Microarray-based Analysis of Anti-angiogenic Activity of Demethoxycurcumin on Human Umbilical Vein Endothelial Cells: Crucial Involvement of the Down-regulation of Matrix Metalloproteinase

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cDNA microarray-based gene expression analysis has been successfully employed to explore the action mechanism and to validate the targets of several drugs. In the present study, we evaluated anti-angiogenic activity of demethoxycurcumin (DC), a structural analog of curcumin, isolated from *Curcuma aromatica*, and investigated the effect of DC on genetic reprogramming in cultured human umbilical vein endothelial cells (HUVECs) using cDNA microarray analysis. Of 1024 human cancer-focused genes arrayed, 187 genes were up-regulated and 72 genes were down-regulated at least 2-fold by DC. Interestingly, 9 angiogenesis-related genes were down-regulated over 5-fold in response to DC, suggesting that the genetic reprogramming was crucially involved in anti-angiogenesis by the compound. To verify the results obtained from cDNA microarray analysis, matrix metalloproteinase-9 (MMP-9), the product of one of the angiogenesis-related genes down-regulated over 5-fold by DC, was investigated using gelatin zymography. DC potentially inhibited the expression of MMP-9, yet showed no direct effect on its activity. These data show that gene expressional change of MMP-9 is a major mediator for angiogenesis inhibition by DC. All genes identified and microarray data are available on the web at <http://dasan.sejong.ac.kr/~bioprobe/>.

Key words: Demethoxycurcumin — Anti-angiogenesis — DNA microarray — Matrix metalloproteinase

Angiogenesis, the formation of capillary sprouts from preexisting blood vessels, is an essential step during embryonic development, reproduction, tissue and organ growth, and wound healing.¹⁾ Under normal conditions, all steps are tightly regulated in order to avoid undesired neovascularization, and many control mechanisms are involved.²⁾ The collapse of this tightly regulated balance triggers pathologic disorder in capillary network formation during the development of many diseases, including cancer, diabetic retinopathy, hemangiomas and vasculitides.³⁾ Angiogenesis is a crucial step in the growth and metastasis of cancers and increased tumor angiogenesis has been associated with an increased incidence of distant metastasis.^{4,5)} Therefore, specific inhibition of angiogenesis has been considered as a powerful way to suppress tumor growth and metastasis.^{6,7)}

Curcuma aromatica, a traditional oriental medicine that has been widely used to treat various diseases, including cancer, throughout India and East-Asian countries, showed a potent anti-angiogenic activity both *in vitro* and *in vivo*.

Using bioactivity-guided fractionation and purification techniques, demethoxycurcumin (DC) was identified as a major active compound of *C. aromatica*. DC is a structural analog of curcumin, which has been entered into phase I clinical trials for chemoprevention by the National Cancer Institute.⁸⁾ DC showed almost the same biological effect as that of curcumin and is a potent anti-angiogenic agent.⁹⁾ However, the action mode of angiogenesis inhibition, particularly the gene transcriptional effect of DC, has not been studied in detail.

In this study, we investigated the effect of DC on angiogenesis using chorioallantoic membrane (CAM) and *ex vivo* rat aortic ring assays. In addition, gene expression analysis of DC-treated cultured human umbilical vein endothelial cells (HUVECs) was conducted using cDNA microarray analysis. DC potentially inhibited the neovascularization of CAM and *ex vivo* capillary formation of rat aortic ring. cDNA microarray analysis showed that DC strongly down-regulated several angiogenesis-related genes including matrix metalloproteinase-9 (MMP-9). Furthermore, gelatin zymogram analysis revealed that DC inhibited the expression of MMP-9, yet had no effect on the enzymatic activity of the protein. These data demonstrate that the suppression of MMP-9 expression is one of

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the major causes of angiogenesis inhibition by DC, and cDNA microarray analysis can be a useful tool to explore the mode of action of bioactive chemicals.

MATERIALS AND METHODS

Materials Basic fibroblast growth factor (bFGF) was obtained from Upstate Biotechnology (Lake Placid, NY). Medium-199 (M-199), fetal bovine serum (FBS), and heparin from Life Technologies (Grand Island, NY), and Matrigel were obtained from Collaborative Biomedical Products (Bedford, MA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), gelatin type B, and Triton X-100 were purchased from Sigma (St. Louis, MO).

Cell culture Early passages (4–8 passages) of human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. Y. G. Kwon at Kangwon Nat'l. Univ. HUVECs were grown onto a gelatin-coated tissue culture flask containing M-199 media supplemented with 20% FBS, 3 ng/ml bFGF, and 5 units/ml heparin. Cells were maintained at 37°C in a humidified incubator adjusted at 5% CO₂.

Cell proliferation assay Cell proliferation was measured by using MTT assay as described previously.¹⁰⁾ Briefly, HUVECs were inoculated at a density of 5×10^3 cells/well in 96-well plates. Various doses of DC were added to each well and incubation was continued for 3 days. MTT (50 μ l, 2 mg/ml) was added and the plate was incubated for an additional 4 h. The absorbance of MTT-formazan was measured using a 540 nm filter-equipped microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Purification of demethoxycurcumin (DC) from *Curcuma aromatica* The rhizome of *Curcuma aromatica* was extracted with 85% methanol for 5 days and concentrated *in vacuo*. Ethyl acetate extract was filtered and separated by silica gel (70–230 mesh) column chromatography (ψ 6 $\times$ 17 cm), with a solvent system of hexane/chloroform/methanol (3:9:1). The active fraction was further purified with preparative thin layer chromatography (silica gel 60 F₂₅₄, Merck, Whitehouse Station, NJ) using a solvent system of hexane/chloroform/methanol (3:9:1). Final purification of DC was achieved by HPLC (Shimadzu, Kyoto, H₂O:acetonitrile=40:60, flow rate: 1 ml/min). The chemical structure and molecular weight (338.4) of DC were analyzed by EI-Mass, ¹H-NMR (500 MHz), and ¹³C-NMR (250 MHz) spectrophotometries, respectively.

Chorioallantoic membrane (CAM) assay Fertilized chick eggs were kept in a humidified incubator at 37°C for 3 days. About 2 ml of egg albumin was removed with a hypodermic needle, allowing the CAM and yolk sac to drop away from the shell membrane. On day 3.5, the shell was punched out and removed and the shell membrane was peeled away. At the stage of 4.5-day-old chick

embryo, a DC-loaded Thermanox coverslip was air-dried and applied to the CAM surface. Two days later, 2 ml of 10% fat emulsion was injected into the chorioallantois and the CAM was observed under a microscope. When sample-treated CAM showed an avascular zone, the response was scored as positive and the score was calculated as the percentage of positive eggs to the total number of eggs tested.

Ex vivo rat aortic ring assay Aorta of rat scarified after CO₂ asphyxiation was flushed gently with Hank's balanced salt solution (HBSS) using a 1-ml syringe fitted with a 23-gauge needle until the aorta was free of clotted blood. The aorta was cut to produce rings 1 mm in width using sterilized scissors. The rings were transferred to a Matrigel-coated 48-well culture plate and 50 μ l of M-199 was added to each well to cover the aorta rings. The plate was incubated in a CO₂ incubator for 30 min and 200 μ l of M-199 containing endothelial cell growth supplement (ECGS) in the presence or absence of DC (10 μ M) was added to each well. After 5 days, the formation of new blood vessels around the aortic rings was observed under a microscope and photographed at a 40 $\times$ magnification.

RNA and probe preparation Total cellular RNA was isolated from HUVECs treated with or without DC (10 μ M) for 24 h using an RNeasy mini kit (Qiagen, Valencia, CA). Cy3 (control)- or Cy5 (treated)-dUTP (Perkin Elmer Life Sciences, Inc., Boston, MA) was incorporated into cDNA during reverse transcription (Superscript II, Life Technologies) and the products were purified using a PCR purification kit (Qiagen). For microarray hybridization, each cyanine-labeled probe was mixed and hybridization blockers containing human Cot-1 DNA (Life Technologies), polyA RNA (Sigma), and yeast tRNA (Life Technologies) were added to the probe mixture. The mixture was concentrated using a Millipore YM-30 tube (Millipore, Bedford, MA).

Microarray hybridization A microarray slide (Genomic Tree, Inc., Taejon, Korea) was pre-hybridized with blocking solution containing 3.5 $\times$ saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) and 10 mg/ml of bovine serum albumin (BSA) at 50°C for 20 min. The slide was then washed with deionized water followed by isopropanol, and dried by centrifugation at 600 rpm for 5 min. The prepared probe mixture was applied to a microarray slide, covered with a cover slip, and hybridized in a GT Hyb-Chamber II (Genomic Tree, Inc.) at 65°C for 16 h. **Microarray scanning and data analysis** A fluorescence image of the microarray was obtained using a GenePix 4000B scanner (Axon Instruments, Foster, CA). Data obtained from the scanner were further analyzed using GeneSpring data analysis software (Silicon Genetics, Redwood, CA).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis Total cellular RNA from HUVECs treated

with or without DC (10 μ M) for 24 h was reversely transcribed by Molony murine leukemia virus reverse transcriptase (Life Technologies) using Oligo-d(T)₁₅ primer (Life Technologies). For the determination of the mRNA content of each transcript, a standard PCR was performed using sense and antisense primer pairs as follows; 5'-AAT-CAGCGCCGAAGTCCA-3' and 5'-TTTGACGAGTAAC-CAAGCC-3' for angiopoietin 1, 5'-CACTGTCCACCC-CTCAGAGC-3' and 5'-GCCACTTGTGCGCGATAAGG-3' for matrix metalloproteinase-9, 5'-GAAGGTGCCATG-GACTTG-3' and 5'-GGTGAAGAGGTTTCCTCC-3' for Tek tyrosine kinase, 5'-GGACTATCCACCTGCAAG-3' and 5'-CTCAATTTCCCCTCCACG-3' for transforming growth factor β 1, 5'-AGAACTGGGACGAGGCC-3' and 5'-TCAATCTCCCATCCGTTGA-3' for von Hippel-Lindau syndrome, 5'-ATGCTGCTGGAGGAGTT-3' and 5'-CTGAGCCCCCTCTCTGCA-3' for cyclin-dependent kinase inhibitor 2D and 5'-ATGCCGCCAAAACCCC-3' and 5'-ACTCCTGTTCTGACCTCG-3' for retinoblastoma 1. Actin mRNA amounts were used to normalize the cDNA content. The PCR products were resolved in a 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Fig. 1. The chemical structure of demethoxycurcumin (DC; C₂₀H₁₈O₅, MW=338.12).

Fig. 2. Effect of DC on the proliferation of HUVECs. HUVECs were treated with DC and the cell growth was measured at various time points. The absorbance of MTT-formazan (Y-axis) represents the cell growth. ■ control, ● DC (7 μ M), ▲ DC (15 μ M).

Gelatin zymography HT1080 cells were seeded at a density of 1×10^6 cells/well in a 6-well plate. After 12 h, cells were incubated in 1 ml of serum-free medium with or without phorbol-12-myristate-13-acetate (PMA) and DC for an additional 12 h. The supernatant of each well was analyzed by SDS-PAGE using 10% acrylamide copolymerized with gelatin (0.33 mg/ml). After electrophoresis, the gel was rinsed three times with 2.5 % Triton X-100 for 30 min and incubated for 24 h at 37°C in substrate buffer (0.05 M Tris-Cl, pH 7.5, 0.15 M NaCl, 0.01 M CaCl₂, 1 μ M ZnCl₂, and 0.02% NaN₃). MMP-9 (gelatinase B) was identified following staining of the gel in Coomassie brilliant blue. The digested area appeared as a clear band on a blue background, indicating the location of MMP-9. To examine the effect of DC on the enzymatic activity of MMP-9 *in vitro*, culture supernatants of HT1080 cells stimulated with PMA were collected as an enzyme source and gelatin zymography was conducted as described above.

RESULTS

DC inhibits the proliferation of HUVECs In a part of our program to discover potent anti-angiogenic agents from natural products, we isolated DC as a major active compound from the rhizome of *Curcuma aromatica* (Fig. 1). Bioactivity-guided fractionation and purification procedures afforded DC as a potent inhibitor of endothelial cell proliferation. DC inhibited the proliferation of HUVECs in a time- and dose-dependent manner (Fig. 2). Cytotoxicity was not observed at up to 15 μ M DC during 72 h treatment. Thus, angiogenesis assays were performed in the concentration range of 1–15 μ M.

DC inhibits *in vivo* neovascularization of CAM We next examined the ability of DC to inhibit *in vivo* neovascularization of CAM. A DC-loaded Thermanox coverslip was placed on a CAM surface and neovascularized zones were observed under a microscope. The inhibition of the angiogenesis by retinoic acid, used as a positive control, was 75% ($n=16$), and that of empty coverslips was 0% ($n=15$). However, DC potently inhibited the neovascularization of chick embryo (85%, $n=20$) without affecting pre-existing vessels (Table I).

Table I. Inhibitory Effect of DC on *in vivo* Neovascularization of CAM

Drug	Inhibited egg/ live egg	Inhibition ratio (%)
Empty	0/15	0
Retinoic acid (1 μ g/egg)	12/16	75
DC (10 μ g/egg)	17/20	85

DC inhibits *ex vivo* capillary formation of rat aortic ring The recently developed *ex vivo* rat aortic ring model provides a simple, reproducible assay for discovering angiogenic agonists and antagonists.^{11,12} Therefore, we conducted the *ex vivo* rat aortic ring assay to investigate the anti-angiogenic activity of DC. As shown in Fig. 3, DC potently inhibited ECGS-induced capillary formation of rat aortic ring at the concentration of 10 μ M. This result together with that of CAM demonstrates that DC is a potent inhibitor of angiogenesis *in vivo*.

Gene expression profile of DC-treated HUVECs To explore the anti-angiogenic mechanism of DC, we investigated the gene expression profile of DC-treated HUVECs using cDNA microarray analysis. A 1024 human cancer-focused genes-arrayed cDNA microarray chip was used for microarray hybridization, and obtained data were analyzed by GeneSpring data analysis software. Numerous genes were up- or down-regulated in response to DC, as shown in scatter plots of the expression profile (Fig. 4). Using the gene-filtering function of the software, 187 and 72 genes were collected as genes up- and down-regulated by at least 2-fold, respectively (Table II). These results suggest that genetic reprogramming of endothelial cells was involved in the anti-angiogenic action of DC. Further analysis revealed that among the 72 down-regulated genes, 9 angiogenesis-related genes were significantly down-regulated by DC (Table III). They include Tek/Tie2, angiopoietin-1, matrix MMP-9, mitogen-activated protein kinase kinase (MAPKK), and vascular endothelial growth factor C (VEGF C). Both Tek and its ligand, angiopoietin-1, were potently down-regulated (6- and 10-fold, respectively) by DC, suggesting that angiopoietin signaling may be affected by the compound. VEGF is a specific mitogenic factor for endothelial cells, and is crucially involved in a variety of steps of angiogenic differentiation of the

cells, including cellular migration and proliferation.¹³ Ras and MAPKKs are known to mediate VEGF signaling.¹⁴ MMP-9, a basement membrane-degrading enzyme, which

Fig. 4. cDNA microarray analysis of gene expression profiles in HUVECs. Total RNA was extracted from HUVECs treated with or without DC (10 μ M) for 24 h and cDNA microarray analysis was performed as described in "Materials and Methods." Data are presented as the scatter plot of gene expression in DC-treated HUVECs (Cy5) versus that in control cells (Cy3). Designated genes indicated by arrows are angiogenesis-related genes significantly affected by DC, as shown in Table III. Ang-1, angiopoietin-1; MAPKK, mitogen-activated protein kinase kinase; Tek, Tek/Tie2 tyrosine kinase; MMP-9, matrix metalloproteinase-9; VEGF C, vascular endothelial growth factor C; TGF- α , transforming growth factor α ; TIMP-3, tissue inhibitor of matrix metalloproteinase-3; CBP, cAMP-responsive element binding protein; TGF- β , transforming growth factor β 1; VHL, von Hippel-Lindau syndrome; p19^{INK4D}, cyclin-dependent kinase inhibitor 2D; Rb1, retinoblastoma 1.

Fig. 3. Effect of DC on the *ex vivo* capillary formation of rat aortic ring. Rat aortic ring assay was performed as described in "Materials and Methods." Dense capillary networks were observed in the control panel (A), and the capillary formation was completely inhibited by DC (B). Black arrows indicate microvessels formed from rat aortic rings. Figures were selected as representative scenes from two independent experiments.

is critical for the invasion of endothelial cells, was also down-regulated by DC. On the other hand, many genes were up-regulated in response to DC. Among them, genes related to anti-angiogenic activity are listed in Table III. Tumor suppressor genes including von Hippel-Lindau syndrome (VHL), retinoblastoma 1 (Rb 1), and cyclin-dependent kinase inhibitor 2D (p19^{INK4D}) and known inhibitory genes of angiogenesis, including transforming growth factor β 1¹⁵) and tissue inhibitor of metalloproteinase-3 (TIMP-3), were up-regulated. Some of these genes were further analyzed using RT-PCR analysis. There was a close correlation between the microarray data and RT-PCR analysis (Fig. 5). The combined data suggest that DC inhibits angiogenesis through the suppression or activation of genes involved in several distinct steps of the angiogenic process. **DC inhibits the expression of MMP-9, but not the enzymatic activity of the protein** To verify the results obtained from cDNA microarray analysis, MMP-9, a major mediator of angiogenesis, was selected, and its expression and proteinase activity were examined.

Table II. Gene Expression Profiles in HUVECs Treated with DC

	Missing genes	0.5≤ER ^a ≤2	2<ER	0.5>ER
The number of genes	308	457	187	72

a) ER represents expression ratio (Cy5/Cy3).

HT1080 cells (human fibrosarcoma) were used to investigate the effect of DC on MMP-9 activity. HT1080 cells were treated with various concentrations of DC in the presence or absence of PMA, and the culture supernatant containing secreted proteins was analyzed by SDS-PAGE containing 0.3% gelatin. As shown in Fig. 6a, gelatinolytic activity was seen at 92 kDa (MMP-9), 72 kDa (pro-MMP-2), and 62 kDa (active-MMP-2). Control HT1080 cells exhibited a basal level activity of both MMP-2 and -9, whereas PMA-treated cells showed a significant enhancement of the gelatinolytic activities of both proteinases. However, DC strongly decreased the gelatinolytic activity of MMP-9. To investigate whether the decrease in gelatinolytic activity originated from down-regulation of the protein expression, we conducted an enzymatic analysis of MMP-9 using conditioned media from HT1080 cells. As shown in Fig. 6b, DC did not directly inhibit the enzymatic activity of MMP-9. These data demonstrate that the suppression of *MMP-9* gene expression is a major factor in angiogenesis inhibition by DC. We also confirmed that the migration and invasion activities of HUVECs were strongly inhibited by DC, verifying the anticipated cellular consequences of reduced expression of MMP-9 by DC (data not shown).

DISCUSSION

Curcuminoids, including curcumin, DC and bisdemethoxycurcumin (BDC), possess potent chemopreventive activity.¹⁶⁻¹⁹⁾ Extensive studies have shown that cur-

Table III. Angiogenesis-related Genes Significantly Affected by DC

	Genes	Cy5/Cy3 ratio	Description
Down-regulated genes	AA443302	0.192	<i>ras</i> homolog gene family, member E
	H07878	0.051	G protein-coupled receptor 19
	A1740827	0.152	cadherin 12 (N-cadherin 2)
	AW044503	0.097	angiopoietin 1
	H07920	0.065	mitogen-activated protein kinase kinase
	H02848	0.170	Tek tyrosine kinase, endothelial
	T72581	0.168	matrix metalloproteinase-9 (gelatinase B)
	H07899	0.142	vascular endothelial growth factor C
	A1823651	0.071	transforming growth factor α
Up-regulated genes	AA426341	2.116	von Hippel-Lindau binding protein 1
	AA099153	2.375	tissue inhibitor of metalloproteinase-3
	W89077	3.000	CREB ^{a)} binding protein (Rubinstein-Taybi's)
	AA045192	3.048	retinoblastoma 1 (including osteosarcoma)
	R77517	3.311	cyclin-dependent kinase inhibitor 2D (p19 ^{INK4D})
	R54176	3.641	von Hippel-Lindau syndrome
	AA428365	5.409	retinoblastoma-binding protein 4
	R36467	10.571	transforming growth factor β 1

a) cAMP-responsive element binding protein.

cumin, DC and BDC have similar biological activities, though their potencies differ.^{20, 21)} Interestingly, in some cases, DC showed the highest potency among the three curcuminoids. Such cases include anti-proliferative activity against MCF-7 breast carcinoma cells,²²⁾ nematocidal activities,²³⁾ and the inhibition of mouse corneal neovascularization.⁹⁾ These data suggest that DC may have a different activity profile from that of curcumin. However, detailed activity profiles, especially the gene transcriptional effects of purified DC, have not been established. In the present study, we investigated the anti-angiogenic activity of DC isolated from *Curcuma aromatica*, an oriental herbal medicine. DC potently inhibited *in vivo* neovascularization of CAM without showing any toxicity. Moreover, it also inhibited capillary formation of rat aortic ring, indicating that DC is a potent inhibitor of angiogenesis *in vivo*.

We next investigated the effect of DC on the gene expression profile of cultured HUVECs using cDNA microarray analysis. The microarray-based gene expression analysis of HUVECs revealed that a large number of genes is involved in anti-angiogenesis induced by DC. Interestingly, 9 angiogenesis-related genes, including MMP-9, were strongly down-regulated and some known angiogenesis-inhibitory genes were significantly increased, suggesting that these genes may be critical mediators of DC-induced anti-angiogenesis. Among the genes, we investigated the role of MMP-9 in the anti-angiogenic

activity of DC for the following reasons. 1) MMPs are a family of proteolytic enzymes, whose physiological functions include tissue remodeling and embryogenesis.^{24, 25)} 2) The importance of this group of proteins in tumor invasion and metastasis is now widely understood, and MMP-2 and -9 are known to be crucially involved in the process of angiogenesis.^{4, 5)}

In our further investigation, gelatin zymography for MMP-9 verified the results obtained from cDNA microarray analysis. Moreover, *in vitro* migration and invasion of HUVECs were strongly inhibited by DC, implying that DC inhibited angiogenesis, at least in part, via the suppression of MMP-9 expression. As shown in the zymography, MMP-2 in HT1080 cells was also down-regulated by DC, which, however, had no effect on its post-translational modification or enzymatic activity, suggesting that the activation of some transcription factor which is commonly involved in the expression of the MMPs may be inhibited by DC. A conserved AP-1 transcription factor-binding site is found in the promoter region of many MMP genes.²⁶⁾ In a previous report, it was demonstrated that curcuminoids including DC, inhibit the expression of MMP-9 via an AP-1-dependent mechanism.²⁷⁾ Thus, the down-regulation of MMP-9 by DC is thought to be the result of the suppression of AP-1 activation by the compound. In this respect, even if there are some dose-efficacy differences between DC and curcumin, the anti-angiogenic mechanism of both compounds seems to be the same.

In conclusion, our study demonstrated potent anti-angiogenic activity of DC and elucidated the genetic

Fig. 5. RT-PCR analysis of angiogenesis-related genes in HUVECs. Total RNA was extracted from HUVECs treated with or without DC (10 μ M) for 24 h and RT-PCR was performed using specific primer sets for each transcript as described in "Materials and Methods." Con denotes control HUVECs and DC denotes DC-treated HUVECs. Fold represents the intensity of RT-PCR results analyzed by densitometry.

Fig. 6. Effect of DC on the expression of MMP-9. (A) HT1080 cells were treated with DC and gelatin zymogram analysis was performed as described in "Materials and Methods." (B) Effect of DC on the enzymatic activity of MMP-9. Conditioned media from HT1080 cells were treated with or without DC and gelatin zymogram analysis was performed.

reprogramming in HUVECs induced by the compound. Several genes closely related to angiogenesis were up- or down-regulated and further studies on these genes or transcription factors whose expression is modulated will be valuable for our understanding of the drug's action mechanism, as well as for discovering new cellular mediators of angiogenesis.

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