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ARTICLE

Anthocyanins from Black Soybean Seed Coats Preferentially Inhibit TNF-α-Mediated Induction of VCAM-1 over ICAM-1 through the Regulation of GATAs and IRF-1

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Adhesion molecules have a key role in pathological inflammation. Thus, we investigated the effect of anthocyanins on the induction of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by TNF-α and the possible molecular mechanisms by which anthocyanins differentially regulate ICAM-1 and VCAM-1 expression. Stimulation of cells with TNF-α increased ICAM-1 and VCAM-1 expression, and pretreatment with anthocyanins inhibited VCAM-1 expression, but not ICAM-1 expression. We found that IRF-1 and GATAs, especially GATA-4 and -6, were involved in the TNF-α-mediated expression of VCAM-1 but not ICAM-1, and anthocyanins decreased nuclear levels of GATA-4 and GATA-6 as well as IRF-1. Moreover, pretreatment with a Jak/STAT inhibitor decreased TNF-α-induced VCAM-1 expression and nuclear GATA-4, GATA-6, and IRF-1 levels. Furthermore, anthocyanins efficiently inhibited the phosphorylation of STAT-3. This suggests that anthocyanins differentially regulate TNF-α-mediated expression of VCAM-1 and ICAM-1 through modulation of the GATA and IRF-1 binding activity via Jak/STAT-3 activation.

KEYWORDS: Anthocyanins; VCAM-1; ICAM-1; GATA; IRF-1; STAT-3

INTRODUCTION

Cell adhesion molecules (CAMs) play a key role in several pathologies, such as cancer, especially metastasis, as well as inflammatory diseases. Initially, tumor cells have to detach from the primary tumor, migrate through the tissue, and invade the lymphatic system or blood vessels. As a next step, circulating tumor cells temporarily adhere to endothelial cells and then extravasate by infiltrating the underlying basement membrane. Finally, cells migrate to a suitable location, where they form metastases (*1−3*). A recent study showed that some highly metastatic human melanoma cells have a high affinity conformation at the cell surface. These cells adhered and migrated to vascular cell adhesion molecule-1 (VCAM-1) rather than to intercellular adhesion molecule-1 (ICAM-1) (*4*). In addition, CAMs mediate different steps of leukocyte migration from the bloodstream toward the inflammatory foci and plays a central role in pathological inflammation such as atherosclerosis (*5*). Although expression of both VCAM-1 and ICAM-1 is regulated in atherosclerotic lesions, VCAM-1 has been reported to play a major role in the initiation of atherosclerosis (*6*). Therefore, recently it has become more important to discover therapeutic agents which have specific suppression effects on adhesion molecules, such as VCAM-1.

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Anthocyanins are polyphenols that are responsible for many fruit and floral colors. Their basic skeleton consists of the 2-phenylbenzopyrylium of flavylium glycoside. Anthocyanins are especially abundant in the epidermis palisade layer of the black soybean seed coat (*7−10*). Three main anthocyanins, cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside, have been characterized in black soybean seed coats (*7*, *8*, *10*). Many studies have reported health-promoting benefits of anthocyanins. These benefits include potential antioxidant effects, inhibition of some inflammatory genes associated with ischemia-reperfusion injury, reduction of the risk of coronary heart disease, and prevention of some chronic diseases (*11−14*). Interestingly, we found that anthocyanins from black soybean seed coats differentially regulate TNF-α-induced VCAM-1 and ICAM-1 expression.

Concerning the differential mechanism that regulates VCAM-1 and ICAM-1 expression, it has been reported that functional transcription factor binding motifs for nuclear factor-kappaB (NF-κB), interferon regulatory transcription factor-1 (IRF-1), activator protein-1 (AP-1), and transcription factor genes binding to DNA sequence GATA (GATAs) exist in the VCAM-1 gene promoter region (*15−20*). The ICAM-1 promoter also has NF-κB, AP-1, and specificity protein-1 (SP-1) binding sites; however, it lacks the octamer binding protein IRF and GATA-binding motifs (*21*). Thus, in this study, we aimed to investigate the differential effect of anthocyanins on the induction of VCAM-1

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and ICAM-1 by TNF-α and the possible molecular mechanisms by which anthocyanins differentially regulate VCAM-1 and ICAM-1 expression.

MATERIALS AND METHODS

Materials and Chemicals. Tissue culture medium 199, fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), glutamine, and collagenase were supplied by Gibco-BRL (Rockville, MD). IRF-1, GATA-4, and GATA-6 small interfering RNA (siRNA), siRNA transfection kit, anti-IRF-1, anti-GATA-4, anti-GATA-6, anti-ICAM-1, anti-VCAM-1, and antiproliferating cell nuclear antigen (PCNA) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p-STAT-3 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Enhanced chemiluminescence (ECL) Western blotting detection reagent was from Amersham (Buckinghamshire, U.K.). All other chemicals, including endothelial cell growth supplements (ECGS) and heparin, were supplied by Sigma-Aldrich (St. Louis, MO).

Anthocyanin Extraction and Purification. Anthocyanins from black soybean (*Glycine max* (L.) *Merr*) were extracted, purified, and quantified as described by Kim et al. (*12*). Briefly, the seed coats of soybean accessions (200 g) were extracted for 24 h at 4 °C with methanol. The solution containing anthocyanins was subjected to an Amberlite XAD-7 (Aldrich, St. Louis, MO) column and washed with deionized water and eluted with methanol containing 1% HCl. The solution was applied to a column packed with Sephadex LH-20 (Amersham Biosciences, Sweden) and eluted using 30% aqueous methanol containing 1% HCl. Cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside were isolated from the seed coats of black soybean and used as the anthocyanins source. The purity (≥99%) and the compositions of anthocyanins were analyzed using HPLC. Anthocyanins consisted of cyanidin-3-glucoside (72%), delphinidin-3-glucoside (20%) and petunidin-3-glucoside (6%).

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment (*22*) and grown in medium 199 supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 5 U/mL heparin, 100 IU/mL penicillin, 10 μg/mL streptomycin, and 50 μg/mL ECGS. Endothelial cells were cultured in 100 mm dishes and grown in a humidified 5% CO_2 incubator. HUVECs were plated at a density of 1×10^7 cells per 100 mm dish. Cells were used between passage numbers 3 and 6.

Western Blot Analysis. Total cell extracts were obtained according to the procedure described previously (*23*). Nuclear proteins were extracted as previously described (*22*). Protein concentration was determined by the Bradford method (*22*). Aliquots of 30 μg of protein were subjected to 10% sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis for 1 h 30 min at 110 V. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane for 2 h at 20 mA with a SD Semidry Transfer Cell (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris-buffed saline (TBS) containing 0.05% Tween 20 (TBS-T) for 2 h at room temperature. Then, the membranes were incubated with primary antibodies in 5% skim milk in TBS-T overnight at 4 °C, and the bound antibody was detected with a horseradish peroxidase-conjugated antirabbit IgG. The membranes were washed and then developed using a Western blotting luminol reagent system (Amersham).

Plasmid Constructions. For construction of the pVCAM1-258-luc reporter plasmid or the pICAM-277-luc reporter plasmid, the human VCAM-1-promoter region spanning (−258/+42) or ICAM-1-promoter region spanning (−277/−9) was cloned into the *Kpn*I/*Hind*III or *Xho I*/*HindIII* site of a pGL3-basic vector (Promega, Madison, WI), respectively (*19*). Polymerase chain reaction was used to amplify the fragment, with primers as follows: forward primer with *Kpn*I site (5′-CAAGGTACCTTTATCTTTCCAGTAAAGATAGCC-3′) and reverse primer with a *Hind*III site (5′-GATAAGCTTAGCTCCTGAAGCCAGTGAG-3′), or forward primer with *Xho I* site (5′-GATCTCGAGGGGGTCATCGCCCTGCCAC-3′) and reverse primer with a *Hin*dIII site (5′-CAAAAGCTTATAGCGAGGCTGAGGTTGC-3′). pVCAM1-258-luc was double digested with *Kpn*I/*Hind*III, and the VCAM-258 fragment was inserted into the pUC19 plasmid according to the enzyme sites to construct the pUC19-VCAM1-258 plasmid. Recombinant pUC19-VCAM1-258 plasmid was used as a template for GATA1 and IRF mutants to obtain pUC19-VCAM1-258-mGATA and pUC19-VCAM1-258-mIRF. pUC19-VCAM1-258-mGATA1 was used as a template for the GATA2 mutant and provided the pUC19-VCAM1-258-mGATA1-mGATA2 plasmid. pUC19-VCAM1-258-mGATA1 was further used as a template for the GATA&IRF double mutant to provide pUC19-VCAM1-258-mGATA1-mGATA2-mIRF. The GATA site was mutated in both the GATA1 and GATA2 binding sequences. PCR and subsequent steps were performed according to the manufacturer's protocol for the QuikChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). All point mutations and insert directions were confirmed by automated DNA sequencing. GATA1 mutant primers were as follows: forward, (5′-CTTTATCTTTCCAGTAAA**tcg**AGCCTTTTGGAGTCGAAG-3′); reverse, (5′- CTTCGACTCCAAAAGGCT**cga**TTTTCTGGAAAGATAAAG-3′). GATA2 mutant primers were as follows: forward, (5′-CTCGGTACCTTT**cga**TTTCCAGTAAAT-3′); reverse, (5′- ATTTACTGGAAA**tcg**AAAGGTACCGAG-3′). IRF mutant primers were as follows: forward, (5′-TATAAAGCACAGACTT**g**CTATTT**a**ACTCCGCGGTATCTG-3′); reverse, (5′-CAGATACCGCGGAGT**t**AAATAG**c**AAGTCTGTGCTTTATA-3′). Substituted bases are indicated in lowercase.

Transfection. Transient transfection of a wild type VCAM-1-luciferase and VCAM-1-luciferase with a mutated IRF-1 site (mIRF-1) or mutated GATA site (mGATA) was performed using Lipofectin (Gibco-BRL) according to the manufacturer's protocol (*12*). Briefly, 5×10^5 cells were plated into 60-mm dishes the day before transfection and grown to roughly 70% confluence. Cells were transfected with an empty vector (pGL3) or 1 μg of a reporter gene construct + 0.5 μg of p-RL-TK-luciferase. Transfections were allowed to proceed for 12 h. The transfected cells were then washed with 4 mL of PBS and then stimulated with 10 ng/mL TNF-α. The cells were continually cultured in serum-free medium 199 until they were harvested. Luciferase activity was normalized using pRL-TK-luciferase activity (*Renilla* luciferase activity) in each sample.

Luciferase Assay. After these treatments, the cells were washed twice with cold PBS, lysed in a passive lysis buffer provided in the dual luciferase kit (Promega, Madison, WI), and assayed for luciferase activity using a TD-20/20 luminometer (Tuner Designs, Sunnyvale, CA) according to the manufacturer's protocol. All transfections were done in triplicate. Data are presented as the ratio between Firefly and *Renilla* luciferase activities.

Statistical Evaluations. Values are expressed as means ± SD. Treatment groups were compared using one-way analysis of variance (ANOVA), and the Newman−Keuls test was used to locate any significant differences identified in the ANOVA. $P < 0.05$ or $p < 0.01$ was accepted as significant.

RESULTS

Anthocyanins Inhibit TNF-α-Induced Expression of VCAM-1 but Not ICAM-1. Anthocyanins reduced TNF-α-mediated VCAM-1 induction in a concentration-dependent manner (10, 50, and 100 μg/mL), and they completely inhibited VCAM-1 at 100 μg/mL but not ICAM-1 in HUVEC (**Figure 1A**). To determine whether anthocyanins act transcriptionally to inhibit TNF-α-induced VCAM-1 gene expression and to understand the molecular mechanism behind the inhibitory actions of anthocyanins, we conducted a promoter reporter assay. Cells were transfected with wild type VCAM-1 and ICAM-1 luciferase promoters and then stimulated with TNF-α with and without anthocyanins in a concentration-dependent manner. As shown in **Figure 1B**, anthocyanins significantly inhibited VCAM-1 luciferase activity, but not ICAM-1 luciferase activity induced by TNF-α in a concentration-dependent manner.

GATA and IRF-1 Motifs Are Important for the Induction of TNF-α-Stimulated VCAM-1. Because GATA and IRF-1 motifs were shown to play a role in mediating TNF-α induction of VCAM-1 (*24*, *25*), we generated a VCAM-1 promoter with mutant motifs at the IRF-1 and GATA sites to better understand the differential regulation of VCAM-1 between ICAM-1 by anthocyanins. The mutation in the IRF-1 (mIRF-1) and GATA (mGATA) sites resulted in a significant decrease in VCAM-1 luciferase activity induced by TNF-α (3-fold to 2.5-fold and 2-fold, respectively). Interestingly, the GATA mutant decreased

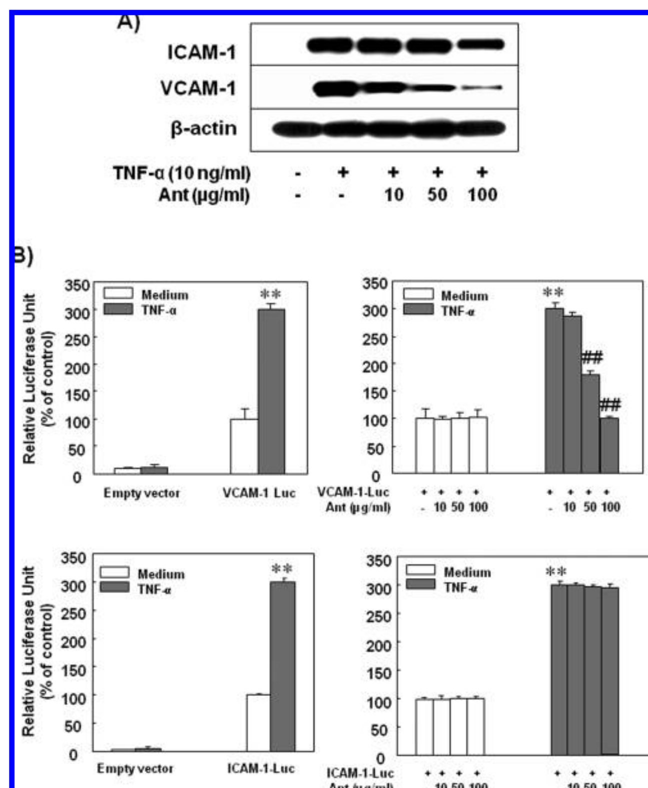

Figure 1. Preferential inhibition of TNF-α-mediated induction of VCAM-1 over ICAM-1 by anthocyanins. HUVECs were treated with anthocyanins for 24 h and then cotreated with TNF-α for 6 h. (**A**) The protein level of ICAM-1 and VCAM-1 was detected by Western blot analysis, as detailed in Materials and Methods. Data were confirmed by three independent experiments. (**B**) Cells were transfected with an empty vector or 1 μg of VCAM-1- or ICAM-1-luciferase + 0.5 μg of pRL-TK-luciferase. Cells were allowed to recover for 24 h and then treated with 10 ng/mL of TNF-α with/without anthocyanins (Ant) (10, 50, and 100 μg/mL). Luciferase activities are presented as the fold activation relative to that of the untreated cells. Data are presented as means ± SD from three independent experiments. Significance compared with control, $^{**}P < 0.01$; significance compared with TNF-α, $^{\#\#}P < 0.01$.

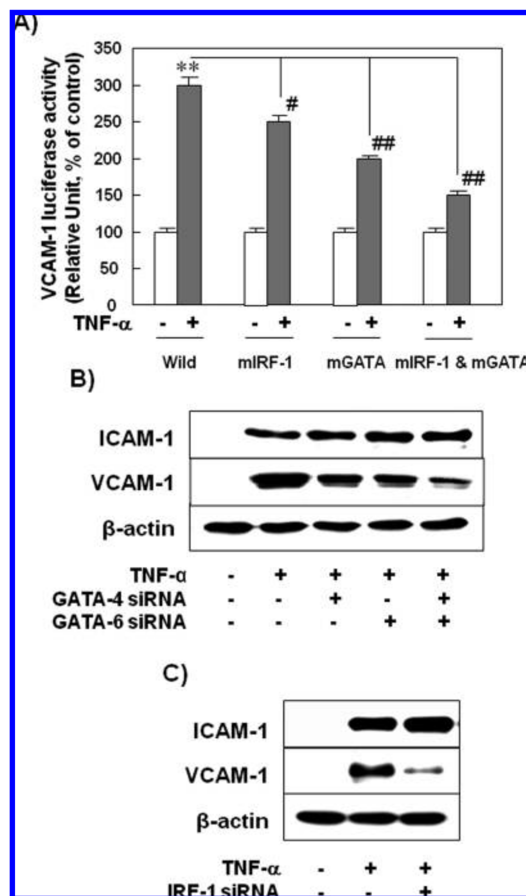

Figure 2. GATA and IRF-1 motifs are important for the full induction of TNF-α-stimulated VCAM-1. (**A**) Cells were transfected with 1 μg of wild type VCAM-1-luciferase or VCAM-1-luciferase containing an IRF-1 motif mutant (mIRF-1), a GATA motif mutant (mGATA), or both. Cells were allowed to recover for 24 h and then stimulated with TNF-α (10 ng/mL) for 6 h. Data are presented as means ± SD from three independent experiments. Significance compared with control, $^{**}P < 0.01$; significance compared with TNF-α, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$). (**B**) Cells were stimulated in a time-dependent manner with TNF-α. Thereafter, the nuclear fraction was extracted, and the GATA-4, GATA-6, or IRF-1 level was determined by Western blot analysis. Then, cells were pretreated anthocyanins for 24 h and treated with TNF-α for either 30 min (for the detection of GATA-4 or GATA-6) or 2 h (for the detection of IRF-1). (**C**) To confirm the role of GATA-4, GATA-6, or IRF-1 in VCAM-1 induction by TNF-α, GATA-4, GATA-6, or IRF-1 was knocked-down with siRNA. Induction of VCAM-1 and ICAM-1 by TNF-α was determined by Western blot analysis. Data were confirmed by three independent experiments.

the induction of VCAM-1 by TNF-α more than the IRF-1 mutant (**Figure 2A**). Then, we determined the exact subtype of GATAs involved in the mechanism. According to the references (*25−27*), we examined the involvement of GATA-4 and GATA-6 in the induction of VCAM-1 using siRNA against GATA-4 and GATA-6. Transfection with GATA-4 or GATA-6 siRNA did not affect ICAM-1 induction but suppressed the expression of VCAM-1 (**Figure 2B**). Moreover, the combination of GATA-4 and GATA-6 siRNA resulted in a little stronger inhibitory effect than GATA-4 or GATA-6 alone on VCAM-1 induction. Transfection with IRF-1 siRNA also affected only VCAM-1 induction by TNF-α (**Figure 2C**). Therefore, we suggest that anthocyanins inhibit TNF-α-induced VCAM-1 expression in a GATA (particularly GATA-4 and GATA-6)- and IRF-1-dependent manner.

Anthocyanins Inhibit TNF-α-Induced Nuclear Level of GATA-4, GATA-6, and IRF-1. Then, we further studied the regulation of GATA-4, GATA-6, and IRF-1 by anthocyanins. In a preliminary study, the maximum level of nuclear GATA-4 and GATA-6 or IRF-1 was detected 30 min or 2 h after TNF-α stimulation, respectively. Thus, cells were pretreated with anthocyanins for 24 h and cotreated with TNF-α for 30 min for the detection of nuclear GATA-4 and GATA-6, or for 2 h for the detection of nuclear IRF-1. Anthocyanins, in a dose-dependent manner, decreased the nuclear level of GATA-4. At high doses (100 μg/mL), they completely suppressed the nuclear level of GATA-4. They significantly inhibited the nuclear GATA-6 level in response to TNF-α stimulation even at low doses (10 μg/mL) (**Figure 3A**). Also, anthocyanins dose-dependently reduced nuclear IRF-1 levels and completely inhibited the nuclear level of IRF-1 at high doses (100 μg/mL) (**Figure 3A**). Then, we tested the effect of anthocyanins on VCAM-1 promoters with GATA and IRF-1 mutant motifs. In contrast to the inhibitory effect of anthocyanins on TNF-α-induced wild type VCAM-1 luciferase activity, anthocyanins could not completely inhibit the luciferase activity of mIRF-1 or mGATA. Moreover, anthocyanins failed to inhibit VCAM-1 luciferase activity of constructs with both mutations (mIRF-1&mGATA) (**Figure 3B**). These results suggest that the

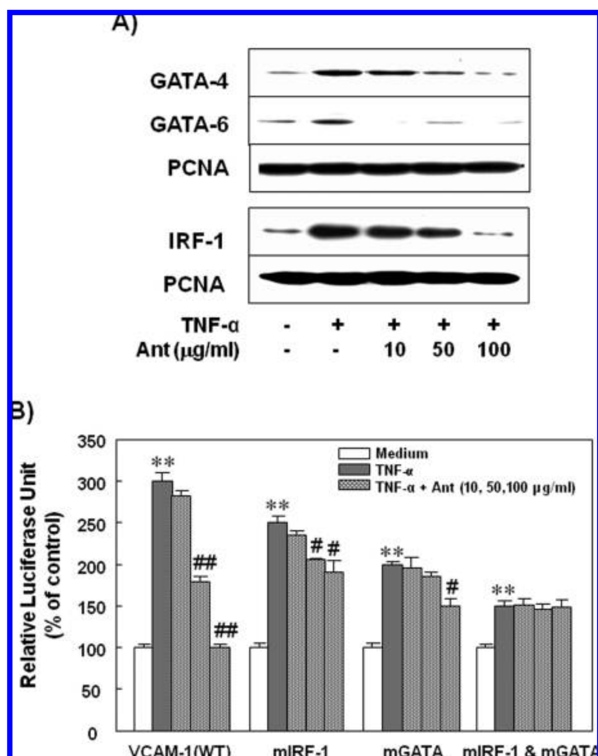

Figure 3. Inhibitory effect of anthocyanins on TNF-α-mediated VCAM-1 induction through the regulation of GATA-4, GATA-6, and IRF-1. (**A**) Cells were stimulated in a time-dependent manner with TNF-α. Thereafter, the nuclear fraction was extracted, and the GATA-4, GATA-6, or IRF-1 level was determined by Western blot analysis. Then, cells were pretreated anthocyanins for 24 h and treated with TNF-α for either 30 min (for the detection of GATA-4 or GATA-6) or 2 h (for the detection of IRF-1). (**B**) Cells were transfected with 1 μg of wild type VCAM-1-luciferase or VCAM-1-luciferase containing an IRF-1 motif mutant (mIRF-1), a GATA motif mutant (mGATA), or both as described in **Figure 2A**. Cells were stimulated with TNF-α (10 ng/mL) for 6 h with or without anthocyanins (10, 50, and 100 μg/mL). Data are presented as means ± SD from three independent experiments. Significance compared with control, $^{**}P < 0.01$; significance compared with TNF-α, $\#P < 0.05$; $\#\#P < 0.01$).

inhibitory effect of anthocyanins on VCAM-1 expression occurs through suppression of the IRF-1 and GATA transcription factors.

TNF-α-Induced VCAM-1 and ICAM-1 Expression Are Differentially Regulated by the Jak/STAT Inhibitor. We clearly showed that the contribution of GATA and IRF-1 elements in TNF-α-induced VCAM-1 expression. This may be the result of the activation of kinase cascades, which could lead to nuclear accumulation and increase of DNA-binding activity of GATA and IRF-1. Previously, we reported that ICAM-1 and VCAM-1 are differentially regulated by PKC and PI3K inhibitors (*28*). Additionally, in this study, we tested the effect of a well-characterized Jak inhibitor, AG490, at doses from 1 to 30 μM. As shown in **Figure 4**, AG490, in a dose-dependent manner, inhibited VCAM-1 protein induction (**Figure 4A**) and luciferase activity (**Figure 4B**). Interestingly, TNF-α-induced ICAM-1 expression was not affected by AG490 at any dose. To confirm whether the Jak/STAT pathway affects VCAM-1 expression through the GATA and IRF-1 motifs, we performed a VCAM-1 luciferase assay with wild type and mutated forms of VCAM-1 (**Figure 4C**). Our data demonstrate that AG490 (a Jak/STAT inhibitor) had a very significant inhibitory effect on VCAM-1 (WT)-luciferase activity (activity decreased from 3-fold to 1.7-fold). Cells transfected

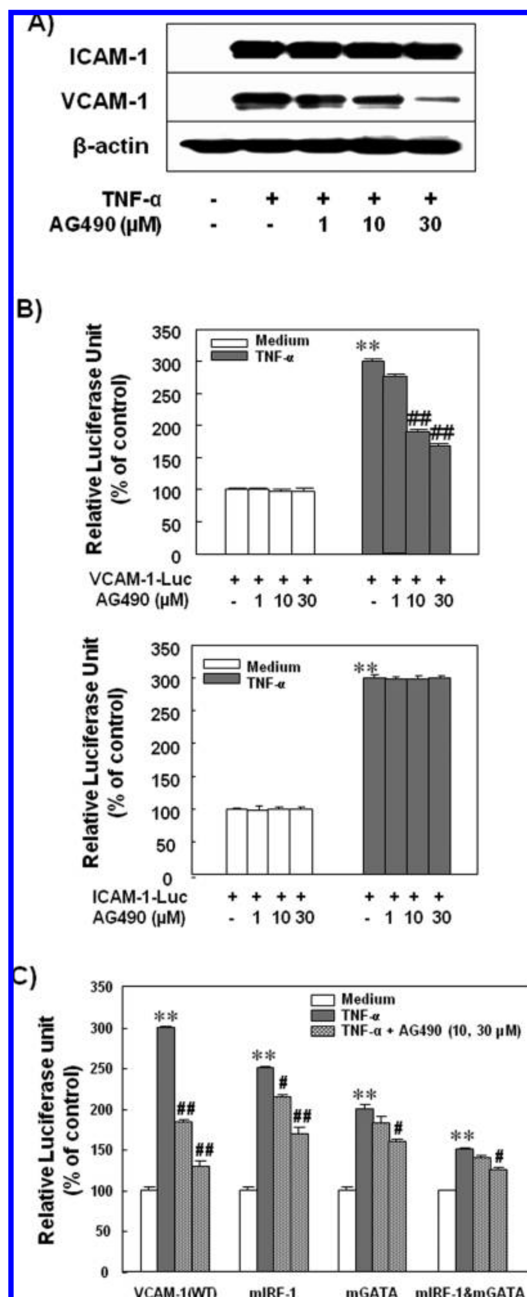

Figure 4. Differential role of the Jak/STAT pathway on ICAM-1 and VCAM-1 production induced by TNF-α. (**A**) Cells were pretreated with the Jak kinase inhibitor AG490 (1, 10, and 30 μM) for 1 h and later cotreated with TNF-α for 6 h for the detection of ICAM-1 and VCAM-1 protein expression by Western blot analysis. Data were confirmed by three independent experiments. (**B**) VCAM-1 or ICAM-1 luciferase activity was determined as described in the legend for **Figure 1B**. Cells were pretreated with the Jak kinase inhibitor AG490 (1, 10, and 30 μM) for 1 h before treatment with anthocyanins. Data are presented as means ± SD from three independent experiments. Significance compared with control, $^{**}P < 0.01$; significance compared with TNF-α, $\#\#P < 0.01$. (**C**) Cells were transfected with 1 μg of wild type VCAM-1 or VCAM-1 containing an IRF-1 motif mutant (mIRF-1), GATA motif mutant (mGATA), or both as described in **Figure 2A**. Before stimulation with TNF-α, cells were pretreated with AG490 (10 or 30 μM). Data are presented as means ± SD from three independent experiments. Significance compared with control, $^{**}P < 0.01$; significance compared with TNF-α, $\#P < 0.05$; $\#\#P < 0.01$.

with mIRF-1, mGATA, or both mIRF-1 and mGATA showed very weak effects of these inhibitors on TNF-α-induced luciferase

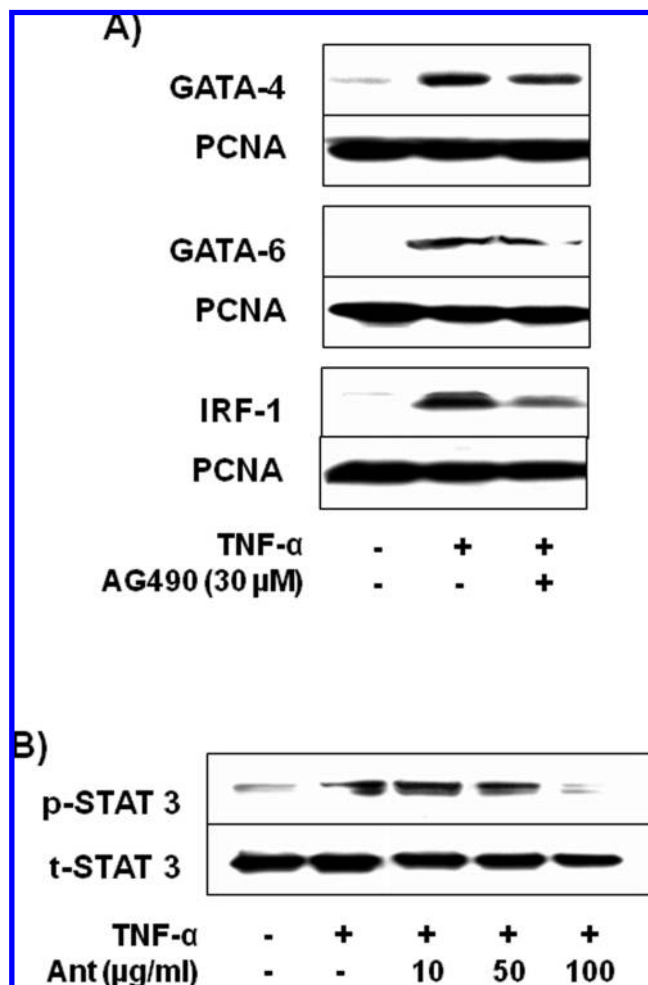

Figure 5. (**A**) Effects of the Jak/STAT signaling pathways on GATA-4, GATA-6, and IRF-1 activation by TNF-α in HUVECs. Cells were pretreated with AG490 (30 μM) for 1 h and later cotreated with TNF-α for either 30 min (for the detection of GATA-4 and GATA-6) or 2 h (for the detection of IRF-1). (**B**) Effect of anthocyanins on STAT-3 activation by TNF-α in HUVECs. Cells were pretreated with anthocyanins (10, 50, and 100 μg/mL) for 24 h and then treated with TNF-α for 1 h (for the detection of phosphor-STAT-3). The nuclear fraction or cell lysate was extracted, and protein levels were determined by Western blot analysis. Data were confirmed by three independent experiments.

activity (activity decreased from 1.5-fold to 1.25-, 1.3-, and 1.4-fold, respectively). These results suggest that the Jak/STAT inhibitor inhibits VCAM-1 in a GATA- and/or IRF-1-dependent manner.

Involvement of Jak/STAT in the Activation of GATA-4 and GATA-6 in TNF-α-Stimulated HUVECs. We further examined the effect of the Jak/STAT pathway on the nuclear level of GATA-4 and GATA-6 increased by TNF-α. HUVECs were pretreated with the inhibitor for 1 h and then cotreated with TNF-α for either 30 min (for the detection of GATA proteins) or 2 h (for the detection of IRF-1). This inhibitor reduced the nuclear GATA-4, GATA-6, and IRF-1 levels increased by TNF-α (**Figure 5A**), suggesting that the Jak/STAT signaling pathway is involved in the regulation of GATA-4, GATA-6, and IRF-1. Next, we determined whether the effects of anthocyanins on VCAM-1 expression were due to the inhibition of the Jak2/STAT3 signaling pathway. In preliminary data, we examined the effect of anthocyanins on STAT-1 phosphorylation by TNF-α; however, anthocyanins did not show significant inhibition of TNF-α-induced STAT-1 phosphorylation. After pretreatment with anthocyanins for 24 h, HUVECs were exposed to 10 ng/mL TNF-α for 1 h (to detect STAT-3 phosphorylation). **Figure 5B** shows that anthocyanins significantly inhibited phosphorylation of STAT-3 in TNF-α-stimulated HUVECs. These findings suggest that TNF-α-induced activation of STAT-3 is differentially involved in the expression of VCAM-1 and ICAM-1; furthermore, these pathways can be regulated by anthocyanins.

DISCUSSION

A potential role of specific cell adhesion molecules (CAMs) in different disease states including cardiovascular disease, cancer, inflammatory disease, ocular pulmonary disease, bone disease, central nervous system disease, kidney disease, and gastrointestinal system disease have been implicated. Besides their key function in various pathological processes, CAMs are ubiquitously expressed proteins playing a central role in physiological maintenance of tissue integrity. Thus, although compounds that suppress the expression of several of the CAMs may be more effective at blocking leukocyte adhesion in a variety of inflammatory settings, reducing the expression of all endothelial cell adhesion molecules (ECAMs) may cause detrimental side effects.

VCAM-1 has been reported to play a major role in the initiation of atherosclerosis (*6*). In addition, many human tumor cells do not express β3 integrin (ICAM-1 receptor), and only the highly metastatic melanoma cell lines MV3 and BLM (*4*) but not the low metastatic cell lines IF-6 and 530 bind to VCAM-1 via the α4β1 integrin (VCAM-1 receptor). Therefore, blocking VCAM-1 on endothelial cells may be a very valuable approach for interfering with tumor metastasis and the atherosclerotic process.

Recently, it has been reported that anthocyanins suppress VCAM-1 but not ICAM-1 expression (*29*). However, the report did not suggest the molecular mechanisms where anthocyanins differentially regulate VCAM-1 but not ICAM-1. In the present study, we have found that (1) anthocyanins from black soybean seed coats differentially regulate adhesion molecule expression by TNF-α; anthocyanins dramatically inhibit TNF-α-induced VCAM-1 expression, but not ICAM-1 expression; (2) anthocyanins fail to suppress mIRF-1 and/or mGATA luciferase activity, thus suggesting that their inhibitory effect on VCAM-1 arises because of IRF-1- and GATA-dependent mechanisms; (3) GATA-4, GATA-6, and IRF-1 are important in the induction of VCAM-1, and anthocyanins significantly inhibit such protein expression in HUVECs; (4) the Jak/STAT-3 signaling pathway is upstream in the regulation of IRF-1 and GATA transcription factors; and (5) anthocyanins markedly suppress phosphorylation of STAT-3 in TNF-α-stimulated HUVECs. Our study showed for the first time that anthocyanins can differentially regulate TNF-α-induced VCAM-1 and ICAM-1 expression and suggests possible mechanisms by which anthocyanins accomplish this differential regulation of VCAM-1 and ICAM-1 induced by TNF-α.

It has been reported that there are several GATA subtypes; GATA-1 was reported to be absent in HUVECs (*26*), and mRNA levels of GATA-6, GATA-2, and GATA-3 but not GATA-5 and GATA-4 were detected in HUVECs (*25*). In response to TNF-α, the mRNA expression of GATA-2 very slightly increased, and the level of GATA-6 significantly increased. In contrast, expression of GATA-3 was reduced by TNF-α. However, GATA-4 expression was detected in the mouse heart. The GATA-4 transcription factor was recently reported to be important in the regulation of αT-catenin, a member of the α-catenin family of cell–cell adhesion molecules (*27*). Therefore, to determine (1) whether anthocyanins inhibit VCAM-1 expression via a GATA-dependent

mechanism and (2) the exact subtype of GATAs involved the mechanism, we further studied the regulation of GATA-6 by anthocyanins. Interestingly, we successfully detected protein expression of GATA-4 in TNF-α-stimulated HUVECs. Thus, we also selected GATA-4 for our study.

Anthocyanins from several sources are a group of naturally occurring phenolic compounds and are known to have health-promoting benefits including potent antioxidant effects, antiinflammatory effects, and the reduction of risk of coronary heart disease. Especially, anthocyanins from black soybean seed coats have been reported to have antiproliferative activity in human colon adenocarcinoma cells (*30*). The present study suggests that anthocyanins from black soybean seed coats have an inhibitory effect of VCAM-1 expression, which is promising for its use as a therapeutic treatment for pathological processes involving VCAM-1 (e.g., atherosclerosis and metastases) without severe side effects.

ABBREVIATIONS USED

ANOVA, analysis of variance; AP-1, activator protein-1; EC, endothelial cell; ECGS, endothelial cell growth supplements; ECL, enhanced chemoluminescence; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IRF, interferon regulatory factor; Jak/STAT, janus kinase/signal transducer and activator of transcription; NF-κB, nuclear factor-kappa B; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SP-1, specificity protein-1; TBS-T, Tris-buffered saline/Tween 20; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

SAFETY

Anthocyanins have no toxicity when they are exposed to humans and animals.

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