Silymarin inhibits TNF-α-induced expression of adhesion molecules in human umbilical vein endothelial cells

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Abstract Silymarin is known to have an anti-atherosclerotic activity, but the mechanism responsible for it remains unclear. Here, we demonstrate a possible mechanism involved in the anti-atherosclerotic activity of silymarin. Silymarin inhibited THP-1 cell adhesion to human umbilical vein endothelial cells (HUVECs). Silymarin also suppressed the TNF-α-induced protein and mRNA expression of adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin, in HUVECs. Moreover, silymarin suppressed the TNF-α-induced DNA binding of NF-κB/Rel in HUVECs. Taken together, these results demonstrate that silymarin exerts an anti-atherosclerotic activity, at least in part, by inhibiting the expression of adhesion molecules.

Key words: Silymarin; VCAM-1; ICAM-1; E-selectin; NF-κB/Rel

1. Introduction

Atherosclerosis and its complications are the most common health problem in the developed countries. Atherosclerosis was considered as a lipid storage disease, but it is now regarded as a chronic inflammatory disease and characterized by the infiltration of mononuclear lymphocytes into the intima [1]. The adhesion of monocytes to the arterial wall and their subsequent infiltration and differentiation into macrophages are key events in the development of atherosclerosis [2].

The recruitment of monocytes to the arterial wall is mediated by cell adhesion molecules expressed on endothelial cells in response to inflammatory stimuli. The most important cell adhesion molecule involved in this process is vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 interacts with the monocyte integrin VLA-4 and is expressed on endothelial cells at the sites of inflammation [3–5]. Intercellular adhesion molecule-1 (ICAM-1) is also implicated in the adhesion of monocytes to endothelial cells, and Collins and coworkers reported that deletion of the ICAM-1 gene resulted in significant reductions in monocyte recruitment to atherosclerotic lesions in apo E-deficient mice [6]. It has been reported that deletion of the gene of E-selectin, another key cell adhesion molecule in apo E-deficient mice, resulted in a substantial decrease in the severity of atherosclerosis [7].

Nuclear factor-κB/Rel (NF-κB/Rel) is known to play a critical role in the development of inflammatory response by upregulating the expression of many inflammatory mediators [8]. It has also been reported that the transcriptional activation of NF-κB/Rel is important for the expression of proinflammatory cell adhesion molecules listed above. Iademarco and coworkers characterized two distinct NF-κB/Rel binding sites in the upstream of VCAM-1 gene and showed that their responsiveness is cell-specific [9]. NF-κB/Rel binding sites were also identified in the upstream of the transcription start sites of the ICAM-1 and E-selectin genes [10,11].

Silymarin is a flavonoid antioxidant isolated from the fruits and seeds of the milk thistle, *Silybum marianum* [12]. Silymarin has been clinically used to treat various liver diseases because of its hepatoprotective effect [13,14]. In addition, recent studies demonstrated an anti-inflammatory effect of silymarin [15,16]. It has been reported that silymarin also has an anti-atherosclerotic effect [17] and its anti-atherosclerotic activity of silymarin is mediated by the inhibition of low density lipoprotein (LDL) oxidation and cholesterol biosynthesis [18,19].

The objective of the present study was to investigate the effect of silymarin on monocyte adhesion to endothelial cells and the expression of cell adhesion molecules and to elucidate the mechanism involved in the anti-atherosclerotic effect of silymarin.

2. Materials and methods

2.1. Chemicals and cell culture

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Silymarin was dissolved in dimethyl sulfoxide and freshly diluted in culture media for all experiments. The human umbilical vein endothelial cells (HUVECs) were grown in EGM-2 Bulletkit (Clonetics, BioWhittaker, Inc., MD, USA) and the human monocyte cell line THP-1 cells (ATCC TIB202) were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 humidified air.

2.2. Cell adhesion assay

Assay was performed as described previously [20]. Briefly, HUVECs were plated in 48-well plate, grown to 70% confluence and pretreated with silymarin (6.25, 12.5, 25 or 50 μg/ml) for 1 h, followed by stimu-

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Abbreviations: VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; HUVECs, human umbilical vein endothelial cells; NF-κB/Rel, nuclear factor-κB/Rel; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-polymerase chain reaction

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lated with TNF-α (10 ng/ml) for 24 h. The cells were rinsed three times with serum free medium, and THP-1 cells were added to each well. After 1 h incubation, the non-adherent THP-1 cells were rinsed off and the adherent cells were collected by treatment with Trypsin-EDTA for 1 min and counted directly under a light microscope. Monoclonal antibodies for functional blocking of adhesion molecules were purchased from R&D systems, Inc (Minneapolis, MN, USA).

2.3. Modified enzyme-linked immunosorbent assay (ELISA) for measurement of the cell surface expressions of adhesion molecules

The cell surface expressions of VCAM-1, ICAM-1 and E-selectin on HUVECs were quantified using cell-ELISA by modification of previously published methods [21]. Briefly, HUVECs were grown to 70% confluence in 96-well, gelatin-coated plates and treated with silymarin (6.25, 12.5, 25 or 50 μg/ml) for 1 h before being treated with TNF-α (10 ng/ml) for 24 h. Following incubation, the cells were washed with phosphate-buffered saline pH 7.4 (PBS) and fixed with 4% paraformaldehyde for 30 min at 4°C. Non-fat dry milk (3.0% in PBS) was added to the monolayers to reduce non-specific binding. After washing three times with PBS, cells were incubated with anti-VCAM-1, anti-ICAM-1 or anti-E-selectin monoclonal antibody overnight at 4°C, washed with PBS and followed by incubation with peroxidase-conjugated goat anti-mouse secondary antibody. Following this, the cells were washed with PBS and exposed to the peroxidase substrate. Absorbance was determined at 490 nm by an automated microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

The expressions of the mRNA transcripts of VCAM-1 (forward primer: 5′-ATGACATGCTTGAGCCAGG-3′, reverse primer: 5′-GTGTCTCCTTCTTTGACACT-3′), ICAM-1 (forward primer: 5′-CAGTGACCATCTACAGCTTTCCGG-3′, reverse primer: 5′-GCTGCTACCACAGTGATGATGACAA-3′), E-selectin (forward primer: 5′-GATGTGGGCATGTGGAATGATG-3′, reverse primer: 5′-AGGTACACTGAAGGCTCTGG-3′) and β-actin (forward primer: 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, reverse primer: 5′-CTAGAAGCATTTGCGGGGACGATGGAGGG-3′) were evaluated by RT-PCR as described previously with slight modifications [22]. Briefly, total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) as described previously [23]. Equal amounts of RNA were reverse transcribed into cDNA using oligo(dT)$_{15}$ primers. Samples were heated to 94°C for 5 min and cycled 25 times at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and this was followed by an additional extension step at 72°C for 5 min. PCR products were electrophoresed in a 1.5% agarose gel and followed by ethidium bromide staining and photography.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously [22]. The protein content of the nuclear extracts was determined using a Bio-Rad protein assay kit according to the manufacturer's instructions. The oligonucleotide sequence for NF-κB/Rel was 5′-GATCTCAGAGGGGACTTTCCGAGAGA-3′ [24]. Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]-ATP. Nuclear extracts (5 μg) were incubated with 2 μg of poly(dI-dC) and a ^{32}P-labeled DNA probe, and DNA binding activity was analyzed using a 4.8% polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography. The specificity of binding was examined by competition with an unlabeled oligonucleotide.

2.6. Statistical analysis

The mean ± S.D. was determined for each treatment group in each experiment. Significance was determined by either Dunnett's two-tailed *t* test for comparison between two groups or by ANOVA (analysis of variance), followed by Dunnett's test for multiple comparisons.

3. Results

3.1. Inhibitory effect of silymarin on THP-1 cell adhesion to HUVECs stimulated with TNF-α and involvement of adhesion molecules in THP-1 cell adhesion

As described previously, monocyte adhesion to endothelial cells is an important event in the development of atherosclerotic lesion. Therefore, we examined THP-1 cell adhesion to HUVECs stimulated with TNF-α to assess anti-atherogenic potential of silymarin. When unstimulated, the binding of THP-1 cells to HUVECs was lower than 20% of the TNF-α-treated group. However, THP-1 cell adhesion was substantially increased when HUVECs were stimulated with TNF-α (10 ng/ml). Treatment of HUVECs with silymarin (6.25, 12.5, 25 or 50 μg/ml) dramatically inhibited THP-1 cell adhesion to TNF-α-stimulated HUVECs in a dose-dependent manner (Fig. 1). Several cell adhesion molecules are implicated in monocyte adhesion to endothelial cells and VCAM-1, ICAM-1 and E-selectin have been known to play an important role in this process (reviewed in [2]). To further confirm the involvement of these adhesion molecules in the adhesion of THP-1 cells, we examined the effect of monoclonal antibodies of VCAM-1, ICAM-1 and E-selectin on THP-1 cell adhesion to TNF-α-stimulated HUVECs. Treatment of anti-VCAM-1 antibody caused 50% inhibition of THP-1 cell adhesion to HUVECs compared to TNF-α-treated group. Anti-ICAM-1 and anti-E-selectin antibodies also blocked THP-1 cell adhesion to HUVECs (Fig. 2).

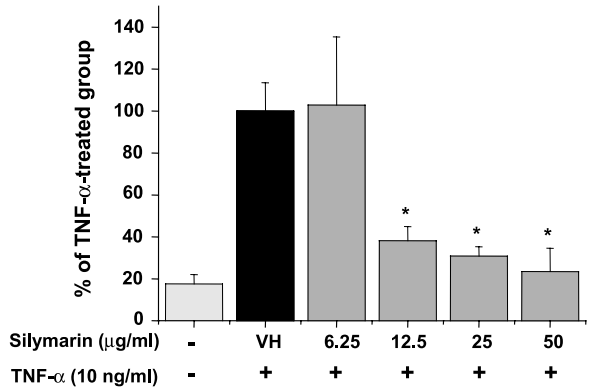

Fig. 1. Effect of silymarin on THP-1 cell adhesion to HUVECs stimulated with TNF-α. HUVECs were pretreated with silymarin (6.25, 12.5, 25 or 50 μg/ml) for 1 h, followed by stimulated expression of adhesion molecules with TNF-α (10 ng/ml) for 24 h. The wells, containing HUVECs, were rinsed, and THP-1 cells were added to each well. After 1 h, non-adherent cells were rinsed off, the adherent cells were collected by treatment with Trypsin-EDTA, and counted under a light microscope.

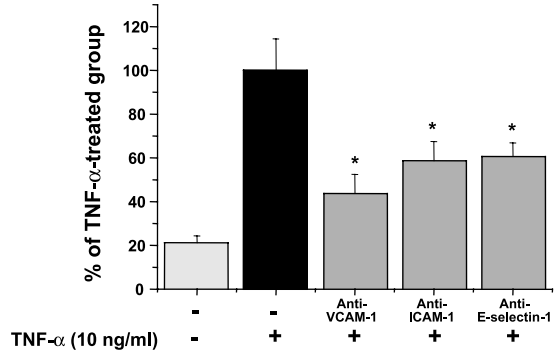

Fig. 2. Involvement of adhesion molecules in THP-1 cell adhesion to HUVECs. HUVECs were stimulated with TNF-α (10 ng/ml) for 24 h and treated with anti-VCAM-1, anti-ICAM-1 and anti-E-selectin antibodies for 1 h. THP-1 cell adhesion was analyzed as described in Section 2.

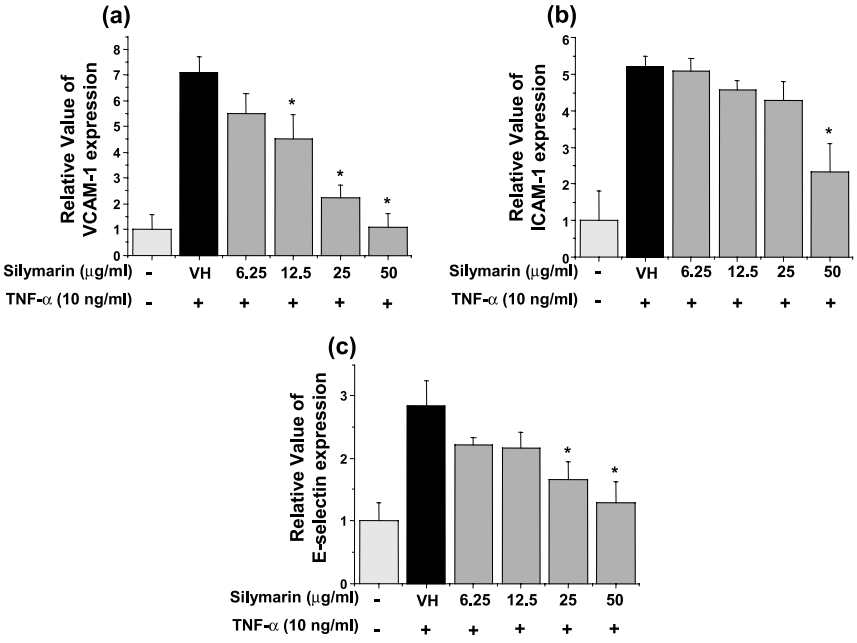

Fig. 3. Inhibition of cell surface expressions of VCAM-1, ICAM-1 and E-selectin by silymarin in TNF-α-stimulated HUVECs. HUVECs were pretreated with the indicated concentrations of silymarin for 1 h before being incubated with TNF-α (10 ng/ml) for 24 h. The cells were fixed with 4% paraformaldehyde and the cell surface expressions of VCAM-1 (a), ICAM-1 (b) and E-selectin (c) were analyzed as described in Section 2. Each column shows the mean ± S.D. of triplicate determinations. *, response that is significantly different from the control group as determined by Dunnett's two-tailed *t* test at $P < 0.05$.

3.2. *Effect of silymarin on the cell surface expressions of VCAM-1, ICAM-1 and E-selectin in TNF-α-stimualted HUVECs*

To further investigate the reason why the treatment of HUVECs with silymarin inhibited THP-1 cell adhesion to HUVECs stimulated with TNF-α, we examined the effect of silymarin on the cell surface expressions of VCAM-1, ICAM-1 and E-selectin in TNF-α-stimulated HUVECs. As shown in Fig. 3a, TNF-α (10 ng/ml) alone increased VCAM-1 expression by seven times the basal level in HUVECs, and this induction of VCAM-1 expression was concentration dependently suppressed by silymarin. The cell surface expressions of ICAM-1 and E-selectin were also significantly increased by TNF-α, but the inhibitory effect of silymarin on ICAM-1 and E-selectin expression was less sensitive than that on the VCAM-1 expression (Fig. 3b,c). The inhibitory effect of silymarin on TNF-α-induced ICAM-1 and E-selectin expression was smaller than that on VCAM-1 expression at low concentrations (6.25, 12.5 and 25 μg/ml), but the effect was significant at a high concentration (50 μg/ml).

3.3. *Effect of silymarin on the mRNA expression of VCAM-1, ICAM-1, and E-selectin in TNF-α-stimulated HUVECs*

To assess the effect of silymarin on the mRNA expression of VCAM-1, ICAM-1, and E-selectin, we measured the mRNA levels by RT-PCR. In unstimulated HUVECs, the mRNA expression was hardly detectable for all of VCAM-1, ICAM-1 and E-selectin. When stimulated with TNF-α (10 ng/ml) for 4 h, however, HUVECs expressed high levels of VCAM-1, ICAM-1 and E-selectin mRNA. TNF-α-induced VCAM-1 mRNA expression was suppressed by silymarin in a dose-dependent manner (Fig. 4). Consistent with the previous results, ICAM-1 mRNA expression was downregulated by silymarin only at a high concentration (50 μM) in TNF-α-stimulated HUVECs (Fig. 4). Furthermore, TNF-α-induced E-selectin mRNA expression was also significantly inhibited by silymarin treatment in HUVECS (Fig. 4).

3.4. *Inhibition of NF-κB/Rel DNA binding by silymarin in TNF-α-stimulated HUVECs*

NF-κB/Rel is known to have a critical role in the expression of proinflammatory cell adhesion molecules. To examine whether the inhibitory effect of silymarin on the gene expression of VCAM-1, ICAM-1 and E-selectin was mediated by suppressing the activation of NF-κB/Rel, the DNA binding of NF-κB/Rel was measured in HUVECs in the presence and absence of TNF-α and/or silymarin. Treatment of HUVECs with TNF-α (10 ng/ml) caused a significant increase in the

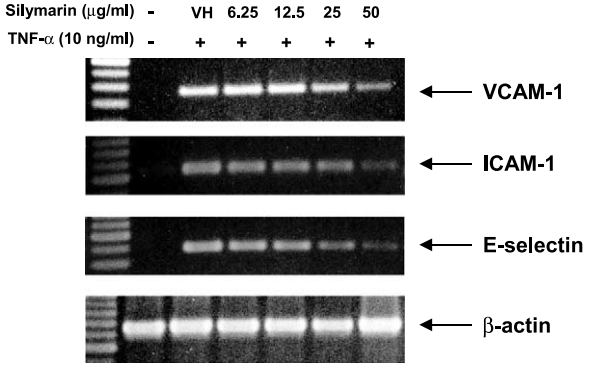

Fig. 4. Inhibition of the mRNA expression of VCAM-1, ICAM-1 and E-selectin by silymarin in TNF-α-stimulated HUVECs. HUVECs were pretreated with silymarin (6.25, 12.5, 25 or 50 μg/ml) for 1 h before being incubated with TNF-α (10 ng/ml) for 12 h. Total RNA was isolated and the mRNA expression of VCAM-1, ICAM-1, E-selectin and β-actin was determined by RT-PCR. One of two representative experiments is shown.

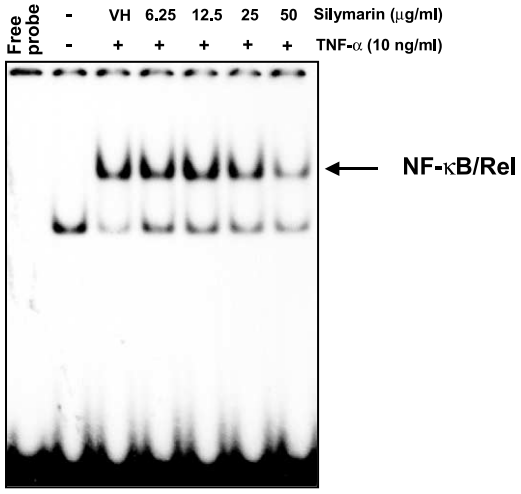

Fig. 5. Effect of silymarin on NF-κB/Rel DNA binding in TNF-α-stimulated HUVECs. HUVECs were pretreated with the indicated concentrations of silymarin for 1 h before being incubated with TNF-α for 30 min. Nuclear extracts were then prepared, and NF-κB/Rel DNA binding was determined by EMSA. The binding specificity was determined using the unlabeled wild-type probe (100-fold in excess) to compete with the labeled oligonucleotide. The results presented are representative of three independent experiments.

DNA binding activity of NF-κB/Rel within 30 min. In the presence of silymarin, TNF-α-induced NF-κB/Rel DNA binding was markedly suppressed in a concentration-dependent manner (Fig. 5)

4. Discussion

Silymarin is known to block the activation of the transcription factor NF-κB/Rel stimulated by various stimuli in a variety of cell types [15,16,25]. As previously described, it is also well known that the transcription factor NF-κB/Rel is essential to the gene expression of cell adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin [9–11]. Therefore, we hypothesized that silymarin has the ability to modulate the expression of cell adhesion molecules on activated endothelial cells. To characterize this hypothesis, we examined the ability of silymarin to inhibit the adhesion of monocytes to endothelial cells and to suppress the gene expression of cell adhesion molecules. In the present study, silymarin was found to inhibit monocyte adhesion to endothelial cells and gene expression of cell adhesion molecules on endothelial cells.

The recruitment of monocytes to the arterial wall and their infiltration and differentiation to macrophages play a protective role by removing cytotoxic and proinflammatory oxLDL particles or apoptotic cells. However, accumulation of macrophages and their uptake of oxLDL ultimately lead to the development of atherosclerotic lesion. Moreover, oxLDL itself has inflammatory and atherogenic properties and accelerates the progression of atherosclerosis. Locher and coworkers reported the inhibitory effect of silymarin on LDL oxidation, suggesting one of the possible mechanisms responsible for the anti-atherosclerotic activity of silymarin [18]. We clearly show here that silymarin directly inhibits the expression of cell adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin, which mediate the recruitment of monocytes to the arterial wall. NO is a potent oxidant and exerts critical and diverse functions in the cardiovascular system and an impaired production of NO plays a key role in the development of cardiovascular diseases [26]. NO is known to have both atherogenic and vascular protective effects, dependent on the source and amount of production. NO produced by endothelial NO synthase (eNOS) has a vasodilator function and has a protective effect. However, inducible NO synthase (iNOS) in macrophages produces a large amount of NO in response to stimuli, and potent oxidative properties of NO produced by iNOS appear to induce atherosclerosis. Our previous study that reported the inhibitory effect of silymarin on iNOS expression in LPS-stimulated macrophages also explains the anti-atherosclerotic activity of silymarin [16]. Therefore, all of these properties of silymarin seem to contribute to its anti-atherosclerotic activity.

Flavonoids are naturally occurring compounds and have a wide range of biological activities. There have been numerous reports demonstrating anti-atherosclerotic activity of flavonoids. Resveratrol, which is a flavonoid present in wine and grape juice, was found to inhibit LDL oxidation [27,28]. Ferrero and coworkers reported that resveratrol inhibited VCAM-1 and ICAM-1 expression [29]. A novel flavonoid, PD098063, was also reported to have an anti-atherosclerotic activity and have an inhibitory effect on TNF-α-induced VCAM-1 expression [30]. Quercetin, a strong anti-inflammatory flavonoid, was reported to inhibit LDL oxidation and have an anti-atherosclerotic effect [31,32]. However, despite its potent anti-atherosclerotic properties, quercetin is not absorbed into the body when orally administered [33]. On the other hand, silymarin was reported to be distributed rapidly to various tissues after oral administration to mice [34]. Furthermore, silymarin has been clinically used for a long time to treat various liver diseases demonstrating its good bioavailability.

In conclusion, the present study demonstrates the effect of silymarin on the gene expression of proinflammatory cell adhesion molecules and indicates a new insight into the mechanism responsible for the anti-inflammatory and anti-atherosclerotic property of silymarin.

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