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Inhibition of cytokine-induced vascular cell adhesion molecule-1 expression; possible mechanism for anti-atherogenic effect of *Agastache rugosa*

Jung-Joo Hong[a], Jae-Hoon Choi[a], Sei-Ryang Oh[b], Hyeong-Kyu Lee[b], Jae-Hak Park[c], Kun-Yeong Lee[a], Jung-Jae Kim[a], Tae-Sook Jeong[d], Goo Taeg Oh[a,*]

[a] *Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), P.O. Box 115, Yusong, Taejon 305-600, South Korea*
[b] *Immunomodulator Research Laboratory, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yusong, Taejon 305-600, South Korea*
[c] *College of Veterinary Medicine, Seoul National University, Suwon 441-744, South Korea*
[d] *Cardiovascular Research Laboratory, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yusong, Taejon 305-600, South Korea*

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Abstract Adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) play an important role during the early stages of atherogenesis. *Agastache rugosa* has an anti-atherogenic effect in low density lipoprotein receptor −/− mice. Moreover, *A. rugosa* reduced macrophage infiltration and VCAM-1 expression has been localized in aortic endothelium that overlies early foam cell lesions. This study ascertained that tilianin (100 μM), a major component of *A. rugosa*, inhibits the tumor necrotic factor-α (TNF-α)-induced expression of VCAM-1 by 74% in cultured human umbilical vein endothelial cells (HUVECs). Also, tilianin (100 μM) reduced TNF-α-induced activation of nuclear factor-κB in HUVECs. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Atherosclerosis; Tilianin; Vascular cell adhesion molecule-1; Macrophage; Nuclear factor-κB; *Agastache rugosa*

1. Introduction

The adhesion of monocytes to the vascular endothelium and their subsequent recruitment into the artery wall are key features in the pathogenesis of atherosclerosis [1,2]. Vascular cell adhesion molecule-1 (VCAM-1), an adhesion molecule expressed on the endothelial cell surface, may be partly responsible for the recruitment of monocytes during atherogenesis [3–5]. VCAM-1 expression has been demonstrated in human coronary atherosclerotic plaques, and this is consistent with the belief that this adhesion molecule plays a role in the disease [6,7].

Nuclear factor-κB (NF-κB) is a heterodimer of two proteins, p50 and p65, that are localized in the cytoplasm of unstimulated cells in an inactive form [8]. On stimulation of cells, for example by tumor necrotic factor-α (TNF-α), the heterodimer translocates to the nucleus and transactivates gene expression [8,9]. The VCAM-1 promoter consists of two NF-κB binding sites [10]. The cell-surface expression of VCAM-1 correlates with an increase in mRNA levels, which indicates transcriptional activation of this gene by TNF-α. Flavonoids, such as PD 098063 (2-(3-amino-phenyl)-8-methoxy-chromene-4-one), and gallates, have been shown to block the TNF-α-stimulated NF-κB activation, transcription, and cell-surface expression of VCAM-1 in endothelial cells [11–13].

The plant species *Agastache rugosa* (Labiatae) contains several kinds of flavonoids (acacetin-7-*O*-β-D-glucopyranoside (tilianin), acacetin, linarin, and agastachoside) [14,15], and tilianin is a main constituent of *A. rugosa*. Both the extract and the constituents of this plant have a variety of physiological and pharmacological activities on inflammatory immune responses such as anti-complement [16], and the inhibition of *Haemophilus influenzae* adhesion to human cells [17].

In the present study, we investigated the effect of *A. rugosa* on the atherosclerosis, a chronic inflammatory disease [1], using low density lipoprotein receptor deficient (Ldlr−/−) mice. To gain some insight into the potential molecular mechanisms of the anti-atherosclerotic properties of *A. rugosa*, the investigation was extended to examine the effect of tilianin on TNF-induced VCAM-1 expression in endothelial cells, an early step in the pathophysiology of atherosclerosis.

2. Materials and methods

2.1. A. rugosa extract and tilianin

The extract (2.5 kg) was obtained by extracting the aerial parts of *A. rugosa* (30 kg) three times with methanol (120 l). It was then suspended in H_2O and partitioned with *n*-hexane, chloroform, and *n*-butanol, sequentially, and the chloroform fraction (590 g) was subject to column chromatography on silica gel (230–400 mesh, 20×75 cm, Merck, USA) using a chloroform–methanol mixture (10:1, v/v) to give five fractions. Tilianin (49.5 g) was obtained as a pale yellow precipitate in methanol from the fourth fraction (Fig. 1).

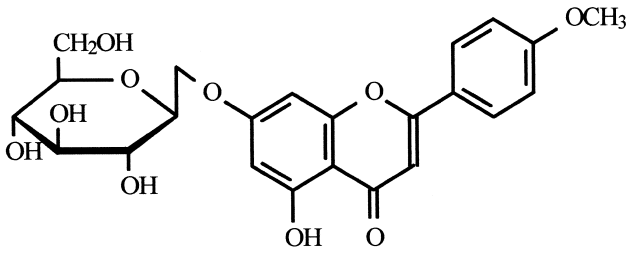

Fig. 1. Structure of tilianin.

*Corresponding author. Fax: (82)-42-860 4609.
E-mail: gootaeg@mail.kribb.re.kr

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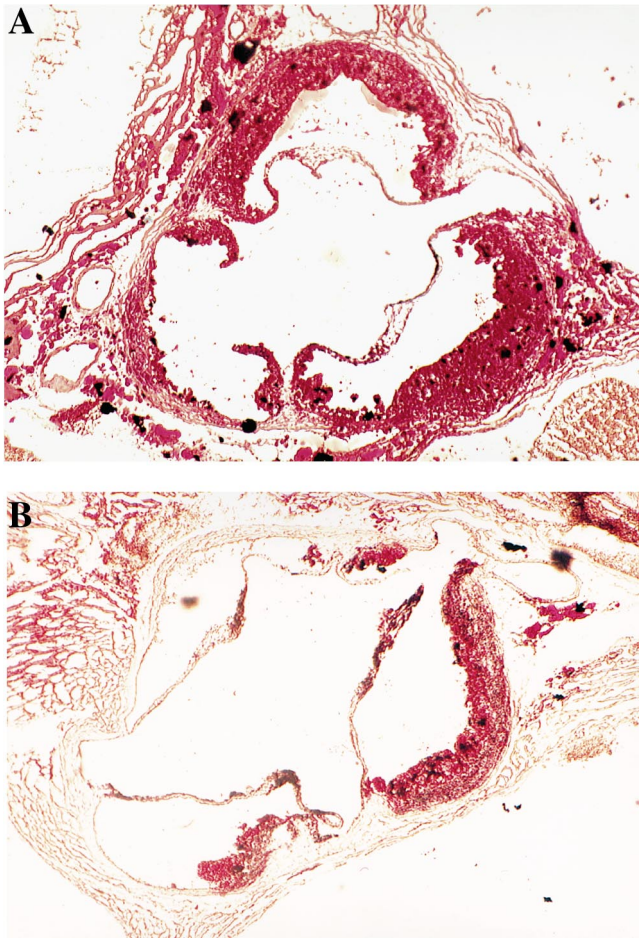

Fig. 2. Effect of *A. rugosa* extract on atherosclerotic lesions in Ldlr−/− mice fed a cholesterol diet for 8 weeks. Representative cross sections of aortic valves (×100) in hearts from (A) a control group and (B) a 1% (w/w) extract-treated group. (C) Oil red O-stained aortic valve lesion areas were quantified by computer-associated morphometry. All results are shown as mean ± S.D., $n = 8$.

2.2. Animals and blood chemical analysis

Three breeding pairs of homozygous Ldlr-null (B6,129-*Ldlr*^{tm1Her} −/−) mice [18] were purchased from the Jackson Laboratory (ME, USA), and bred at the Korea Research Institute of Bioscience and Biotechnology under specific pathogen free conditions. Twenty female Ldlr−/− mice were randomly divided into two groups of 10 animals and fed on a high cholesterol diet (CRF-1 supplemented with 15% fat, 1.25% cholesterol, and 0.5% Na-cholate, Oriental Yeast Co. Ltd., Japan), the first group without additional supplementation (control), and the second group supplemented with 1% (weight/diet weight (w/w)) of the methanol extract of *A. rugosa*. The mice had ad libitum access to water and food. Following treatment with extract for 8 weeks, the mice were sacrificed by cervical dislocation, and plasma total cholesterol was measured using an automatic blood chemical analyzer (CIBA Corning, OH, USA). Lipoprotein fractions were separated by FPLC from the pooled plasma of each group [19].

2.3. Quantification of aortic sinus lesions and immunohistochemical analysis of VCAM-1 and macrophage

Fatty streak lesions were quantified by evaluating the lesion size in the aortic sinus, as previously described [20], with minor modifications. Atherosclerotic plaque was stained with Oil red O and counter-stained with Harris hematoxylin. The lesion area was then quantified by computer-assisted morphometry (Image pro plus, MD, USA) and the average lesion size calculated for each animal. Frozen sections were stained with a rat monoclonal antibody to mouse macrophages (MOMA-2, Serotec Inc., NC, USA) and a rabbit anti-mouse polyclonal VCAM-1 antibody (diluted 1:100, Santa Cruz Biotechnology, CA, USA) using the ABC method (Novastatin Super ABC kit, Novocastra Lab. Ltd., UK). Macrophage infiltration was quantified by determining the MOMA-2-stained area [21]. The sections stained with VCAM-1 antibody were evaluated with light microscopy and graded by two independent observers, each of whom was blinded to the identity of the sections. Staining intensity was scored semi-quantitatively [22]: 0 indicated no staining; 1, rare positive cells or staining barely visible at low power (×100); 2, focal staining or faint diffuse staining clearly visible at low power; 3, multifocal staining or moderate diffuse staining; and 4, intense diffuse staining. The scores of the two observers were highly correlated ($r^2 = 0.96$; $P < 0.0001$). For convenience, therefore, scores from only one observer were used for calculation of vessel mean scores.

2.4. Flowcytometry analysis of VCAM-1

Following treatment of human umbilical vein endothelial cells (HUVECs) with tilianin, the cells were trypsinized, collected in tubes, and washed three times with phosphate-buffered saline (PBS). The cells (5×10^5) were then resuspended in 100 μl of PBS, and mouse anti-human monoclonal antibody (Rb 1/9; 1 μg) added, whilst the tubes were continuously shaken for 40 min on ice. The cells were then washed three times with cold PBS and incubated with FITC-

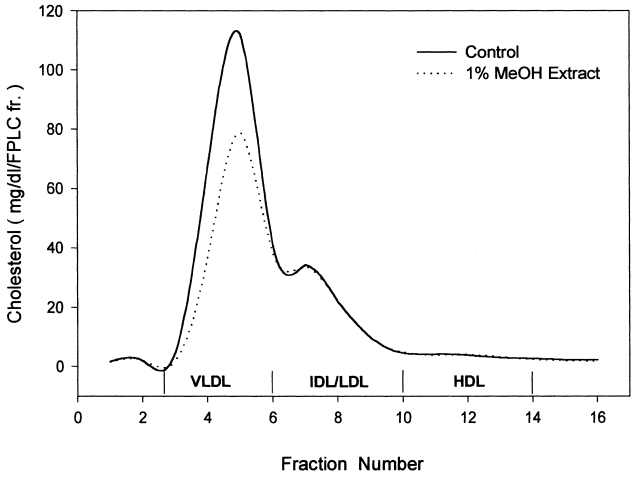

Fig. 3. Plasma lipoprotein cholesterol distribution in Ldlr−/− mice fed a 1% (w/w) methanol extract of *A. rugosa*. Plasma was obtained from Ldlr−/− mice 8 weeks after the administration of 1% (w/w) MeOH extract of *A. rugosa*. Equal volumes of plasma from 10 animals were pooled, and lipoproteins were size-fractionated by FPLC using a Superose 6 HR column. Total cholesterol content of each fraction (1 ml/fraction) was measured using enzymatic colorimetric method kits (Sigma Chemical Co.) according to the manufacturer's instructions. The retention times for mouse VLDL ($d < 1.006$ g/ml), IDL/LDL ($d = 1.02$–1.055 g/ml) and HDL ($d = 1.07$–1.21 g/ml) are indicated.

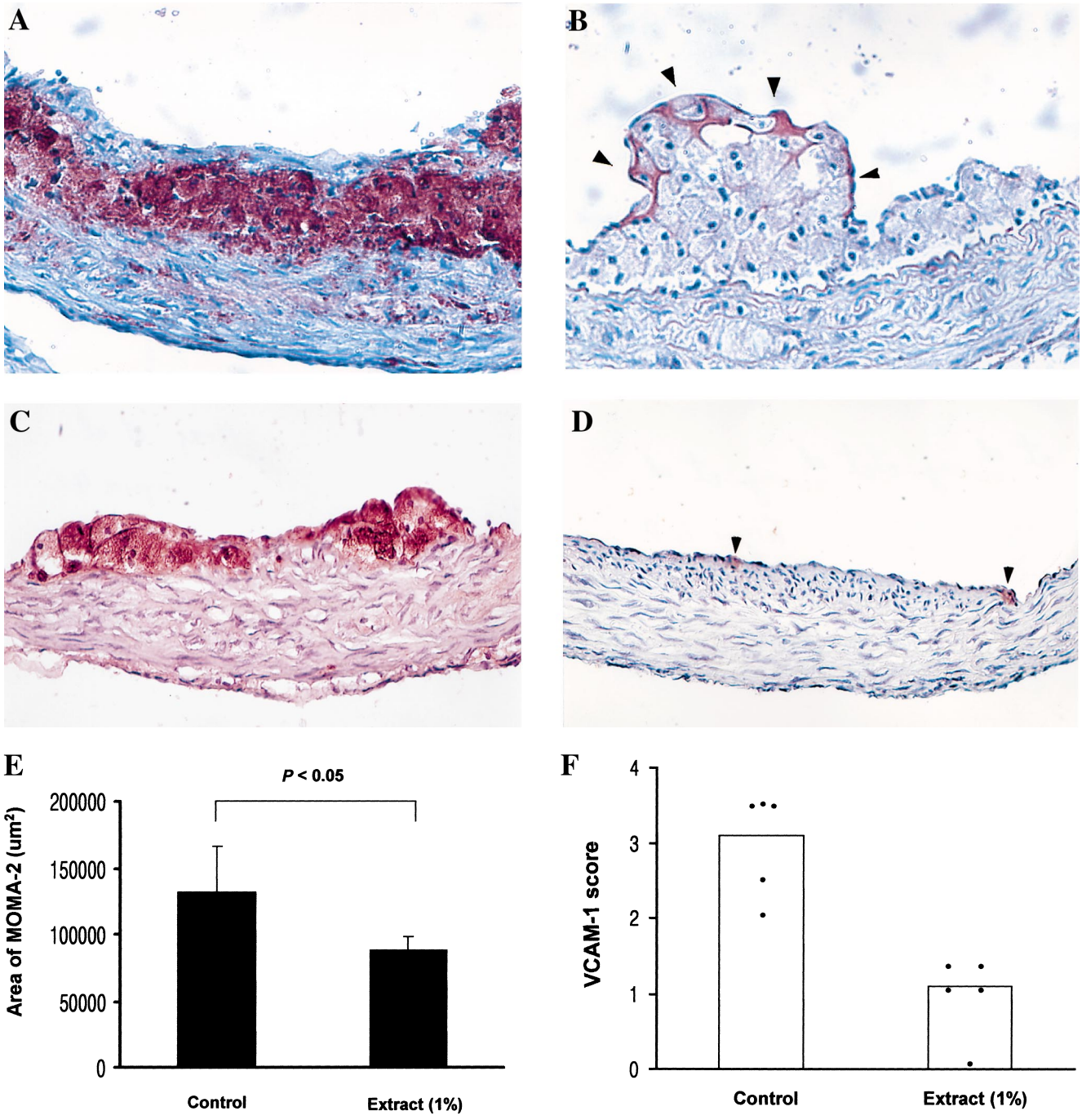

Fig. 4. Quantitative analysis of macrophage content and VCAM-1 in the aortic arteries of Ldlr−/− mice fed a 1% (w/w) methanol extract of *A. rugosa*. A–D: Immunohistochemical staining of aortic valve lesions (×200) with the macrophage marker MOMA-2 (A, C) and VCAM-1 antibody (B, D). Representative frozen sections from the aortic arches of Ldlr−/− mice fed a high cholesterol diet (A, B) and 1% (w/w) extract-supplemented high cholesterol diet (C, D). E: Quantitative analysis of macrophage infiltrating area. The area of MOMA-2 staining was determined by computer-associated morphometry. All results are shown as mean ± S.D., $n = 8$. F: Intensity of histochemical staining for detection of VCAM-1 expression. See Section 2 for description of 0–4 intensity scales. Each data point represents a single artery. Bars indicate the group medians.

conjugated goat F(ab′)2 anti-mouse IgG at a dilution of 1:25 in PBS for 1 h in ice. The cells were then fixed with 1% paraformaldehyde and analyzed by FACScan (Bio-Rad, USA) analysis. Results were plotted as fluorescence intensity vs. cell numbers.

2.5. Electrophoretic mobility shift assay (EMSA)

HUVECs were maintained in a EGM-2 Bullet kit (Clonetics, Bio-Whittaker, Inc., MD, USA), and pre-incubated with 1, 10, and 100 μM of tilianin for 2 h. After pre-incubation, HUVECs were activated by 10 ng/ml of TNF-α for 15 min in the presence of tilianin. Nuclear protein (10 μg) was mixed with a double-stranded oligonucleotide (5 pM) corresponding to the NF-κB/Rel [23] binding sequence. The 5 μg of nuclear protein was incubated with 2 μl of radio-labeled probe (20 000 cpm) to make the protein–DNA complex.

2.6. Statistics

Results are expressed as mean ± S.D. and statistical significance was determined using Student's *t*-test.

3. Results

3.1. Analysis of atherosclerotic lesion

Fig. 2 shows sections of aortic roots from Ldlr−/− mice in the two groups that showed oil-red O positive atherosclerotic lesions on light microscopic examination (Fig. 2A,B). The aortic sinus of the control group showed lipid-enriched foam cells in the vessel walls and an increased amount of extracellular matrix in the presence of sheaths apparently composed of proliferative smooth muscle cells. However, the lesions in the 1% extract-supplemented group were smaller and less advanced, and these were characterized by a less prominent necrotic core and less smooth muscle cell proliferation. Computer-associated morphometry was used to quantify lesion areas. The mean lesion area of the aortic sinus was significantly reduced in the 1% extract-supplemented group compared with the control group (162 629.6 ± 28 362.1 μm^2 vs. 304 644.0 ± 76 602.6 μm^2, $P < 0.01$) (Fig. 2C).

3.2. Effects of tilianin on plasma lipoprotein profiles

Following the 8 weeks high cholesterol diet, plasma cholesterol levels were markedly elevated, and about 3.5 times that of the chow diet-fed Ldlr−/− mice. Total plasma cholesterol levels were reduced slightly (1064.6 ± 31.5 mg/dl vs. 985.6 ± 14.7 mg/dl) after treatment with *A. rugosa* extract, and the cholesterol content of VLDL was also lower in the *A. rugosa* extract-supplemented group, however, this was not statistically significant (Fig. 3).

3.3. Immunohistochemical staining of VCAM-1 and macrophages

The mechanism of the anti-atherogenic effect of the extract is likely to involve recruitment of monocyte into the arterial wall. To evaluate this possibility, macrophage accumulation and VCAM-1 expression in the aortic arch were examined by immunohistochemical staining with specific antibodies. A marked accumulation of the macrophages was observed in the subendothelial surface of the control group (Fig. 4A), and this accumulation was reduced in the 1% extract-treated group (Fig. 4C). Aortic arches from 1% extract-treated mice had 37% less MOMA-2 staining than arches from control group (88 561.5 ± 9403.5 μm^2 vs. 131 497.3 ± 35 611.1 μm^2, $P < 0.05$) (Fig. 4E). The fact that VCAM-1 expression plays an important role in monocyte recruitment, was evident from the VCAM-1 expression levels in endothelial cells at the edges of and adjacent to the lesions (Fig. 4B). The expression of VCAM-1 was reduced in all aortic arches from the 1% extract-treated group (Fig. 4D,F).

3.4. Effects of tilianin on the expression of VCAM-1 and NF-κB activation

Fig. 5 shows the representative traces of FACS analysis from three separate analyses. To examine whether tilianin reduced the elevated expression of VCAM-1 on HUVECs induced by TNF-α, FACS analysis was conducted using VCAM-1 specific monoclonal antibody (PharMingen, Becton Dickinson, USA). Pretreatment of HUVECs with tilianin markedly suppressed the TNF-α-induced expression of VCAM-1 in a dose-dependent manner. The TNF-α-induced expression of VCAM-1 was reduced by 10 and 100 μM of tilianin, by 80 and 74%, respectively. Also, both the extract

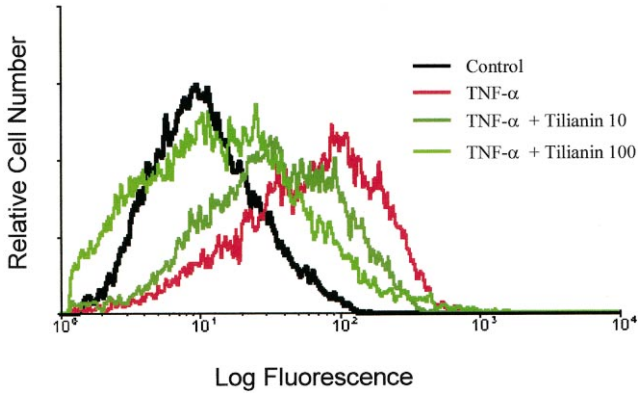

Fig. 5. Dose-dependent effect of tilianin on endothelial adhesion molecule induction. HUVECs were pre-incubated with 10 or 100 μM for 2 h and stimulated with TNF-α (10 ng/ml, 16 h), stained for VCAM-1 or isotype control, and analyzed by flow cytometry.

and tilianin inhibited the VCAM-1 mRNA expression induced by TNF-α assessed by RT-PCR (data not shown).

To determine whether tilianin inhibits activation of NF-κB, the effect of tilianin on NF-κB activation in HUVECs was evaluated by EMSA. The activity of NF-κB was markedly suppressed by the tilianin treatment (100 μM) (Fig. 6A). The identity of the activated NF-κB/oligonucleotide complex in HUVECs was determined by supershift analysis (Fig. 6B). The addition of p65 and/or p50 antiserum during NF-κB/oligonucleotide binding resulted in a shift of the complex in accordance with the findings of a previous study [24]. In the competitive assay, the cold identical oligonucleotide sequences inhibited labeled oligonucleotide binding, whereas the mutant sequences did not inhibit this specific binding. Hence, NF-κB activation in response to TNF-α conclusively resembles that of the p50/p65 heterodimer NF-κB complex.

4. Discussion

In this study, we investigated the anti-atherogenic activity of *A. rugosa* extract in vivo. The extract of *A. rugosa* has been shown previously to show a range of activities upon inflammatory immune responses [16,17] and inhibitory activity on the expression VCAM-1. *A. rugosa* is known to contain a number of flavonoids, such as tilianin, acacetin, and diosmetin, which have shown inhibition activity against the expression of cellular adhesion molecules on the surface of THP-1 leukemic monocyte cells. Among the flavonoids of *A. rugosa*, tilianin was a major constituent (1.96% of the methanol extract), and showed a strong inhibitory activity on the TNF-α-induced expression of adhesion molecules in the THP-1 cells (data not shown). This information was based on our previous studies upon the anti-atherogenic mechanism of *A. rugosa* in vitro.

The present study is the first to investigate the effect of *A. rugosa* extract on the development of vascular proliferative disease in vivo. Fatty streak formation was prevented, to a statistically significant degree, in high-cholesterol dieted Ldlr−/− mice after treatment with the extract for 8 weeks. The pattern of the lipoprotein cholesterol profile was similar to that reported in previous studies [18,25]. The cholesterol profile of VLDL differed slightly in the two groups, as the

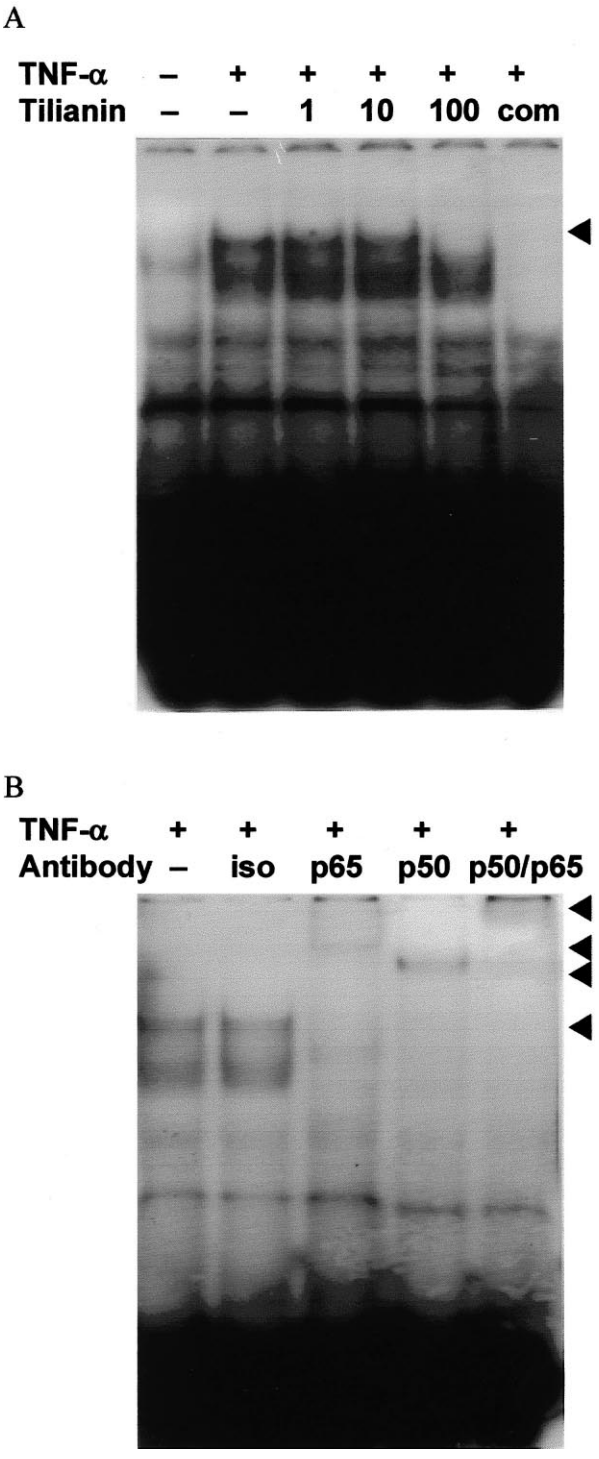

Fig. 6. Effects of tilianin on the activation of NF-κB in a TNF-α-stimulated primary culture of HUVECs. A: Inhibition of NF-κB activation was analyzed by EMSA using a specific (NF-κB) oligonucleotide derived from mouse VCAM-1 promoter. HUVEC cells were pretreated with the indicated concentrations of tilianin for 2 h and stimulated with TNF-α (10 ng/ml) for 15 min. B: Supershift analysis of the active NF-κB complex was performed as described in Section 2. For supershift analysis a p50 antibody or a p65 antibody was included. NF-κB binding without antibody addition (−), competitor (com), and isotype oligonucleotide (iso) are shown.

total cholesterol was slightly reduced in the extract-treated group. Thus, these lowered plasma cholesterol levels by *A. rugosa* extract may help to protect against atherosclerosis. Decreased plasma VLDL levels in animals could have been the result of either decreased hepatic secretion of VLDL particles or increased clearance. The increment of body weight and consumption of the diet were similar in the extract-treated and control animals (data not shown), which excludes any negative effect of the extract on appetite.

Endothelial cell VCAM-1 expression patterns were highly reproducible and similar in rabbits and Ldlr−/− mice fed a high cholesterol diet [26]. Biohumoral correlation studies have shown a significant positive association between increasing plasma concentrations of soluble VCAM-1 and the risk of atherosclerosis [27,28]. Our present study shows that the expression of VCAM-1 and accumulation of macrophages in the lesions were regressed by *A. rugosa* extract treatment.

To determine the possible mechanisms of the anti-atherogenic effect of *A. rugosa*, the flavonoid tilianin, a major component of the methanoic extract of the aerial portion of *A. rugosa*, was investigated. Other naturally occurring flavonoids, including gallates [13], apigenin [29], and PD098063 [12], belong to another group of phenolic compounds that seem to inhibit monocyte adhesion by a variety of mechanisms. Apigenin suppressed cytokine-induced VCAM-1 expression by inhibiting NF-κB activation without affecting the nuclear translocation of NF-κB p65 [29]. PD098063 selectively inhibited VCAM-1 expression by a mechanism independent of NF-κB, and without altering cytokine-induced ICAM-1 expression [12]. Gallates inhibited the cytokine-induced nuclear translocation of NF-κB and the expression of leukocyte adhesion molecules in HUVECs [13]. In accordance with studies on gallates, tilianin inhibits TNF-α-induced VCAM-1 expression with FACS analysis, and also inhibits TNF-α-induced NF-κB activation. Further studies on the effects of tilianin in vivo may extend the possible actions of tilianin on the prevention of atherosclerosis by the inhibition of NF-κB-dependent pro-atherogenic gene expression.

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