

## Genipin Selectively Inhibits TNF- $\alpha$ -activated VCAM-1 But Not ICAM-1 Expression by Upregulation of PPAR- $\gamma$ in Human Endothelial Cells

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Vascular inflammation process has been suggested to be an important risk factor in the development of atherosclerosis. Recently we reported that induction of peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) selectively inhibits vascular cell adhesion molecule-1 (VCAM-1) but not intercellular cell adhesion molecule-1 (ICAM-1) in tumor necrosis factor (TNF)- $\alpha$ -activated human umbilical vein endothelial cells (HUVEC). In this study, we investigated whether genipin inhibits expression of cellular adhesion molecules, which is relevant to inflammation. Pretreatment with genipin reduced reactive oxygen species (ROS) production and expression of VCAM-1, but not ICAM-1 in TNF- $\alpha$ -activated HUVEC. Genipin dose- and time-dependently increased PPAR- $\gamma$  expression and inhibited TNF- $\alpha$ -induced phosphorylation of Akt and PKC with different degrees. Finally, genipin prevented TNF- $\alpha$ -induced adhesion of U937 monocytic cells to HUVEC. Taken together, these results indicate that upregulation of PPAR- $\gamma$  by genipin selectively inhibits TNF- $\alpha$ -induced expression of VCAM-1, in which regulation of Akt and/or PKC play a key role. We concluded that genipin can be used for the treatment of cardiovascular disorders such as atherosclerosis.

**Key Words:** Atherosclerosis, Adhesion molecules, Peroxisome proliferator-activated receptor, Endothelial cells, Tumor necrosis factor

### INTRODUCTION

Vascular inflammatory disorders such as atherosclerosis and stroke are the most common causes of death in humans. Although the pathophysiology of atherosclerosis is not completely understood, inflammation is regarded to play a major role in its development [1,2]. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), one of known inflammatory cytokines, plays a major role for systemic inflammation and immune responses. TNF- $\alpha$  can induce inflammatory responses in the vascular endothelium by enhancing the expression of adhesion molecules and the secretion of inflammatory mediators [3,4]. We [5] and others [6] suggested that activators of peroxisome proliferator-activated receptor (PPAR) can selectively inhibit the induction of vascular cell adhesion molecule-1 (VCAM-1), but not intercellular cell adhesion molecule-1 (ICAM-1) in human umbilical vein endothelial

cells (HUVEC). Moreover, we [7,8] suggested that PI3K/PTEN, mitogen-activated protein kinases (MAPK) signals also differentially regulate the expression of adhesion molecules in lipopolysaccharide- or TNF- $\alpha$ -treated EC. Therefore, it is at least theoretically possible that any chemical which modifies activities of PPAR or PI3K or MAP kinases in EC can selectively inhibits VCAM-1 expression. During the screening of antioxidant and anti-inflammatory activity of traditional herbs, we isolated and identified genipin (Fig. 1) from *Gardenia jasminoides* Ellis [9]. Genipin has long been used in oriental countries to treat inflammation, jaundice and hepatic disorders [10]. Although genipin exerts remarkable anti-inflammatory and antiangiogenic effects [11], no information on the expression of adhesion molecules in HUVECs, which play an important role in the development of atherosclerosis, is available so far. Thus the aim of the present study was to elucidate whether genipin inhibits adhesion molecules in HUVECs when activated with TNF- $\alpha$ , which can provide scientific evidence for the traditional use of genipin in the treatment of inflammatory disorders. We found that genipin showed upregulation of

Received May 2, 2011, Revised June 14, 2011,  
Accepted June 15, 2011

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**ABBREVIATIONS:** DMEM, dulbecco's modified eagle medium; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular cell adhesion molecule-1; MAPK, mitogen-activated protein kinases; PPAR, peroxisome proliferator-activated receptor; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

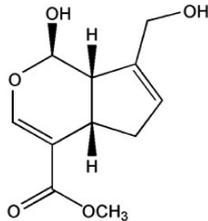


Fig. 1. Chemical structure of genipin.

PPAR- $\gamma$  as well as inhibition of P-Akt and P-PKC which resulted in selective inhibition of expression of VCAM-1 but not ICAM-1 in HUVECs activated with TNF- $\alpha$ .

## METHODS

### Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) were purchased from Gibco-BRL (Rockville, MD). Anti-ICAM-1, anti-VCAM-1, anti-PPAR- $\gamma$ , anti-phospho-ERK1/2 and anti- $\beta$ -actin antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA), anti-p-Akt and anti-p-PKC antibodies were obtained from Cell Signaling Technology (Beverly, MA). All other chemicals were supplied by Sigma-Aldrich (St. Louis, MO). Genipin was isolated from dried fruits of *Gardenia jasminoides* Ellis as described [9].

### Cell culture

Human umbilical endothelial cells (EA.hy 926 cells) were obtained from ATCC and grown in the DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 10  $\mu$ g/ml streptomycin. Cells were cultured in 100 mm dishes and grown in a humidified 5% CO<sub>2</sub> incubator. Cells were plated at a density of  $1 \times 10^7$  cells per 100 mm dish. Cells were used between passage numbers 6 and 12. For adhesion assay, U937 human monocyte was obtained from Korea Cell Line Bank (KCLB, Seoul, Korea) and grown in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 25 mM HEPES, 25 mM NaHCO<sub>3</sub>, 100 IU/ml penicillin, and 10  $\mu$ g/ml streptomycin.

### MTT assay

Cell viability was determined colorimetrically using the MTT assay. Cells in the exponential phase were seeded at  $1 \times 10^4$  cells per well in 24-well plates. After different treatments, 20  $\mu$ l of 5 mg/ml MTT solution was added to each well (0.1 mg/well), and wells were incubated for 4 h. The supernatants were aspirated, the formazan crystals in each well were dissolved in 200  $\mu$ l of dimethyl sulfoxide for 30 min at 37°C, and optical density at 570 nm was read on a Microplate Reader (Bio-Rad, Hercules, CA).

### Western blot analysis

HUVECs were treated with TNF- $\alpha$  (10 ng/ml) and/or tested compound for 24 h. The cells were then washed two

times with cold PBS and lysed in RIPA buffer (PBS supplemented with 1% NP40, 0.5% sodium deoxycholate, 1 mmol/l phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml aprotinin, and 1 mmol/l sodium orthovanadate). The cell lysates were then incubated at 4°C for 30 min, after which they were cleared by centrifugation at  $10,000 \times g$  for 10 min. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, Rockford, IL). The proteins were then resolved by SDS-PAGE. The gels were transferred to polyvinylidene difluoride (PVDF) membranes by semidry electrophoretic transfer at 15 V for 60~75 min. The PVDF membranes were blocked overnight at 4°C in 5% bovine serum albumin (BSA). The cells were incubated with primary antibodies (PPAR- $\gamma$ , ICAM-1, VCAM-1, p-ERK1/2, p-PKC, p-Akt, and  $\beta$ -actin) diluted 1 : 500 in Tris-buffered saline/Tween 20 (TBS-T) containing 5% BSA for 2 h and then incubated with the secondary antibody at room temperature for 1 h. Anti-rabbit IgG was used as the secondary antibody (1 : 5,000 dilution in TBST containing 1% BSA). The signals were detected by ECL (Amersham, Piscataway, NJ).

### U937 cell adhesion assay

Cells were seeded into two-well chamber slides 48 h before experiments. The medium was refreshed before stimulation with TNF- $\alpha$ . U937 monocytes ( $3 \times 10^7$ ) were incubated in RPMI 1640 medium containing 2% FBS and 10 mg/ml of the fluorescent dye BCECF/AM (Boehringer, Mannheim, Germany) at 37°C for 30 min, as described previously [7]. Fluorescence-labeled cells were pelleted and resuspended ( $7.5 \times 10^7$ /ml) DMEM. Cells were washed three times with DMEM before addition of loaded cells and incubated at 37°C. After 30 min, cell suspensions were withdrawn and cells were gently washed with DMEM. Fluorescent images were selected using a high-resolution video camera (DXC-960MD; Sony) mounted on a BH-2 Olympus microscope (Melville, NY). We then picked images of 0.2 mm in width within these first selected areas and the immunoreactivity of these was measured using SigmaGel 1.0 (Jandel Scientific, Germany). Analyses were repeated three times over the same region and the results are the means of three independent experiments.

### ROS production assay

The intracellular generation of ROS was monitored using dichlorofluorescein diacetate (DCFH-DA), a fluorescent dye. Inside the cells, this compound is oxidized by ROS to form a fluorescent carboxydichlorofluorescein. Briefly, cells were pretreated with different concentration of each compound during 30 min then incubated with or without TNF- $\alpha$  at 10 min, cells were suspended with 1xPBS then DCFH-DA was added at the final concentration of 10  $\mu$ M. Fluorescence was monitored at the excitation and emission wavelength of 485 and 530 nm, respectively using a fluorescence plate reader (50 cycles per 20 s at 37°C). Data were expressed as relative changes to the initial fluorescence.

### Statistical analysis

The data were expressed as the means $\pm$ SD. ANOVA and Student's *t*-test were applied to determine the statistical significance with a  $p < 0.05$ .

## RESULTS

### Cell viability

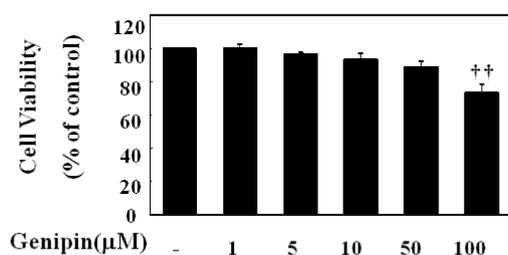
Fig. 2 shows that anti-proliferative effect of genipin. When cell proliferation was measured by MTT assay, genipin did not show harmful effect from 1 to 50  $\mu$ M concentration. However, at 100  $\mu$ M it reduced cell survival rate about 15~30%. Therefore, concentration was limited to maximum of 50  $\mu$ M.

### Effects on ROS production

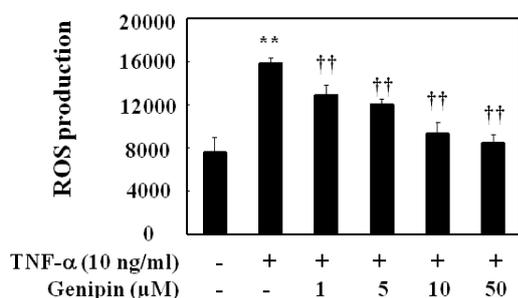
To examine ROS production, cells were treated with genipin with various doses (1, 5, 10, 50  $\mu$ M) along with TNF- $\alpha$  (10 ng/ml) which were incubated for 10 min. Fig. 3 shows that the TNF- $\alpha$  significantly increased production of ROS, which was significantly reduced by depending on genipin concentrations.

### Effects on TNF- $\alpha$ -induced VCAM-1 and ICAM-1 expression

Although genipin showed anti-inflammatory action [9,11], no report is available on adhesion molecules expression. So,



**Fig. 2.** Anti-proliferative effect of genipin in HUVECs. HUVECs were treated as indicated concentrations of genipin for 24 h. Cell proliferation was then assessed by MTT assay. The data were expressed as the means $\pm$ SD of three independent experiments. ††p<0.01 compared with control.



**Fig. 3.** Inhibition of ROS production in TNF- $\alpha$ -activated HUVECs. Different concentration of genipin was added 30 min prior to TNF- $\alpha$  and further incubated for 10 min. Cells were suspended and then DCFH-DA was added at the final concentration of 10  $\mu$ M. Fluorescence was monitored at the excitation and emission wavelength of 485 and 530 nm, respectively using a fluorescence plate reader (50 cycles per 20 s at 37°C). Results were expressed as relative changes to the initial fluorescence. The data were expressed as the means $\pm$ SD of three independent experiments. \*\*p<0.01 compared with control. ††p<0.01, compared with TNF- $\alpha$ .

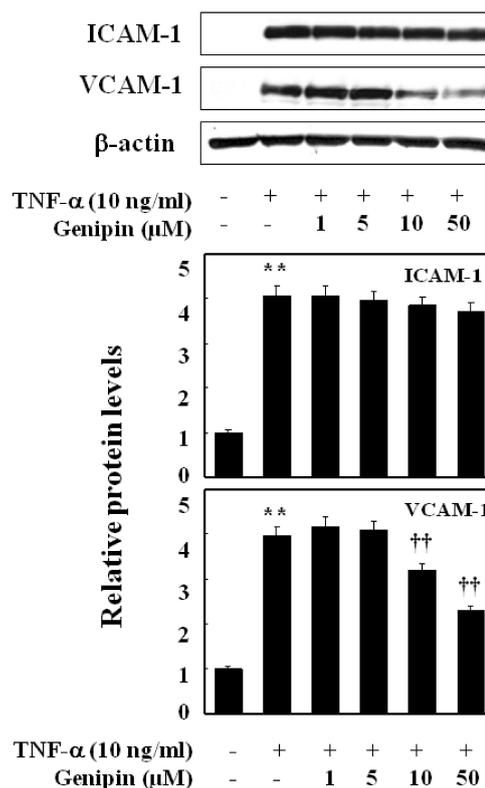
we asked whether genipin inhibits adhesion molecules in EC activated with TNF- $\alpha$ . As shown in Fig. 4, genipin significantly inhibited TNF- $\alpha$ -activated VCAM-1 but not ICAM-1 expression.

### Involvement of PPAR- $\gamma$ in anti-inflammatory effect

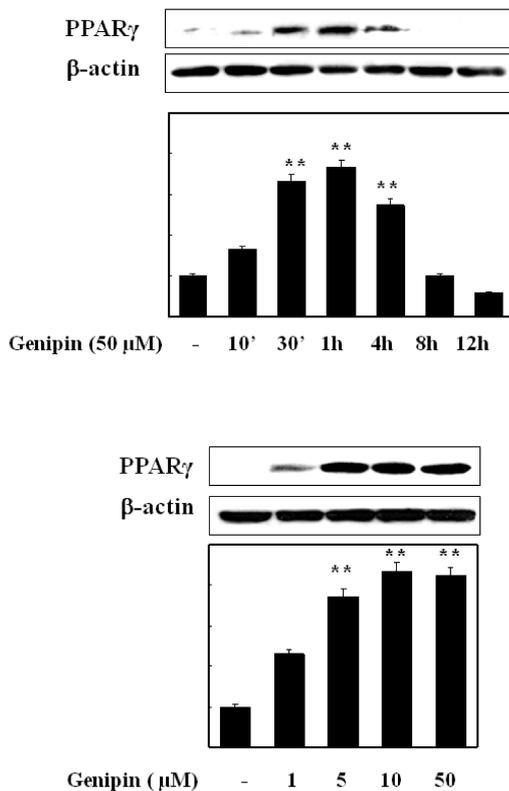
Then we asked whether the differential regulation of expression of adhesion molecules involves PPAR activation as suggested by Jackson et al [6]. Fig. 5 shows that genipin dose- and time-dependently increased PPAR- $\gamma$  expression in HUVEC.

### Effect on phosphorylation of ERK1/2, Akt, and PKC

In addition to PPAR-activators, signal molecules and kinases such as PI3K/Akt, PKC, and ERK1/2 inhibitors also showed differential effects on ICAM-1 and VCAM-1 expression [7,8]. Therefore, we explored the effect of genipin on the TNF- $\alpha$ -induced phosphorylation of these signal molecules. Fig. 6 shows that genipin significantly inhibited phosphorylation of PKC and Akt but not ERK.



**Fig. 4.** Preferential inhibition of TNF- $\alpha$ -mediated induction of VCAM-1 over ICAM-1. HUVECs were pretreated with genipin for 1 h and then treated with TNF- $\alpha$  for 6 h. 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. The expression levels of ICAM-1, VCAM-1 protein were quantified by densitometer. Data are presented as means $\pm$ SD from three independent experiments. Significance compared with control. \*\*p<0.01 compared with control. ††p<0.01, compared with TNF- $\alpha$ .



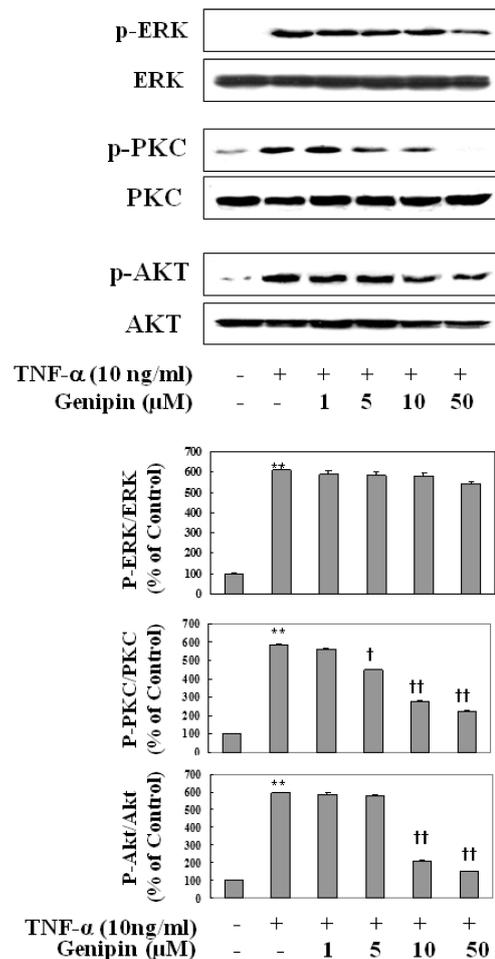
**Fig. 5.** Time- and concentration-dependent induction of PPAR- $\gamma$  protein in TNF- $\alpha$ -induced HUVECs. Cells were treated with different time period with fixed concentration (upper) or different concentration (lower) of genipin. Proteins were isolated and subjected to Western blot for PPAR- $\gamma$  expression. Data were confirmed by three independent experiments. \*\* $p < 0.01$  compared with control.

#### Inhibition of TNF- $\alpha$ stimulated adhesion of monocytes to HUVECs

Finally, we asked whether genipin inhibits adhesion of monocytes to HUVEC due to inhibition of VCAM-1 expression. As shown in Fig. 7, adhesion of human monocytes (U937) to HUVEC increased by 6-fold after stimulation with TNF- $\alpha$ , which was significantly and concentration-dependently reduced by the presence of genipin.

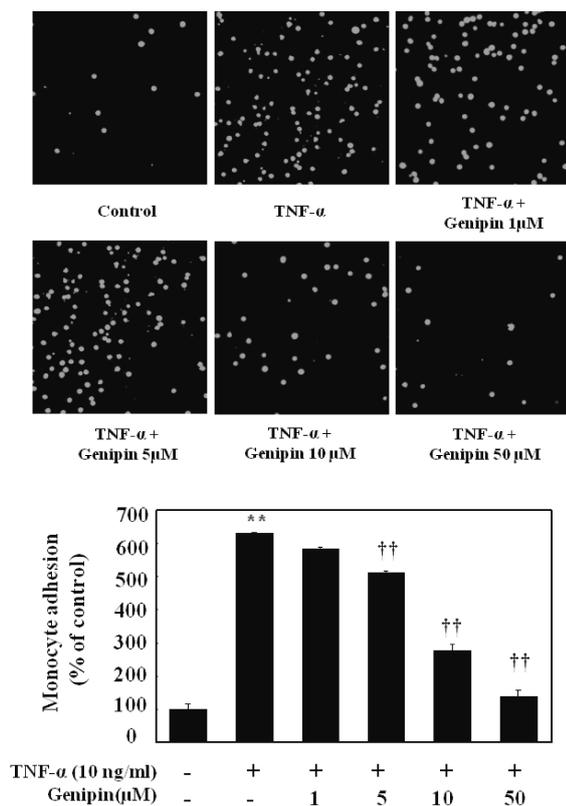
## DISCUSSION

Although genipin showed antioxidant and anti-inflammatory action, no report is available so far on the expression of PPAR- $\gamma$  induction and/or adhesion molecules in human ECs. Therefore, we investigated to gain scientific evidence why extract of fruit of gardenia (*Gardenia jasminoides* Ellis) has been used for chronic inflammatory disorders [10]. In the present study, we clearly demonstrated that genipin isolated from *Gardenia jasminoides* inhibited the expression of TNF- $\alpha$ -induced VCAM-1 protein but not ICAM-1 in EA, hy 926 cells. Previous studies have suggested genipin has remarkable anti-inflammatory and anti-angiogenic effect [11]. A critical step in initiation and progression of atherosclerosis is leukocyte infiltration through



**Fig. 6.** Effect on phosphorylation of ERK1/2, Akt, and PKC activation by TNF- $\alpha$  in HUVECs. Cells were pretreated with different concentration of genipin (1, 5, 10 and 50  $\mu$ M) for 24 h, and then treated with TNF- $\alpha$  for 10 min for detection of phospho-ERK1/2 and PKC or for 30 min for detection of phospho-Akt. Cells were extracted and protein level was detected by Western blot analysis (upper). The blot was quantified by using densitometry and represented as % increase of control (lower). \*\* $p < 0.01$  compared with control. † $p < 0.05$ , †† $p < 0.01$ , compared with TNF- $\alpha$ , respectively. Data were confirmed by two independent experiments.

vascular endothelium into the vessel wall [12]. Inflammation of vascular EC causes induction of adhesion molecules and promotes leukocyte interaction [13]. Expression of adhesion molecules in EC is also essential for angiogenesis in the vasculature. Can inhibition of VCAM-1 by genipin be responsible for traditional use of this chemical for anti-inflammatory and anti-angiogenic effect? If so, then, what is the possible mechanism for the selective inhibition of TNF- $\alpha$ -induced expression of VCAM-1 in HUVEC by genipin? Given PPAR- $\gamma$  activators can decrease expression of adhesion molecules in activated human endothelial cells [6,14,15] and PPAR- $\gamma$  is expressed in vascular cells [16], we asked whether genipin induces PPAR- $\gamma$  in HUVEC. We found that genipin up-regulated PPAR- $\gamma$  expression in HUVEC by a concentration- and time-dependent manner. Although exact mechanism(s) by which genipin exerts selective in-



**Fig. 7.** Effect on adhesion of monocytes to HUVECs stimulated with TNF- $\alpha$ . Cells were pretreated with different concentrations of genipin (1, 5, 10 and 50  $\mu$ M) and then stimulated with 10 ng/ml TNF- $\alpha$  for 6 h. Thereafter, cells were coincubated with fluorescently labeled monocytic cells for 30 min at 37°C. Monocyte adhesion was presented as images and a percentage of U937 cells bound to TNF- $\alpha$ -untreated cells (control). Data represent mean  $\pm$  SD from three independent experiments. \*\* $p$  < 0.01, compared with control, †† $p$  < 0.01 compared with TNF- $\alpha$ , respectively.

Inhibition of TNF- $\alpha$ -induced VCAM-1 expression in HUVECs remains unclear. Induction of PPAR- $\gamma$ , at least, can be a possible mechanism for differential inhibition. Or inhibition of phosphorylation of Akt and PKC could be involved in selective inhibition of VCAM-1 expression. These speculations come from our previous reports that PI3K/Akt and PKC signaling pathways are involved in VCAM-1 induction but not ICAM-1 in HUVECs [7,8]. Therefore, inhibition of phosphorylation of these signal molecules (Akt, PKC) can be another possible mechanism for this differential expression. As shown in the present study, phosphorylation of Akt and p-PKC by TNF- $\alpha$  was concentration-dependently inhibited by genipin. However, it remains further study as to the link between inhibition of these signal molecules and PPAR- $\gamma$  induction.

It seems likely that PKC and Akt signals are important regulators on differential regulation of adhesion molecules by genipin as triterpenoid glycosides do [5]. Lastly, some transcription factors such as GATA or IRF-1 [17,18] also can be target molecules for genipin. Since these transcription factors are not present in ICAM-1 promoters, so these can be target molecules by many medicinal compounds as we demonstrated that anthocyanins isolated

from black soy bean coat selectively inhibited VCAM-1 but not ICAM-1 expression in TNF- $\alpha$ -activated HUVECs by inhibiting GATA-2, GATA-4, and IRF-1 [18]. It should be noted that TNF- $\alpha$  could activate NF- $\kappa$ B in endothelial cells via oxidative stress [19]. Genipin reduced TNF- $\alpha$ -induced ROS production in HUVECs by a fluorescent probe DCFH-DA assay. These results suggest that the inhibitory effect of genipin on adhesion molecule expressions may be due to ROS reduction possibly through inhibition of NF- $\kappa$ B activity, which needs further investigation.

Finally, the inhibitory effect on the adhesion of monocytic U937 cells to TNF- $\alpha$ -treated HUVECs was shown by genipin. This inhibitory effect on the adhesion of monocytes to vascular endothelium seems likely to contribute to the anti-atherosclerotic activity of genipin. Although expression of both VCAM-1 and ICAM-1 is upregulated in endothelial cells by TNF- $\alpha$ , it is indicated that VCAM-1 plays a dominant role in the initiation of atherosclerosis [20]. Thus, genipin may inhibit adhesion of monocytes without hampering on ICAM-1 expression in HUVECs by TNF- $\alpha$ .

Even though we did not evaluate *in vivo* effect of genipin, Kim et al [9] nicely demonstrated that administration of genipin protected lipopolysaccharide-induced liver damage in mice, indicating that genipin showed anti-inflammatory action *in vivo*, too. We are under investigation this protective effect is due to PPAR- $\gamma$  induction *in vivo*.

In conclusion, we clearly demonstrated that genipin selectively inhibited VCAM-1 but not ICAM-1 expression in TNF- $\alpha$ -activated HUVECs via activating PPAR- $\gamma$ . Although the precise mechanism of action awaits further investigation, we provide scientific evidence why extract of gardenia has been used in chronic inflammatory disorders [11]. Therefore, we propose that genipin can be used for the treatment of pathologic inflammatory disorders such as atherosclerosis.

## ACKNOWLEDGEMENTS

This work was supported by a grant from the Korea Food and Drug Administration for Studies on the Identification of the Efficacy of Biologically Active Components from Oriental Herbal Medicines.

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