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Flavonoids Inhibit VEGF/bFGF-Induced Angiogenesis In Vitro by Inhibiting the Matrix-Degrading Proteases

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Abstract Flavonoids have been proposed to act as chemopreventive agents in numerous epidemiological studies and have been shown to inhibit angiogenesis and proliferation of tumor cells and endothelial cells in vitro. Angiogenesis requires tightly controlled extracellular matrix degradation mediated by extracellular proteolytic enzymes including matrix metalloproteinases (MMPs) and serine proteases, in particular, the urokinase-type plasminogen activator (uPA)-plasmin system. In this study, we have investigated the antiangiogenic mechanism of the flavonoids, genistein, apigenin, and 3-hydroxyflavone in a human umbilical vein endothelial cell (HUVEC) model. The stimulation of serum-starved HUVECs with vascular endothelial growth factor/basic fibroblast growth factor (VEGF/bFGF) caused marked increase in MMP-1 production and induced the pro-MMP-2 activation accompanied by the increase in MT1-MMP expression. However, pretreatment with flavonoids before VEGF/bFGF stimulation completely abolished the VEGF/bFGF-stimulated increase in MMP-1 and MT1-MMP expression and pro-MMP-2 activation. Genistein blocked VEGF/bFGF-stimulated increase in TIMP-1 expression and decrease in TIMP-2 expression. Apigenin and 3-hydroxyflavone further decreased TIMP-1 expression below basal level and completely abolished TIMP-2 expression. VEGF and bFGF stimulation also significantly induced uPA expression, most strikingly the level of 33 kDa uPA, and increased the expression of PA inhibitor (PAI)-1. Genistein, apigenin, and 3-hydroxyflavone effectively blocked the generation of 33 kDa uPA, and further decreased the activity of the 55 kDa uPA and the expression of PAI-1 below the basal level. In conclusion, these data suggest that genistein, apigenin, and 3-hydroxyflavone inhibit in vitro angiogenesis, in part via preventing VEGF/bFGF-induced MMP-1 and uPA expression and the activation of pro-MMP-2, and via modulating their inhibitors, TIMP-1 and -2, and PAI-1. J. Cell. Biochem. 89: 529–538, 2003.

Key words: genistein; apigenin; 3-hydroxyflavone; antiangiogenesis; matrix metalloproteinase; urokinase-type plasminogen activator

Flavonoids are the most abundant polyphenols in our diet and are found in soybeans, tea, fruits, and vegetables. They have been suggested to possess chemopreventive property in numerous epidemiological studies [Miller, 1990; Messina et al., 1994; Kennedy, 1995] and to inhibit the proliferation of tumor cells including breast, prostate, and lung cancer cells in vitro [Lian et al., 1998; Shao et al., 1998a; Davis et al., 1999]. They have also been shown to be antiangiogenic in several in vitro studies [Fotsis et al., 1993, 1995, 1997]. Genistein, a soy isoflavone, inhibited the proliferation of endothelial cells and bFGF (or vascular endothelial growth factor/basic fibroblast growth factor (VEGF/bFGF))-induced in vitro angiogenesis [Fotsis et al., 1993, 1995]. Moreover, Fotsis et al. [1997, 1998] have shown that certain structurally related flavonoids including 3-hydroxyflavone and apigenin were more potent inhibitors of proliferation of bovine capillary endothelial cells and in vitro angiogenesis of bovine microvascular endothelial cells than genistein. However, the antiangiogenic mechanism of flavonoids is largely unknown.

Angiogenesis, the generation of new capillaries by sprouting of pre-existing microvessels, is a tightly regulated process and restricted to a

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few conditions including normal reproductive function, embryonic development, and wound healing [Zetter, 1998]. However, aberrant angiogenesis is a crucial factor in the pathogenesis of numerous diseases including rheumatoid arthritis [Bodolay et al., 2002], diabetic retinopathy [Lee et al., 1998], and cancer [Zetter, 1998]. Angiogenic stimulators include, but are not limited to, the bFGF and the VEGF families of cytokines [Kuwano et al., 2001]. Extracellular proteolysis also plays an important role in many aspects of angiogenic process, including basement degradation and cell migration/extracellular matrix invasion, and is mediated by metalloproteinases and serine proteinases [Pepper et al., 1996].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, which are secreted in a latent form and become activated by partial proteolytic cleavage [Nagase and Woessner, 1999]. They are grouped by their substrate preferences and domain structures: collagenases (MMP-1, MMP-8, and MMP-13) degrade fibrillar collagen, gelatinases (MMP-2 and MMP-9) are potent in non-fibrillar and denatured collagen degradation, stromelysins (MMP-3, MMP-10, and MMP-11) prefer proteoglycans and glycoproteins as substrates, and membrane-type MMPs (MT1-, MT2-, MT3-, MT4-, and MT5-MMP) contain C-terminal transmembrane domain directing a cell surface localization [Nagase and Woessner, 1999]. MT1-MMP plays an essential role in basement membrane, degradation by activating pro-MMP-2 [Seiki, 2002]. The activity of MMPs can be inhibited by interaction with the endogenous inhibitors, tissue inhibitors of MMPs (TIMPs) [Nagase and Woessner, 1999].

The urokinase-type plasminogen activator (uPA) is a 55 kDa serine protease that is highly specific for the activation of plasminogen to plasmin which, in turn, can cleave matrix components such as fibrin and fibronectin and activate several MMPs [Rabbani and Mazar, 2001]. The form of uPA initially released from cells is the one-chain zymogen form. This is converted to two-chain uPA (55 kDa, also called high molecular weight (HMW)-uPA) by cleavage of the peptide bond between kringle and serine proteinase domain and is held together by a disulfide bond [Andreasen et al., 1997]. Proteolytic cleavage of uPA in the linker region gives rise to the amino-terminal fragment, (i.e., the growth factor domain and the kringle), and low molecular-weight uPA (LMW-uPA), (i.e., the serine proteinase domain). Like HMW-uPA, LMW-uPA activates plasminogen to plasmin directly [Stump et al., 1986]. There are two main inhibitors of plasminogen activators, plasminogen activator inhibitor (PAI)-1 and PAI-2 which contain a reactive center peptide bond which acts as a pseudo-substrate for uPA and trap the enzyme in a stable inactive complex of 1:1 stoichiometry [Andreasen et al., 1997].

Cornelius et al. [1995] reported that PMA stimulation markedly increased the expression of interstitial collagenase (MMP-1), stromelysin, and MMP-9 from human umbilical vein endothelial cells (HUVECs). HUVECs secrete significant amounts of pro-MMP-2 which can be activated to 64 and 62 kDa bands following either PMA treatment of cells [Foda et al., 1996] or treatment by a cocktail of nine angiogenic agents (VEGF, bFGF, EGF, TNF-α, TGF-β1, TGF-α, HGF/SF, IL-1α, and angiogenin); however, individual angiogenic factors were unable to promote MMP-2 activation [Lafleur et al., 2001]. MT1-MMP is selectively induced in HUVECs in response to PMA treatment, consistent with a major role for MT1-MMP in pro-MMP-2 activation [Foda et al., 1996; Lafleur et al., 2001]. HUVECs express low levels of TIMPs, and respond to PMA stimulation by up-regulating expression of TIMP-1 [Lafleur et al., 2001], but by decreasing TIMP-2 expression [Cornelius et al., 1995].

A study has shown that VEGF (10–100 μg/L) and bFGF (10 μg/L) stimulated the expression of interstitial collagenase at the mRNA and protein level in HUVECs, but not the mRNA of gelatinases or TIMPs [Unemori et al., 1992]; however, the protein concentration of MMP-2 and TIMP-1 in culture supernatants from HUVECs treated for 18 h with VEGF was increased by 63 and 900%, respectively, when compared to media only-treated HUVECs [Zucker et al., 1998]. VEGF alone did not induce pro-MMP-2 activation, but together with coagulation factors resulted in progressive conversion of pro-MMP-2 to activated 62 kDa [Zucker et al., 1998]. VEGF and basic FGF induced cultured bovine endothelial cells to produce uPA and PAI-1 [Montesano et al., 1986; Pepper et al., 1991; Fotsis et al., 1993], and enhanced the mRNA production of uPA in HUVECs [Kumar et al., 1998]. When added individually to microvascular endothelial cells grown on the surface of three-dimensional collagen gels,

VEGF and bFGF induced the cells to form capillary-like tubules in which bFGF was about twice as potent [Pepper et al., 1992]. However, when added simultaneously, VEGF and bFGF induced an in vitro angiogenic response which was synergistic, and which occurred with greater speed than the response to either cytokine alone [Pepper et al., 1992].

In the present study, we have investigated the effects of the flavonoids, genistein, apigenin, and 3-hydroxyflavone, on the expression and activation of MMPs and uPA, and their corresponding inhibitors, TIMPs and PAI-1 in VEGF/bFGF-stimulated HUVECs.

MATERIALS AND METHODS

Reagents and Antibodies

EGM-2® BulletKit® (CC-3162) for HUVEC culture was purchased from BioWhittaker Cell Biology Products (Walkersville, MD) and Opti-MEM from Gibco™ Invitrogen Corporation (Carlsbad, CA). Genistein, apigenin, and 3-hydroxyflavone were purchased from Sigma (St. Louis, MO). VEGF and bFGF were purchased from Chemicon (Temecula, CA). Monoclonal antibodies to MMP-1 (Ab-6), MMP-2 (Ab-3), MT1-MMP (Ab-1), TIMP-1 (Ab-1), and PAI-1 (Ab-1) were purchased from Oncogene (San Diego, CA), monoclonal antibody to TIMP-2 (MAB3310) from Chemicon, and monoclonal antibody to β-actin (clone AC-15) from Sigma.

Cell Culture

HUVECs were obtained from BioWhittaker Cell Biology Products and cultured in EGM-2 complete medium consisting of EGM basal medium supplemented with hydrocortisone, ascorbic acid, heparin, GA-1000, and 2.5% FBS plus growth factors (human recombinant endothelial growth factor (hEGF), VEGF, human recombinant basic-FGF, human recombinant insulin-like growth factor (R^3-IGF-1)) except for serum starvation in which FBS concentration was reduced to 0.1% and no growth factors were added. All cells were grown in a humidified atmosphere, 95% air and 5% CO_2 at 37°C and passaged every 5–7 days. Cells from the third to the seventh passage were used for experiments. Flavonoids were dissolved in DMSO and added at the following concentration: genistein (10 mg/L), apigenin (5 mg/L), and 3-hydroxyflavone (5 mg/L). The final concentration of DMSO did not exceed 0.1% (v/v) in any case.

In Vitro Angiogenesis Assay

Matrigel™ (BD Biosciences, Bedford, MA) was thawed at 4°C, and 250 μl were quickly added to each well of a 24-well plate and allowed to solidify for 1 h at 37°C. Once solid, HUVECs serum-starved for 24 h were added to each well (100,000 cells/well) in EGM-2 complete medium. After cells had adhered to the Matrigel, DMSO, genistein, apigenin, or 3-hydroxyflavone was added, and cells were incubated at 37°C for 24 h. The tube formation of HUVECs was visualized with a Zeiss Photomicroscope 2 (Thornwood, NY) with 4× magnification and a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) using Adobe Photoshop 4.0 LE software.

Preparation of Conditioned Medium and Cell Lysates

HUVECs (10^6 cells/treatment) were serum-starved for 24 h and medium was changed to Opti-MEM (1 ml/10^6 cells). Opti-MEM serum-free medium was routinely used in our laboratory for extracellular proteinase assays in culture supernatants. Cells were either untreated or pretreated with DMSO (0.1%, v/v), genistein, apigenin, and 3-hydroxyflavone for 2 h. VEGF (50 μg/L) + bFGF (50 μg/L) were then added to cells. One group of cells remained untreated for use as an unstimulated control. Culture supernatants were collected after 24 h, centrifuged to remove debris, and frozen at −80°C. For cell lysate preparation, cells were lysed in M-PER® Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) and centrifuged to clear cell debris. The resulting supernatants were frozen at −80°C. Protein concentrations of cell lysates were determined using the bicinchoninic acid-based assay (Sigma) using bovine serum albumin (BSA) as a standard.

Zymography

Culture supernatants were used for the analysis of gelatinase and uPA activity in zymography. For the analysis of gelatinase activity, the non-reduced culture supernatants (25 μl) were resolved in a 10% SDS–polyacrylamide gel (PAGE) containing 2 g/L of gelatin (Sigma). After electrophoresis, the gel was incubated at room temperature for 1 h in

washing buffer (0.05 mol/L Tris-Cl, pH 7.5, 0.005 mol/L $CaCl_2$, 10^{-6} mol/L $ZnCl_2$, 2.5% Triton X-100) and then incubated overnight in the same buffer containing only 1% Triton X-100. For the analysis of uPA activity, the non-reduced culture supernatants (10 μl) were resolved in 10% SDS–PAGE containing 0.2% w/v casein Hammarsten (USB, Cleveland, OH) with or without 2 g/L lys-plasminogen (American Diagnostica, Inc., Greenwich, CT). After electrophoresis, the gel was incubated at room temperature for 1 h in 2% Triton X-100 in H_2O for 2 h and then incubated in 0.1 mol/L glycine–NaOH, pH 8.3 for overnight. Following incubation, gels were stained with 0.25% Coomassie brilliant blue R-250 in 40% methanol, 7% acetic acid for 2 h, and destained in the same buffer without Coomassie as needed.

Immunoblot Analysis

Culture supernatants (30 μl) were used for MMP-2, TIMP-1, TIMP-2, and PAI-1 analysis, and cell lysates (50 μg protein) for MT1-MMP and β-actin analysis. Proteins were separated by 10% SDS–PAGE under reducing conditions and then transferred to a nitrocellulose membrane. The blot was blocked for 1 h in T-PBS (0.1% Tween-20 in PBS) with 5% non-fat dry milk, and incubated with 1 mg/L of the appropriate primary antibodies in T-PBS as indicated in figure legends for overnight at 4°C. After washing, the blot was incubated with peroxidase-coupled goat anti-mouse IgG (H + L) in T-PBS at 1:50,000 dilution (Pierce) for 1 h at room temperature. After extensive washing, the bands were detected using SuperSignal West Femto Chemiluminescent Substrate System (Pierce). The resulting chemiluminescence was visualized by ChemiImager™ 4400 equipped with a ChemiNova™ CCD camera (Alpha Innotech Corporation, San Leandro, CA). Densitometric analysis of protein bands was performed using AlphaEase™ Software (Alpha Innotech Corporation).

MMP-1 ELISA

The amount of MMP-1 protein in culture supernatants was measured using MMP-1 ELISA kit essentially as described in the protocol provided by the manufacturer (Oncogene). Briefly, HUVEC culture supernatants were diluted 100-fold with Opti-MEM and added into wells of MMP-1 microplate coated with a monoclonal antibody against MMP-1 and incubated for 2 h at room temperature. The plate was washed with wash buffer (provided in the kit) five times. MMP-1 conjugate was added and the plate was incubated for 1 h at room temperature. The plate was washed and color reagent was added. Color development was stopped by the addition of stop solution and the absorbance was measured in an $Elx808_{IU}$ Ultra Microplate Reader (BIO-TEK Instruments, Inc., Winooski, Vermont) at wavelength of 450 nm and reference wavelength of 630 nm. A standard curve was constructed using the MMP-1 protein standard provided in the kit. The MMP-1 concentration of each sample was calculated using the equation of standard curve.

RESULTS

Inhibition of In Vitro Angiogenesis of HUVECs by Flavonoids

HUVECs were serum-starved in a medium containing 0.1% FBS and no growth factors for 24 h, then harvested, and seeded on a Matrigel-coated plate in a complete medium containing 2.5% FBS and growth factors (see Materials and Methods). After cells adhered onto Matrigel, DMSO (0.1% v/v), genistein (10 mg/L), apigenin (5 mg/L), or 3-hydroxyflavone (5 mg/L) was added to the wells. The tube formation of HUVECs was examined under a microscope after 24 h. As shown in Figure 1, HUVECs cultured on Matrigel organized into tubular networks, resembling capillaries. Control cells and DMSO-treated cells

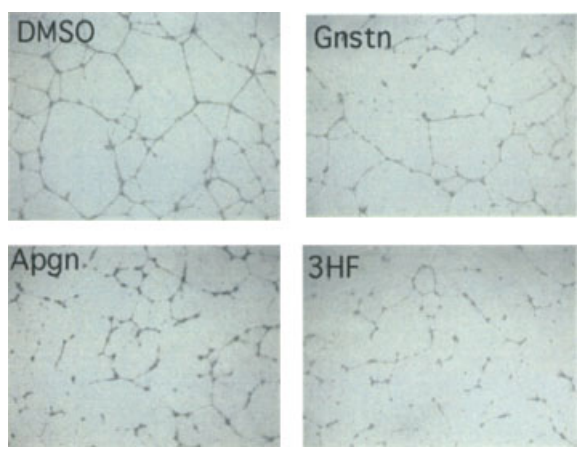

Fig. 1. Inhibition of the in vitro angiogenesis of human umbilical vein endothelial cells (HUVECs) by flavonoids. Representative microscopy photographs of HUVECs cultured for 24 h on Matrigel in the presence of the indicated compounds are illustrated.

formed tubes to the same degree (data not shown and Fig. 1). However, treatment of these cells with genistein, apigenin, or 3-hydroxyflavone significantly inhibited the tube formation of HUVECs. To ensure that the suppressed in vitro angiogenesis of HUVECs was not due to the cytotoxicity of the flavonoids, the presence of apoptotic cells was examined by the propidium iodide staining of cellular DNA followed by flow cytometric analysis. None of the flavonoids at the concentration used for in vitro angiogenesis assay increased apoptosis in HUVECs for 24-h incubation, when compared with control HUVECs (data not shown).

Inhibition of MMP Expression and Activity by Flavonoids

In a previous study, VEGF and bFGF, when added together, acted synergistically to induce a capillary-like tube formation of endothelial cells [Pepper et al., 1992]. To further investigate the antiangiogenic mechanism of flavonoids, the role of flavonoids on the regulation of MMP and TIMP expression induced by VEGF and bFGF was investigated. As shown in Figure 2, VEGF in combination with bFGF (VEGF/bFGF) induced the production of MMP-1 by 3.7-fold, when compared with unstimulated cells (basal level). Induction of MMP-1 was maintained in DMSO treatment with only a slight reduction (three–fold induction). However, the treatment with genistein, apigenin, and 3-hydroxyflavone abolished the VEGF/bFGF-stimulated MMP-1 production, reducing the MMP-1 amount to the basal level. Moreover, apigenin further decreased MMP-1 expression below the basal level.

Next, gelatinase activity and expression were determined by gelatin zymography and immunoblotting. Gelatin zymography of culture supernatants from HUVECs showed the 72 kDa gelatinolytic band (indicated by a top arrow) corresponding to the pro-MMP-2 in unstimulated cells (Fig. 3A, lane 1). VEGF/bFGF-stimulation generated intermediate 64 kDa form (indicated by a middle arrow) and fully activated 62 kDa MMP-2 (indicated by a bottom arrow) in cells (Fig. 3A,B; lane 2). Although Foda et al. [1996] demonstrated MMP-9 activity in HUVEC culture supernatants stimulated by PMA, we were not able to detect MMP-9 activity in VEGF/bFGF-stimulated HUVECs (Fig. 3A). DMSO had no significant effect on the generation of intermediate and fully activated MMP-2 by VEGF/bFGF stimulation (Fig. 3A,B;

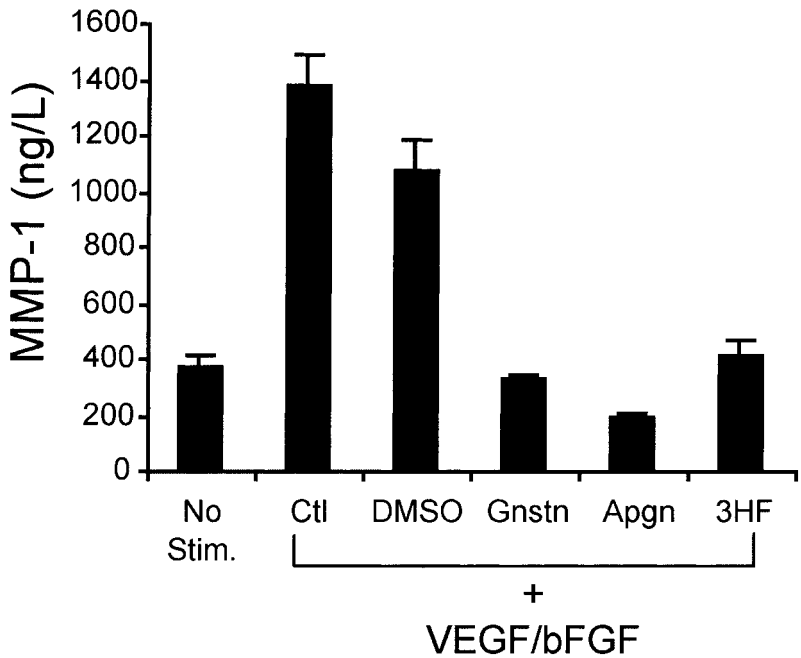

Fig. 2. Inhibition of vascular endothelial growth factor/basic fibroblast growth factor (VEGF/bFGF)-stimulated MMP-1 production by flavonoids. ELISA for MMP-1 was performed on culture supernatants (100-fold diluted) collected from confluent HUVECs pre-treated with none, DMSO, genistein, apigenin, or 3-hydroxyflavone for 2 h followed by the stimulation with VEGF and bFGF for 24 h. HUVEC culture supernatant cultured in medium only was included as control for basal MMP-1 (No Stim.). Triplicate wells were used per sample. MMP-1 amount was calculated according to standard curve and was shown as a bar with SD. Maximum stimulation of MMP-1 was 3.7-fold. Each flavonoid efficiently blocked the MMP-1 stimulation.

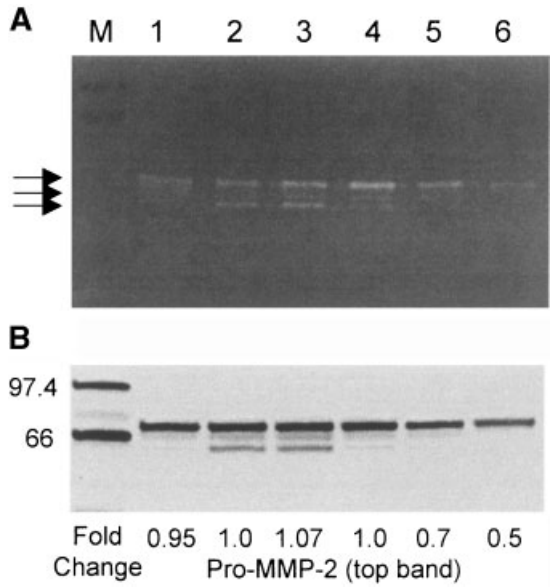

Fig. 3. Inhibition of VEGF/bFGF-stimulated pro-MMP-2 activation by flavonoids. Culture supernatants from HUVECs pre-treated with none (**lane 2**), DMSO (**lane 3**), genistein (**lane 4**), apigenin (**lane 5**), or 3-hydroxyflavone (**lane 6**) for 2 h followed by the stimulation with VEGF and bFGF for 24 h were subjected to gelatin zymography (**A**) or immunoblotting using antibody to MMP-2 (**B**) as described in Materials and Methods. Arrows indicate the pro-MMP-2 at 72 kDa (**top**), the intermediate form at 64 kDa (**middle**), and the active form at 62 kDa (**bottom**). **M** indicates molecular weight standard and **lane 1** indicates unstimulated control supernatants. Densitometric analysis of pro-MMP-2 band (indicated by a top arrow) on immunoblot was done as described in Materials and Methods. Fold change of pro-MMP-2 expression was calculated relative to pro-MMP-2 expression in lane 2 set at 1. Results shown are from one representative experiment of two.

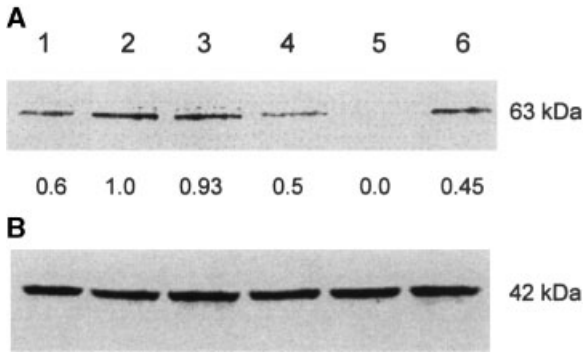

Fig. 4. Inhibition of VEGF/bFGF-stimulated MT1-MMP expression by flavonoids. Cell lysates from HUVECs pre-treated with none (**lane 2**), DMSO (**lane 3**), genistein (**lane 4**), apigenin (**lane 5**), or 3-hydroxyflavone (**lane 6**) for 2 h followed by the stimulation with VEGF and bFGF for 24 h were subjected to immunoblotting with antibody to MT1-MMP (63 kDa) (**A**) or β-actin (42 kDa) (**B**) and densitometric analysis was performed as described in Materials and Methods. **Lane 1** indicates cell lysates from unstimulated control HUVECs. MT1-MMP expression was normalized to β-actin expression and fold change of MT1-MMP expression was calculated relative to MT1-MMP expression in lane 2 set at 1. Results shown are from one representative experiment of two.

lane 3). However, flavonoids inhibited pro-MMP-2 activation as intermediate and fully active MMP-2 bands were abolished in flavonoid-treated cells (Fig. 3A,B; lanes 4–6). Apigenin and 3-hydroxyflavone further downregulated the pro-MMP-2 expression by 30 and 50%, respectively (Fig. 3B; lanes 5, 6).

MT1-MMP is a cellular receptor for and activator of pro-MMP-2, with which it forms a trimolecular complex on the cell surface with TIMP-2 [Seiki, 2002]. VEGF/bFGF-induced activation of pro-MMP-2 in HUVECs was accompanied by modest but significant increase of the MT1-MMP immunoreactive band at 63 kDa in the cell lysate, indicating increased MT1-MMP synthesis (Fig. 4A, lane 2). Apigenin was the most potent inhibitor abrogating the MT1-MMP expression completely, while genistein and 3-hydroxyflavone inhibited the VEGF/bFGF-stimulated increase by 50% (Fig. 4A, lanes 4–6). The overall protein synthesis was not affected by the flavonoids, since there was no significant effect on β-actin level (Fig. 4B).

Previously, VEGF (at 38 μg/L) has been shown to stimulate TIMP-1 secretion from HUVECs by nine–fold [Zucker et al., 1998]. In this study, VEGF in combination with bFGF resulted in a small but significant increase in TIMP-1 expression (1.6-fold) in HUVECs (Fig. 5A, lane 2). On the other hand, the same VEGF/bFGF treatment decreased TIMP-2 expression in HUVECs by 30% (Fig. 5B, lane 2). Genistein abolished the VEGF/bFGF-induced changes in TIMP-1 and -2 secretion by decreasing TIMP-1 and increasing TIMP-2 levels to the basal level (Fig. 5A,B; lane 4). Most noticeably, apigenin and 3-hydroxyflavone down-regulated TIMP-1 secretion by 80% and totally inhibited the TIMP-2 expression (Fig. 5A,B; lanes 5, 6). DMSO had no significant effect on the VEGF/bFGF-induced changes in TIMP-1 and -2.

Inhibition of uPA and PAI-1 by Flavonoids

To investigate the effect of flavonoids on uPA activity and expression from HUVECs, we performed casein zymography with or without plasminogen incorporation. Plasminogen-dependent lysis zones were found in culture supernatants of none-stimulated HUVECs,

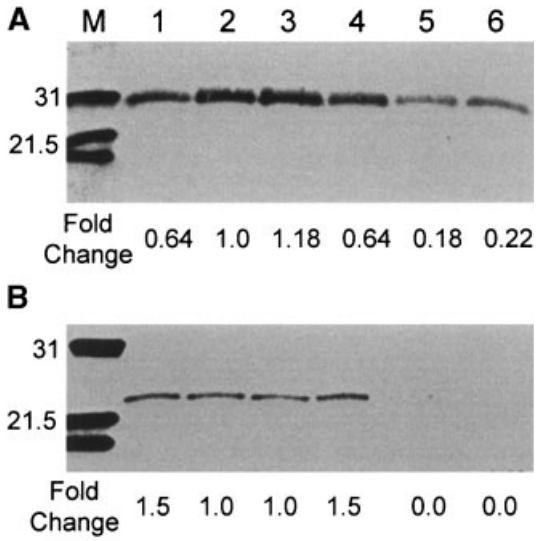

Fig. 5. Effects of flavonoids on TIMP expression modulated by VEGF/bFGF stimulation. Culture supernatants from HUVECs pre-treated with none (**lane 2**), DMSO (**lane 3**), genistein (**lane 4**), apigenin (**lane 5**), or 3-hydroxyflavone (**lane 6**) for 2 h followed by the stimulation with VEGF and bFGF for 24 h were subjected to immunoblotting with antibody to TIMP-1 (**A**) or TIMP-2 (**B**) and densitometric analysis was performed as described in Materials and Methods. **M** indicates molecular weight standard and **lane 1** indicates unstimulated control supernatants. Fold change of TIMP-1 and -2 expression was calculated relative to TIMP-1 and -2 expression in lane 2 set at 1. Results shown are from one representative experiment of two.

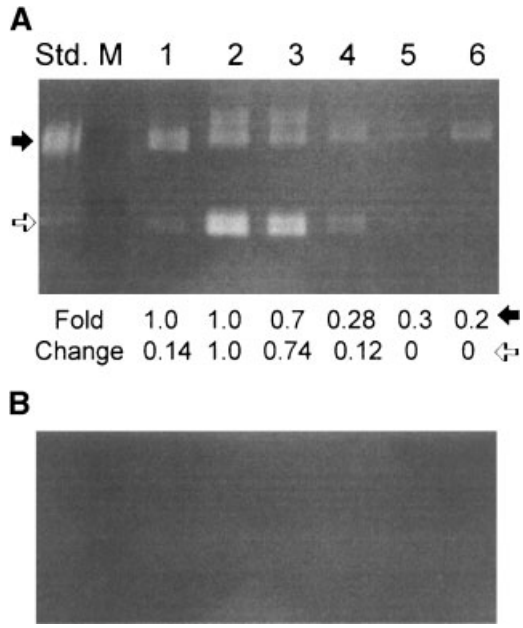

Fig. 6. Inhibition of VEGF/bFGF-induced uPA expression by flavonoids. Culture supernatants from HUVECs pre-treated with none (**lane 2**), DMSO (**lane 3**), genistein (**lane 4**), apigenin (**lane 5**), or 3-hydroxyflavone (**lane 6**) for 2 h followed by the stimulation with VEGF and bFGF for 24 h were subjected to casein zymography with plasminogen (**A**) or without plasminogen (**B**). **M** indicates molecular weight standard and **lane 1** indicates unstimulated control supernatants. **Std.** indicates uPA standard. Arrows indicate HMW-uPA (⬅) and LMW-uPA (⇦). The zymography gel was visualized by ChemiImager™ 4400 and densitometric analysis of uPA bands was performed using AlphaEase™ Software. Fold change of uPA expression was calculated relative to uPA expression in lane 2 set at 1. Results shown are from one representative experiment of two.

which corresponded to the molecular weights of standard uPA, 55 kDa (⬅) and 33 kDa (⇦) (Fig. 6A; lanes Std., 1). The 55 kDa band (two chain active HMW-uPA) was the major form of uPA present in unstimulated cells constituting 75% of total uPA, while the 33 kDa (LMW-uPA) band was the minor form, constituting 25% of total uPA. Casein zymography without plasminogen did not yield any lysis zones, confirming that the lytic bands were dependent on the presence of plasminogen in the SDS–PAGE and corresponded to uPA (Fig. 6B). VEGF/bFGF stimulation markedly induced uPA expression, specifically that of LMW-uPA, increasing the intensity of lytic band by seven–fold, while HMW-uPA relatively remained unchanged. DMSO had no significant effect on the VEGF/bFGF-induced increase in uPA expression (lane 3). Genistein completely reduced VEGF/bFGF-induced increase in LMW-uPA to the basal level and downregulated HMW-uPA by 70% (Fig. 6A, lane 4). Apigenin and 3-hydroxyflavone completely abated LMW-uPA and downregulated HMW-uPA by 70 and 80%, respectively (lanes 5, 6).

PAI-1 expression was also upregulated by VEGF/bFGF stimulation along with increased uPA activity (Fig. 7, lane 2). It has been suggested a functional interplay between uPA and PAI-1 during angiogenesis in which PAI-1 protects neovascularized tissues from excessive proteolysis [Pepper, 2001]. DMSO treatment further stimulated PAI-1 expression by an as yet unknown mechanism (lane 3). However, flavonoids efficiently blocked the VEGF/bFGF-induced increase in PAI-1 expression and further decreased PAI-1 synthesis below the basal level (lanes 4–6).

DISCUSSION

Flavonoids have been proposed to act as cancer chemopreventive agents in Asian populations [Miller, 1990; Messina et al., 1994; Kennedy, 1995]. Fotsis et al. [1997] have

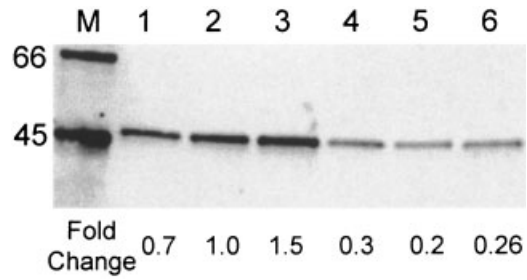

Fig. 7. Inhibition of VEGF/bFGF-stimulated PAI-1 production by flavonoids. Culture supernatants from HUVECs pre-treated with none (**lane 2**), DMSO (**lane 3**), genistein (**lane 4**), apigenin (**lane 5**), or 3-hydroxyflavone (**lane 6**) for 2 h followed by the stimulation with VEGF and bFGF for 24 h were subjected to immunoblotting with antibody to PAI-1. **M** indicates molecular weight standard and **lane 1** indicates unstimulated control supernatants. Fold change of PAI-1 expression was calculated relative to PAI-1 expression in lane 2 set at 1. Results shown are from one representative experiment of two.

determined that certain structurally related flavonoids including genistein, 3-hydroxyflavone, fisetin, apigenin, and luteolin, were potent inhibitors of endothelial cell proliferation and VEGF/bFGF-stimulated in vitro angiogenesis. However, the mechanism of anti-angiogenesis of these flavonoids was largely unknown.

Tumor angiogenesis is a vital process for the progression of a neoplasm from a small localized tumor to an enlarging tumor with the ability to metastasize. Among the proangiogenic factors known to this date, bFGF and VEGF are the best characterized. They stimulate endothelial cells to secrete several MMPs and uPA, resulting in the degradation of the vessel basement membrane, which in turn allows the cells to invade the surrounding matrix. VEGF increased the expression of MMP-1, MMP-2, and TIMP-1 proteins, and uPA mRNA in HUVECs [Unemori et al., 1992; Kumar et al., 1998; Zucker et al., 1998]. VEGF and basic FGF induced cultured bovine endothelial cells to produce the increased amount of uPA and PAI-1 proteins [Montesano et al., 1986; Pepper et al., 1991; Fotsis et al., 1993]. Moreover, VEGF and bFGF induced in vitro angiogenesis synergistically when added together to endothelial culture [Pepper et al., 1992].

Genistein and apigenin have been shown to downregulate MMPs and uPA in several studies [Fotsis et al., 1993; Yu et al., 1997; Shao et al., 1998c; Lindenmeyer et al., 2001]. Genistein has been shown to inhibit both constitutive and epidermal growth factor (EGF)-stimulated invasion of estrogen receptor (ER)-negative human breast carcinoma cells mediated through a downregulation of MMP-9 and an upregulation of TIMP-1 [Shao et al., 1998b,c]. Genistein also inhibited ConA-induced MT1-MMP expression and MMP-2 activation in MDA-MB-231 breast carcinoma cells [Yu et al., 1997]. Genistein has been shown to inhibit the bFGF-induced expression of uPA and PAI-1 [Fotsis et al., 1993, 1995]. Lindenmeyer et al. [2001] have shown that apigenin decreased uPA expression and totally abolished PMA-stimulated MMP-9 secretion in MDA-MB231 breast carcinoma cells. Apigenin also significantly reduced EGF-induced MMP-9 secretion and invasion of SKBR-3 metastatic breast carcinoma cells through MAPK inhibition [Reddy et al., 1999]. On the contrary, there is a very limited number of studies regarding to 3-hydroxyflavone. In one study, 3-hydroxyflavone has been shown to induce cell cycle arrest at different stages depending on cell type, increase in G_2/M population in SF295 human central nerve system cancer cell line, S population in HCT15/CL02 colorectal cancer multidrug resistant subline cells, and S and G_2/M population in HCT15 parental cells [Choi et al., 1999]. In addition, Fotsis et al. [1997] have reported that 3-hydroxyflavone was the most potent among flavonoids tested in inhibiting in vitro angiogenesis.

To the best of our knowledge, this is the first study to determine the inhibitory activity of apigenin and 3-hydroxyflavone on VEGF/bFGF-induced MMP and uPA expression and activation. In the present study, we have determined that the flavonoids, genistein, apigenin, and 3-hydroxyflavone, exert their antiangiogenic effect, in part via the inhibition of VEGF/bFGF-stimulated expression and activation of MMPs and uPA, and via the regulation of their endogenous inhibitors, TIMP-1 and -2, and PAI-1. The stimulation of HUVECs with VEGF in combination with bFGF induced an increase in MMP-1 and its endogenous inhibitor, TIMP-1 (see Figs. 2 and 5A and Unemori et al. [1992], Zucker et al. [1998]). MMP-1, also known as interstitial collagenase, has been shown to be necessary for angiogenesis in vitro through its ability to degrade type I collagen, the major component of the perivascular extracellular matrix [Fisher et al., 1994]. During the activation (sprouting) phase of angiogenesis,

increased expression of TIMP-1 and -2 inhibited tumor-associated angiogenesis [Pepper, 2001]. However, inhibition of MMP activity is required in the resolution phase of angiogenesis to stabilize newly formed vessels and allow basement membrane deposition [Pepper, 2001]. In this study, we analyzed MMP-1 and TIMP-1 expression after 24 h stimulation, at which time the vessels were already formed and stabilized. VEGF/bFGF-stimulated MMP-1 and TIMP-1 expression was effectively inhibited by genistein, and further downregulated below the basal level by apigenin and 3-hydroxyflavone. However, more detailed kinetic studies are needed to dissect the time course of MMP-1 and TIMP-1 expression.

Moreover, we have documented that VEGF/bFGF stimulation induced pro-MMP-2 activation accompanied by MT1-MMP increase and TIMP-2 decrease (see Figs. 3, 4, and 5B). Genistein completely inhibited VEGF/bFGF-induced pro-MMP-2 activation and MT1-MMP increase without affecting the basal level expression of pro-MMP-2. Genistein also inhibited the VEGF/bFGF-induced decrease in TIMP-2, increasing its expression back to the basal level. Apigenin and 3-hydroxyflavone had the most prominent effect, not just inhibiting pro-MMP-2 activation but also decreasing basal pro-MMP-2 expression as well as completely abolishing TIMP-2 expression. Since TIMP-2 is required as a receptor for pro-MMP-2 and its activation, complete inhibition of TIMP-2 expression would result in the inhibition of pro-MMP-2 activation.

In addition to MMPs, the serine protease, uPA plays an important role during angiogenesis by activating plasminogen to plasmin which, in turn, degrades basement membranes and activates pro-MMPs [Pepper, 2001]. In this study, we have determined that the VEGF/bFGF stimulation resulted in marked induction in LMW-uPA without significant effect on HMW-uPA, and a modest increase in PAI-1 expression (see Figs. 6, 7). In previous studies, VEGF and bFGF induced 48 kDa uPA from bovine endothelial cells, which was most likely equivalent to HMW-uPA in human, and uPA mRNA in HUVEC [Montesano et al., 1986; Pepper et al., 1991; Fotsis et al., 1993; Kumar et al., 1998]. Studies have shown that proteases such as trypsin, plasmin, or MMP-7 cleaved the link between the C-terminal serine proteinase domain and the kringle of uPA, and generated LMW-uPA, consisting of the serine protease domain [Andreasen et al., 1997; Marcotte et al., 1992]. LMW-uPA was still able to activate plasminogen to plasmin to initiate the protease cascade during angiogenesis [Stump et al., 1986]. Flavonoid treatment, however, completely abolished LMW-uPA and PAI-1 induction by VEGF/bFGF stimulation, as well as downregulated HMW-uPA.

In conclusion, flavonoids, genistein, apigenin, and 3-hydroxyflavone inhibited VEGF/bFGF-induced angiogenesis, in part via preventing the VEGF/bFGF-induced MMP-1, MT1-MMP, and uPA expression and the activation of pro-MMP-2, and via modulating their inhibitors, TIMP-1, TIMP-2, and PAI-1. More studies are needed to further pursue their antiangiogenic potential for the clinical application.

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