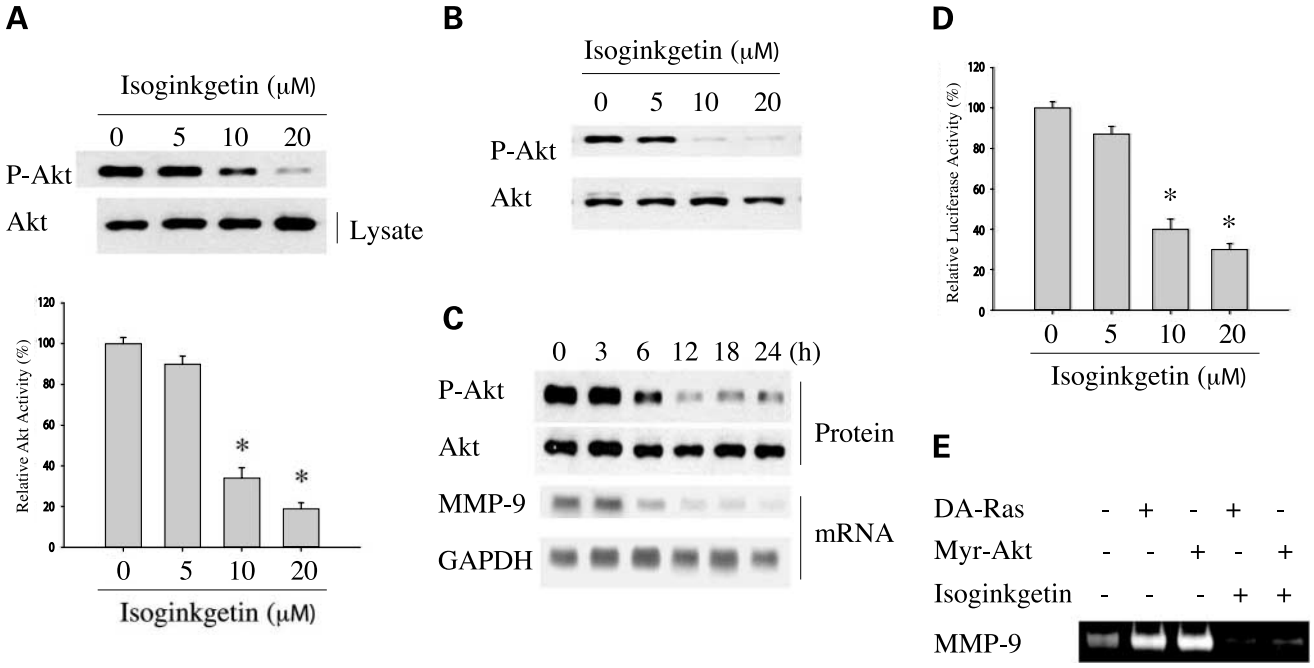

Figure 4. Isoginkgetin inhibits the activities of Akt and NF-κB. **A,** cells were treated with isoginkgetin and Akt activity was measured as described in Materials and Methods. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test. **B,** cells were treated with isoginkgetin for 24 h. The expression of phospho-Akt and Akt was detected by immunoblot analysis. **C,** cells were treated with 20 μmol/L isoginkgetin for indicated times. The protein levels of phospho-Akt and Akt were detected by immunoblot analysis and the levels of mRNA of MMP-9 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were detected by Northern blot analysis. **D,** cells were cotransfected with a luciferase construct driven by NF-κB–binding sites and a control renilla luciferase reporter vector. After 24 h, cells were treated with the indicated concentration of isoginkgetin for another 24 h in the absence of serum. Cells were lysed and luciferase activity was measured. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test. **E,** cells were transfected with either dominant-active H-Ras or myr-Akt. After 24 h, cells were treated with isoginkgetin for another 24 h in the absence of serum. Conditioned medium was collected and gelatin zymography was done.

concentrations of these inhibitors against the kinases were first determined by kinase assay and immunoblot analysis (data not shown). As shown in Fig. 3A, treatment with FPTI III (Ras processing inhibitor; ref. 15) and LY294002 (PI3K inhibitor; ref. 16) exhibited decreased MMP-9 activity. However, SP600125 (c-Jun-NH_2-kinase inhibitor; ref. 17), PD98059 (mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase–ERK pathway inhibitor; ref. 18), and SB203580 (p38 inhibitor; ref. 19) did not change MMP-9 activity. Based on these findings, we further examined the effects of H-Ras and PI3K on the MMP-9 activity. As shown in Fig. 3B, expression of dominant-active H-Ras and p85, a subunit of PI3K, increased MMP-9 activity. In contrast, dominant-negative H-Ras and kinase-dead p85 decreased MMP-9 activity. To evaluate whether H-Ras increased MMP-9 activity through PI3K, we expressed dominant-active H-Ras and treated cells with LY294002 and PD98059. Treatment of cells with LY294002 resulted in decreased phospho-Akt level as well as MMP-9 activity (Fig. 3C). However, PD98059 did not inhibit H-Ras–induced MMP-9 activity. This suggests that H-Ras increases MMP-9 activity through PI3K. Based on our previous reports that Akt increases MMP-9 activity through NF-κB–dependent pathway in HT1080 cells (20), we examined the possibility that H-Ras may increase MMP-9 activity through Akt/NF-κB–dependent pathway. Indeed, expression of dominant-active H-Ras and constitutively active Akt (myr-Akt) increased NF-κB activity, whereas H-Ras did not increase NF-κB activity in the presence of PI3K inhibitor, LY294002 (Fig. 3D). In addition, H-Ras expression did not increase MMP-9 activity in the presence of the NF-κB inhibitor, SN50 (Fig. 3E).

Isoginkgetin Inhibits MMP-9 Expression through the Inhibition of PI3K/Akt/NF-κB Pathway in HT1080 Cells

Based on the findings that isoginkgetin inhibited MMP-9 expression, and PI3K/Akt/NF-κB is the major pathway of H-Ras–induced MMP-9 activity, we hypothesized that isoginkgetin may regulate this pathway. This hypothesis was evaluated by assessing the effect of isoginkgetin on the activities of Akt and NF-κB. Isoginkgetin dramatically decreased Akt activity (Fig. 4A) and phosphorylation of Akt on serine 473 (Fig. 4B) as determined by Akt kinase assay and immunoblot analysis with a phosphospecific antibody, respectively. We next examined the time course effect of isoginkgetin on phospho-Akt and MMP-9 expression. As shown in Fig. 4C, isoginkgetin decreased phospho-Akt protein level and MMP-9 mRNA level around 6-hour time point. In addition, NF-κB activity was also inhibited by isoginkgetin (Fig. 4D). We next investigated the effects of H-Ras and Akt on MMP-9 activity in the presence of

isoginkgetin. Cells transfected with dominant-active H-Ras and myr-Akt did not exhibit increased MMP-9 activity in the presence of isoginkgetin (Fig. 4E).

Isoginkgetin Inhibits Carcinoma Invasion

To evaluate the contribution of Ras/PI3K/NF-κB pathway to the cell invasion, we did invasion assay in the presence of the inhibitors of these signaling molecules. Treatment of cells with Ras inhibitor (FPTI III), PI3K pathway inhibitor (LY294002), and NF-κB inhibitor (SN50) resulted in marked decrease in cell invasion (Fig. 5A). Given that MMP-9 is involved in cell invasion, we assessed the effect of Ras/Akt–induced MMP-9 activity on the invasion of cells. The cells transfected with dominant-active H-Ras, myr-Akt, and p65 (a subunit of NF-κB) were treated with MMP-9 inhibitor, and invasion assay was done. In the presence of MMP-9 inhibitor, the ability of cells to penetrate Matrigel was dramatically decreased (Fig. 5B), indicating that MMP-9 is important for Ras/Akt/NF-κB-induced cell invasion. We next examined the effect of isoginkgetin on cell invasion. As shown in Fig. 5C, cells that were treated with isoginkgetin exhibited marked decrease in invasion. Moreover, dominant-active H-Ras, myr-Akt, and p65 overexpression did not increase cell invasion in the presence of isoginkgetin (Fig. 5D).

Isoginkgetin Inhibits MMP-9 Activity and Invasion of Other Cells

To evaluate the effect of isoginkgetin on the MMP-9 activity and invasion in other cells, we used MDA-MB-231 breast carcinoma and B16F10 melanoma cells. Both MMP-9 activities (Fig. 6A) and invasive abilities (Fig. 6B) were decreased substantially by isoginkgetin. MMP-9 inhibitor was also effective at inhibiting the invasion of these cells (Fig. 6C). The ability of isoginkgetin to influence Akt activity was investigated by immunoblot analysis. As shown in Fig. 6D, isoginkgetin decreased phosphorylation of Akt on serine 473. Further studies exhibited that PI3K pathway is important for invasion of these cells, as evidenced by the finding that LY294002 profoundly decreased invasion of these cells (Fig. 6E).

Discussion

Isoginkgetin is one of the biflavonoids that are the unique and minor classes of flavonoids distributed mainly among several plant families. Although isoginkgetin was isolated from *M. glyptostroboides* in our studies, it was mostly purified from *Ginkgo biloba*, a ginkgo tree. Ginkgo has been used for medical applications to promote blood circulation, alleviate allergy reactions, reduce peripheral arterial occlusion and heart attack, and counteract a variety of disorders, such as Alzheimer's disease, depression, short-term memory loss, and hearing loss (21). Modern work with flavonoids, the components that affect blood circulation, was initiated by the isolation of ginkgetin, but there are only a few reports on the effects of ginkgetin and isoginkgetin on cell physiology. Ginkgetin has been shown to decrease cyclooxygenase (COX)-2 expression, and consequently, inhibits COX-2–dependent phases of prostaglandin D2 generation and leukotriene C4 (22). Ginkgetin

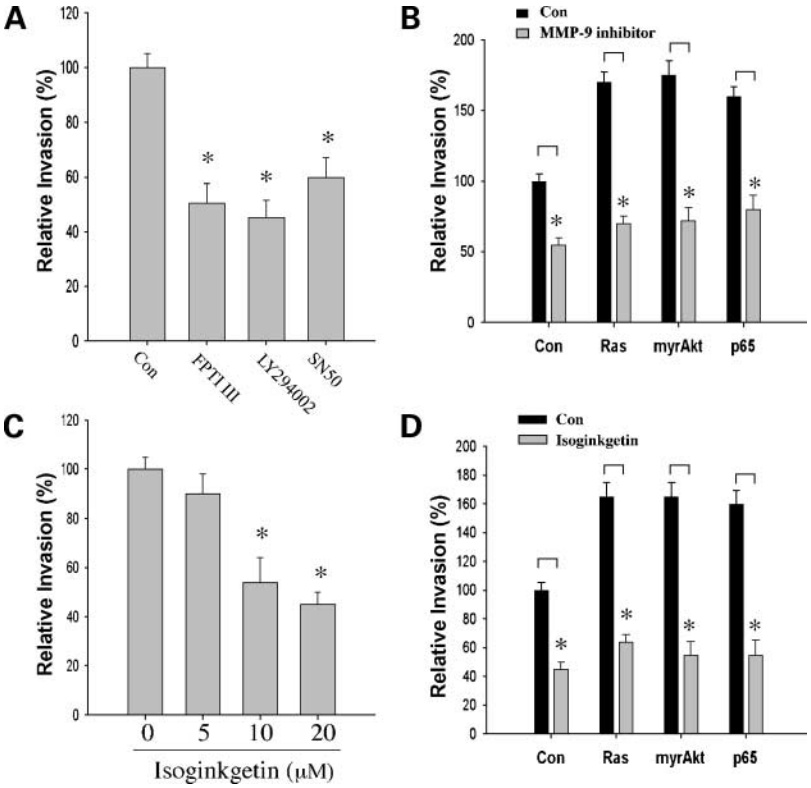

Figure 5. Isoginkgetin inhibits cell invasion. **A,** invasion assay was done in the presence of FPTI III, LY294002, or SN50. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test. **B,** cells were transfected with dominant-active H-Ras, myr-Akt, or p65. After 24 h, invasion assay was done in the presence of MMP-9 inhibitor. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test. **C,** the ability of cells to invade Matrigel was evaluated in the presence of the indicated concentration of isoginkgetin. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test. **D,** cells were transfected with dominant-active H-Ras, myr-Akt, or p65. After 24 h, invasion assay was done in the presence of 20 μmol/L isoginkgetin. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test.

also reduced the arthritic inflammation without any side effects, which was evidenced by i.p. injection in a chronic inflammatory animal model (23). Isoginkgetin exhibited strong neuroprotection against cytotoxic insults induced by oxidative stress, amyloid β, and DNA-damaging agent (24). Mixture of ginkgetin and isoginkgetin (1:1) inhibited production of prostaglandin E2 and skin inflammation by down-regulating COX-2 expression (25). Here, we reveal that isoginkgetin is effective at inhibiting tumor cell invasion through inhibition of the Akt/NF-κB pathway. There is a strong correlation between inflammation and tumor invasion. COX-2–derived bioactive lipids, including prostaglandin E2, are potent inflammatory mediators that promote tumor growth and metastasis through the stimulation of cell proliferation, invasion, and angiogenesis (26). Of interest, PI3K/Akt pathway is important for COX-2 expression and inflammation (27, 28). Therefore, our findings that isoginkgetin could inhibit PI3K/Akt pathway–dependent tumor cell invasion may explain why isoginkgetin has shown to inhibit COX-2 expression, prostaglandin production, and finally inflammation in other studies.

PI3K/Akt is the major pathway for tumor invasion (13). For example, the PI3K pathway can regulate the molecules that are important in cell migration and invasion, such as Rac, Cdc42, p70 S6K1, GSK-3β, etc. In addition, this pathway is involved in cell survival/apoptosis, cell growth, cell size regulation, migration, angiogenesis, etc. (13). Therefore, the inhibition of this pathway is therapeutically important. Although our study highlights MMP-9 as a target of isoginkgetin, it is more likely that several other proteins that are involved in invasion are also targets of isoginkgetin considering the potent function of this toward PI3K/Akt pathway. Our findings that isoginkgetin inhibits the activity of PI3K/Akt is intriguing in light of the importance of this kinase. Therefore, it is tempting to speculate that isoginkgetin may regulate other downstream molecules of PI3K/Akt that are involved in tumor cell invasion, cell size/growth, metastasis, and angiogenesis.

We showed that LY294002, a specific inhibitor of PI3K pathway, completely blocks Akt phosphorylation, whereas isoginkgetin showed ~70% to 80% reduction of Akt phosphorylation. However, their effects on cell invasion are similar. This indicates that isoginkgetin could inhibit other pathways that regulate cell invasion. Although mitogen-activated protein kinases, such as ERK and p38, do not regulate MMP-9 expression in HT1080 cells, they have shown to be important for cell invasion and migration (29, 30). Therefore, we examined the effect of isoginkgtin on mitogen-activated protein kinases and found that

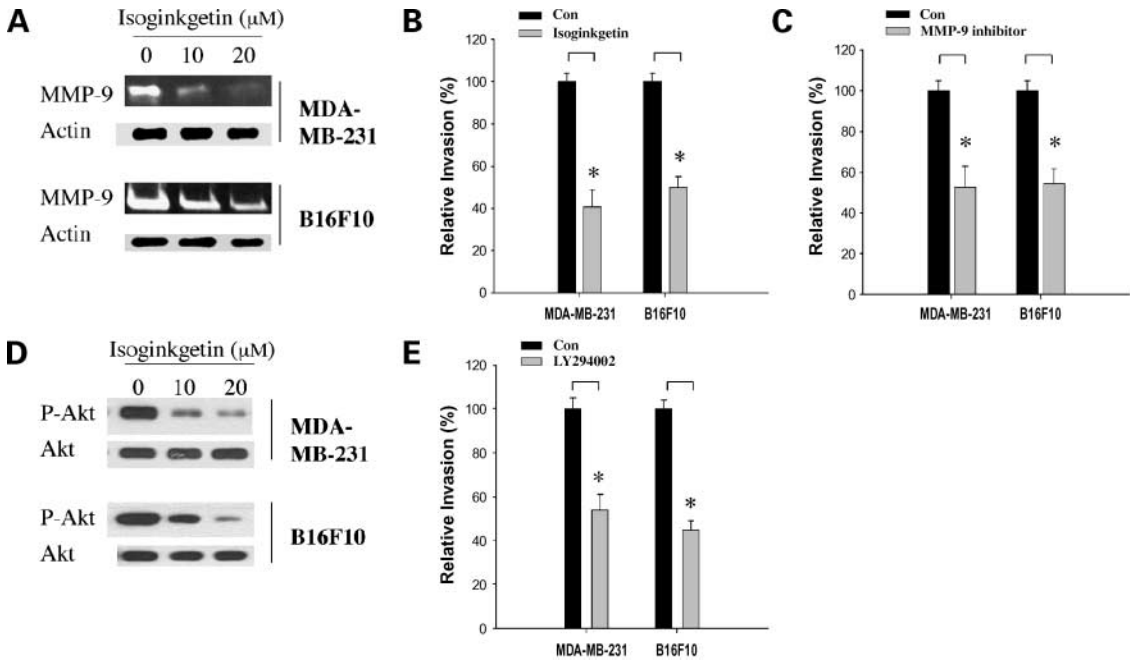

Figure 6. Isoginkgetin inhibits PI3K/Akt/NF-κB pathway-dependent invasion of MDA-MB-231 and B16F10 cells. **A,** MDA-MB-231 breast carcinoma and B16F10 melanoma cells were treated with the indicated concentration of isoginkgetin for 24 h and conditioned medium was collected. MMP-9 activity was measured by gelatin zymography. Actin was detected in total cell lysates by immunoblot analysis to normalize protein loadings. **B,** the ability of cells to invade Matrigel was evaluated in the presence of the indicated concentration of isoginkgetin. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test. **C,** invasion assay was done in the presence of MMP-9 inhibitor. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test. **D,** MDA-MB-231 and B16F10 cells were treated with isoginkgetin. Expression of phospho-Akt and Akt was detected by immunoblot analysis. **E,** invasion assay was done in the presence of LY294002. *Columns,* mean of three independent experiments; *bars,* SD. Results were statistically significant (*, $P < 0.01$) using Student's *t* test.

isoginkgetin inhibited phosphorylation of ERK and p38 ~20%.[5] These suggest that the anti-invasive effect of isoginkgetin is mainly from the inhibition of PI3K/Akt pathway and partly from the inhibition of ERK and p38 mitogen-activated protein kinases. Further studies are required for the identification of other kinases that are affected by isoginkgetin.

It is of interest that isoginkgetin decreased MMP-9 expression while increasing its inhibitor, TIMP-1. Regulation of TIMP-1 expression has not been well elucidated up to now. Although TIMP-1 promoter contains activator protein-1 binding site (31), inhibition of activator protein-1 does not always lead to TIMP-1 down-regulation. For example, in hepatic stellate cells, induction of c-Fos and c-Jun is unlikely to result in transactivation of the TIMP-1 promoter (32). Therefore, it is suggested that unidentified factors may be involved in the regulation of TIMP-1 expression (32–34). Some reagents exhibit similar activity like isoginkgetin toward MMP-9 and TIMP-1. Genistein (35), selenite (36), ursolic acid (37), 1α,25-dihydroxyvitamin D3, and its analogues (38) decrease MMP-9, but increase TIMP-1 production. From our studies, it is likely that isoginkgetin has lots in common with genistein in terms of functions. Both reagents inhibit COX-2 activity, decrease inflammation, down-regulate MMP-9, up-regulate TIMP-1, and inhibit tumor invasion. It is likely that these same functions come from the inhibitory effects of these reagents on PI3K/Akt pathway.

Flavonoids are natural polyphenolic compounds in plants. Some flavonoids possess strong pharmacologic properties against inflammation, neurodegenerative disorders, cardiovascular disease, microbial and fungal action, mutagenesis, cancer growth, metastasis, and angiogenesis (11). As many as 70% of therapeutic drugs currently in use are derived from plants (39). It has been shown that conventional flavonoids, such as quercetin, have weak *in vivo* activity. However, biflavonoids, such as isoginkgetin, have potent *in vivo* activity, so that the effects of biflavonoids are strong enough for a clinical trials (40). Our findings suggest that isoginkgetin could be a potential therapeutic candidate against tumor invasion. For this purpose, more detailed mechanistic and functional studies on isoginkgetin are crucial.

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