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Modulation of TNF-α-induced ICAM-1 Expression, NO and H_2O_2 Production by Alginate, Allicin and Ascorbic Acid in Human Endothelial Cells

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Plant nutrients are believed to provide protection against various diseases including inflammation. Since interactions of the cell adhesion molecules are known to play important roles in mediating inflammation, inhibiting adhesion protein upregulation is a possible therapeutic target. In this study, the interacellular adhesion molecule-1 (ICAM-1) was induced in human umbilical endothelial cells (HUVECs) after stimulation with TNF-α. In addition, alginate, ascorbic acid and allicin were demonstrated to inhibit the TNF-α induced expression of ICAM-1 on the HUVECs in a dose-dependent manner. These compounds also inhibited the production of NO and H_2O_2 induced by TNF-α, which suggests that the inhibition of ICAM-1 expression by the three compounds may be due to the modulated production of the reactive oxygen/nitrogen components. Overall, these results indicate that these dietary components have a therapeutic potential in the treatment of various inflammatory disorders associated with an increase in endothelial leukocyte adhesion molecules.

Key words: Alginate, Allicin, Ascorbic acid, TNF-α, Endothelial cells, ICAM-1, NO, H_2O_2

INTRODUCTION

Phytochemicals found in fruits and vegetables have been investigated for their potential role in protecting against preventing pathological diseases, such as inflammation (Middleton *et al.*, 1998). Continued research is being undertaken to further elucidate the biological actions of phytochemicals from dietary sources. Of those phytochemicals investigated, published data have shown that alginate, allicin and ascorbate are physiological antioxidants and have potential anti-inflammatory activity (Darr *et al.*, 1996; Hasegawa *et al.*, 1989; Hobauer *et al.*, 2000).

Alginate, a seaweed extract is linear polymer of polysaccharides with gel-forming properties composed of alternating blocks of β-(1-4)-D-mannosyluronic acid (M), α-(1-4)-L-glucosyluronic acid (G) (Davidson *et al.*, 1976). Alginate has been used to immobilize the Langerhans islets for treating experimental diabetes mellitus in rats (Tze *et al.*, 1982; Soon-Shiong *et al.*, 1994). In addition, it has been suggested that sodium alginate has a theraputic effect on healing radiation-induced stomatitis (Hasegawa *et al.*, 1989). Garlic has long been used as a general food and for therapeutic purposes in Oriental medicine. Previous investigations have shown that garlic plays an important pharmacological role as an anti-microbial (Cellini *et al.*, 1996), anti-thrombotic (Bordia *et al.*, 1996), anti-hypertensive (Mcmahon *et al.*, 1993; Foushee *et al.*, 1982), anti-hyperglycemic (Chang *et al.*, 1980), and anti-hyperlipemic (Yeh and Yeh, 1994; Eilat *et al.*, 1995) agent. It has also been suggested that allium derivatives from garlic regulate the nuclear factors involved in the immune and inflammatory functions, as well as in proliferation (Pinto *et al.*, 2001). Ascorbic acid is the most powerful reducing agent available to cells and is of general importance as an antioxidant because of its high reducing potential (Strain *et al.*, 1999).

An injury to or a dysfunction of the endothelium is believed to be one of the first events in the development of the inflammation, and the inflammatory mechanisms are an integral part of this process (Berliner *et al.*, 1995). An important event in this inflammatory response is the localization of leukocytes at the sites of the inflammatory

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lesions through a multistep process. Endothelial cell adhesion molecules such as E-selectin, the intercellular adhesion molecule-1 (ICAM-1) and the vascular cell adhesion molecule-1 (VCAM-1), play an important role in leukocyte adhesion and transendothelial migration at sites of the inflammation. The endothelial expression of these molecules is known to be higher in the temporal and spatial connection with the inflammatory cell infiltrates (Carlos *et al.*, 1994). When activated by inflammatory cytokines, endothelial cells exhibit an upregulation of the specific adhesion molecules on their surface, the ligands for which are supported on the circulation leukocytes (Bevilacqua *et al.*, 1985 Springer *et al.*, 1990). Indeed, TNF-α or IL-1 is known to induce ICAM-1 in a number of cell types including human umbilical endothelial cells (HUVECs) *in vitro* and ICAM-1 expression is sustained above the baseline for at least 72 h (Myers *et al.*, 1992). A logical target for novel drug development would be the design of compounds that interfere with the adhesion molecule interactions. It has been suggested that various small molecules, such as glucocorticoids, aspirin and pentoxifylline inhibit the increase in adhesion protein expression and have a protective effect against inflammatory diseases (Brostjan *et al.*, 1997; Weber *et al.* 1995; Neuner *et al.*, 1997).

Since reactive oxygen and nitrogen species including nitric oxide, superoxide, hydrogen peroxide and peroxynitrite are involved in acute, and chronic inflammation, there is a need to define the role of these oxidants and free radicals in the pathophysiology of inflammatory diseases. However, despite many studies, the precise roles of these species in the expression adhesion molecules induced by TNF-α are not clearly understood.

The aim of this study was to evaluate the effects of dietary alginate, allicin and ascorbic acid on ICAM-1 expression, NO and H_2O_2 release and the possible mechanism of their effects in human umbilical vein endothelial cells. The results of this study suggest that the dietary components inhibit the upregulation of TNF-α-induced adhesion protein expression. This study also found that these compounds blocked the production of NO and H_2O_2 induced by TNF-α.

MATERIALS AND METHODS

Reagents

The alginate and ascorbic acid were purchased from Sigma Chemical Co. (St Louis, USA). The allicin extract was prepared according to the methods reported by Prasad *et al.* (1995). Briefly, allicin, a diallyl disulfide-oxide, was extracted from a garlic in tablet form, which contained 2500 μg of allicin per tablet (Madaus Murdock Inc., Mountain Spring Parkway, Springville, Utah, 84663 USA). The uncoated tablet was crushed (0.75 g) and homogenized in a test tube containing 5.0 mL of distilled water. The homogenate was then centrifuged for 10 min at 13,000×g. The supernatant was recentrifuged for 5 min at 13,000×g and the concentration was adjusted to 500 μg/mL of allicin. The 2',7'-dichlorofluroescein diacetate (DCFH-DA) were obtained from Molecular Probes, USA. The TNF-α and anti-ICAM-1 (BBA3) antibodies were purchased from R & D Systems, USA. The anti-mouse IgG-HRP and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. The fetal bovine serum was purchased from Gibco, USA.

Cells and cell culture

The HUVECs were purchased from Clonetics (San Diego, CA) and grown in EGM-2 medium (Clonetics) in gelatin coated tissue culture flasks. For subculturing, the cells were detached using 0.125% trypsin containing 0.01 M EDTA. The cells used in this study were obtained from the first to third passage.

Cytotoxicity evaluation

Alginate, allicin, ascorbic acid and TNF-α at the concentrations presented herein were shown to be non-toxic by the following procedure: The viability of the HUVECs treated with the three test compounds and TNF-α, respectively, was determined using a MTT assay. The HUVEC were cultured in gelatin coated 96-well microplates (Costar Products, Cambridge, MA) until confluent. The cells were treated with or without the test compounds in quadruplicate for the indicated time, and subsequently 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) was added for 4 h. In addition, a visual inspection of the cells was performed by trypan blue exclusion staining under an inverted microscope.

ELISA for measurement of adhesion molecules

The cell surface expression of the adhesion molecules on the endothelial monolayers was quantified using ELISA using a modification of the methods described previously (Son *et al.*, 2001). The HUVECs were seeded at a concentration of 2×10^4 cells/well in 96-well, flat bottom, gelatin-coated plates (Nalgen Nunc International, IL). The cells were incubated with the control media, and TNF-α (5 ng/mL) for 20 h in the presence or absence of the various doses of the test compounds in order to measure ICAM-1 expression. In some experiments, inhibitors of the metabolic pathways were included along with TNF-α. The cells were washed with phosphate buffer saline pH 7.4 (PBS) and fixed with 10% glutaraldehyde for 30 min at 4°C. Bovine serum albumin (1.0% in PBS) was added to the cells in order to reduce the non-specific binding. The cells were incubated with anti-ICAM-1 monoclonal antibodies or the isotype matched control antibodies (0.25 mg/mL, diluted

in blocking buffer) overnight at 4°C, washed with PBS followed by incubation with the alkaline phosphatase-conjugated goat anti-mouse secondary antibodies (1 g/mL, diluted in PBS). The cells were then washed with PBS and exposed to the peroxidase substrate (*p*-nitrophenyl phosphate 1 mg/mL in 0.1 M glycin buffer, pH 10.4 containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$). The absorbance was determined at 405 nm using a Molecular device microplate reader (Menlo Park, CA). The absorbance values of the isotype matched control antibodies were taken as blank and were subtracted from the experimental values.

Nitrite determination

The HUVECs were treated with the control media, TNF-α (5 ng/mL) for 20 h in the presence or absence of various doses of the test compounds, and the accumulation of nitrite in the culture supernatant was measured using the assay system reported by Ding *et al.* (1998). Briefly, 100 μL of the supernatant was removed from each well and placed into an empty 96-well plate. After adding 100 μL of a Griess reagent to each well, the absorbance at 540 nm was measured using a Molecular device microplate reader. The nitrite concentration was calculated from a $NaNO_2$ standard curve. The absorbance levels are indicative of NO production. The Griess reagent was prepared by mixing one part 0.1% naphthylethylenediamine dihydrochloride in distilled water plus one part 1% sulfanilamide in 5% concentrated H_3PO_4.

Detection of hydrogen peroxide (H_2O_2) production

The effect of the test compound on (H_2O_2) production in the HUVECs was determined by a fluorometric assay using DCFH-DA as the probe (Wan *et al.*, 1993). This method is based on the oxidation by (H_2O_2) of the non-fluorescent DCFH-DA to the flurorescent 2',7'-dichloro-flurescin (DCF). The confluent HUVEC (10^4 cells/well) in the 96-well plates were pretreated with the control media, and TNF-α (5 ng/mL) for 20 h in the presence or absence of various doses of the test compounds. After the cells were washed with HBSS, HBSS containing 10 μM DCFH-DA was added and further incubated for 45 min at 37°C. The fluorescence intensity (relative fluorescence units) was measured at 485 nm excitation and 530 nm emission using a Fluorescence Microplate Reader (Bio-TeK Instruments, Winooski, VT).

Statistical analysis

The data is reported as a means±S.E.M. The statistical difference between the groups was determined by a one-way analysis of variance (ANOVA) and the significant values are represented by an asterisk (*$p<0.05$, **$p<0.01$).

RESULTS

Effect of alginate, allicin and ascorbic acid on TNF-α-induced ICAM-1 expression in endothelial cells

ELISA was used to examine the effects of alginate, allicin and ascorbic acid on the TNF-α stimulated upregulation of ICAM-1 in the HUVECs. All the determinations were made on the same 96-well plate. The incubation time and concentration of these compounds used in these experiments had no effect on the viability, as determined by trypan blue staining and the morphology of the endothelial

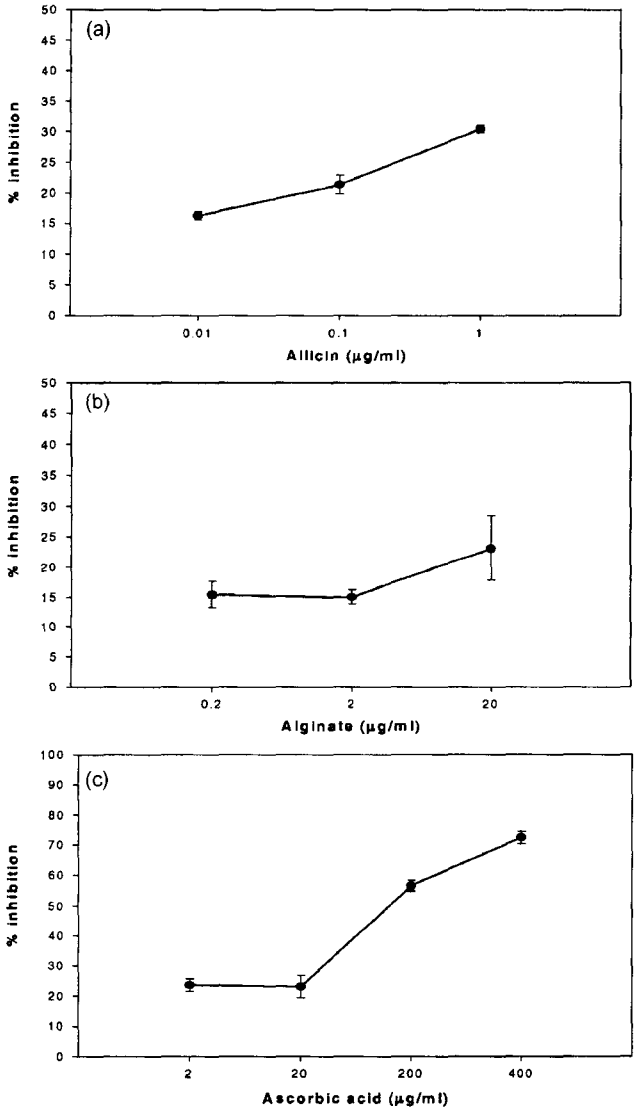

Fig. 1. Dose dependent inhibition of TNF-α-induced ICAM-1 expression by allicin (a), alginate (b) and ascorbic acid (c). Three independent experiments were performed in which the confluent HUVEC were cultured for 20 h in the medium or in the medium supplemented with TNF-α (5 ng/mL) in the presence or absence of the three compounds and ICAM-1 expression were measured by ELISA. The results are a mean±S.E.M from three independent experiments, after calculating the percentage of ICAM-1 expression in comparison with that of control.

cells (data not shown). These concentrations were in complete agreement with the optimum concentrations of the compounds needed in the other system reported (Prasad, K. *et al.*, 1995; Strain *et al.*, 1999; Son *et al.*, 2001). ELISA showed the ICAM-1 was expressed at low levels on the nonstimulated endothelial cells and was significantly induced by TNF-α. The three compounds did not have any effect on the basal ICAM-1 expression level, whereas it led to a reduction in the TNF-α-induced ICAM-1 expression (Fig. 1).

Alginate, allicin and ascorbic acid inhibit TNF-α-induced NO and H_2O_2 production

The effect of the test compounds on NO production in the endothelial cells was determined because NO is an important modulator of the inflammatory response to various stimuli as well as the expression of adhesion

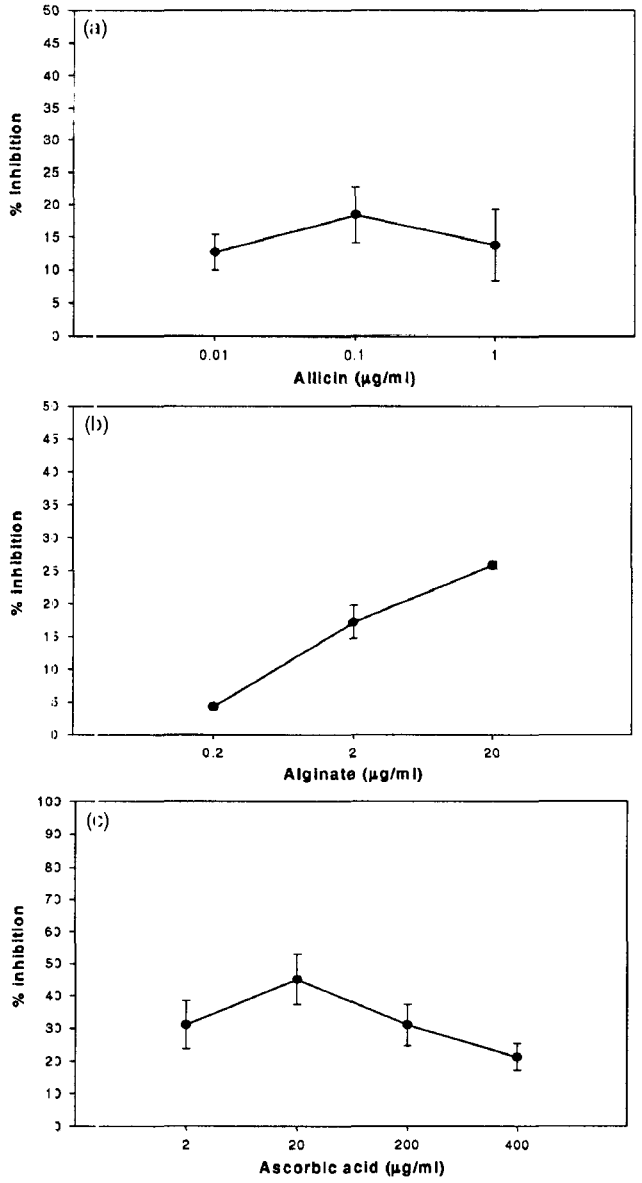

Fig. 2 Inhibition of TNF-α-induced NO production by allicin (a), alginate (b) and ascorbic acid (c). Three independent experiments were performed in which the confluent HUVECs were cultured for 20 h in medium or in the medium supplemented with TNF-α (5 ng/mL) in the presence or absence of the three compounds and the nitrite concentration was determined using the Griess reagent. The results are represented as a mean±S.E.M from three independent experiments, after calculating the percentage of NO production in comparison with that of control

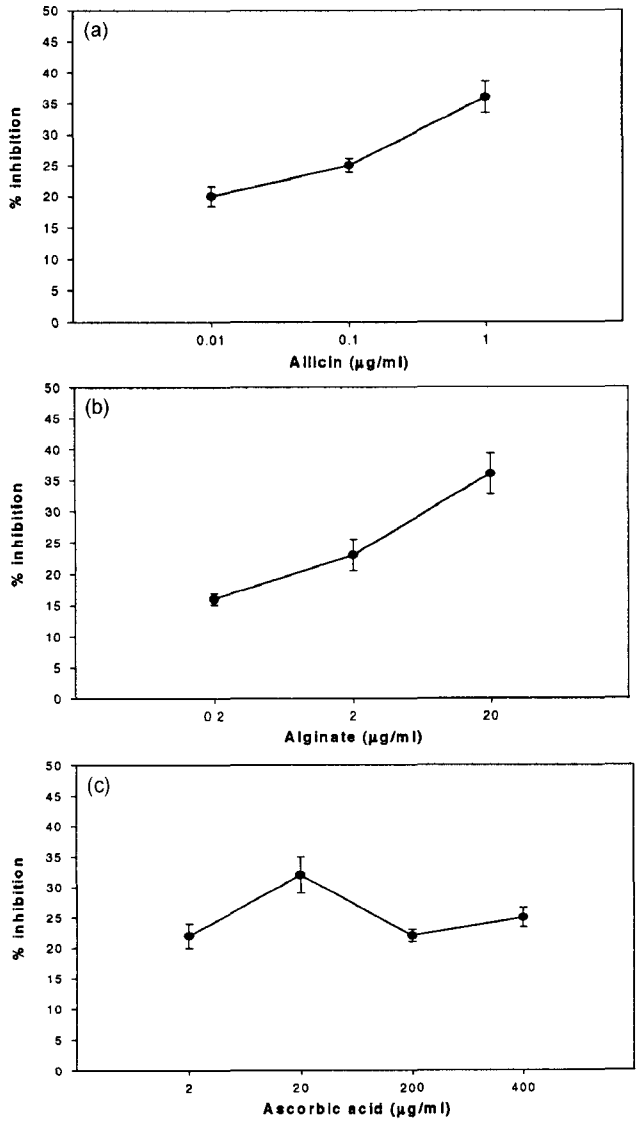

Fig. 3. Inhibition of TNF-α-induced H_2O_2 production by allicin (a), alginate (b) and ascorbic acid (c). The confluent HUVECs were cultured for 20 h in medium or in medium supplemented with TNF-α (5 ng/mL) in the presence or absence of the three compounds. The HUVECs were washed with HBSS to remove the compounds and DCFH-DA was then added to the wells. After 45 min incubation at 37°C, the DCF fluorescence in was measured. The results are represented as a mean ±S.E.M of 3 experiments, after calculating the percentage of H_2O_2 production in comparison with that of control.

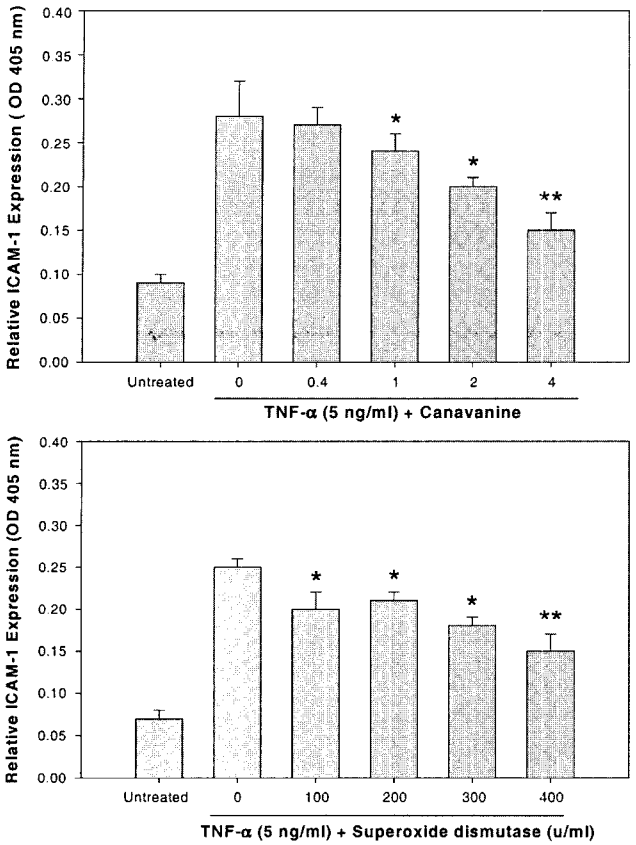

Fig. 4. Effect of Canavanine (a) and superoxide dismutase (b) on TNF-α-induced ICAM-1 expression in HUVECs. Three independent experiments were performed in which the confluent HUVECs were treated for 20 h with or without TNF-α in the presence or absence of the free radical inhibitors. The conditioned medium was collected, and ICAM-1 expression level was measured by ELISA. The results are represented as a mean±S.E.M from three independent experiments. *$p<0.05$, **$p<0.01$; significantly different from TNF-α-treated control.

molecules (Palvick *et al.*, 2002). As shown in Fig. 2, treatment of the HUVECs with TNF-α resulted in an increase in the NO release that was partially inhibited by the three compounds.

This study also examined the effect of these compounds on the production of H_2O_2 since several lines of evidence suggests that reactive oxygen species are implicated in ICAM-1 expression (Cominacini *et al.*, 2002; Peng *et al.*, 2000). Stimulation of the HUVECs with TNF-α resulted in a 31% increase in H_2O_2 release compared to the control without the TNF-α treatment. The simultaneous treatment with the test compounds inhibited partially the H_2O_2 release and the inhibition was concentration dependent except for ascorbic acid (Fig. 3).

In addition, when the HUVECs that were treated with TNF-α was exposed to either canavanine, an inhibitor of the L-arginine-dependent synthesis of the nitrogen derivatives, or superoxide dismutase, a reactive oxygen scavenger, the ICAM-1 expression level was decreased when compared to control. The dose selection of inhibitors for these experiments was based on the results reported by other groups (Aoki *et al.*, 1997; Bence *et al.*, 2002; Morandini *et al.*, 1999). These results indicate that the mechanism by which the three compounds from the dietary sources decrease the ICAM-1 expression level may be similar to the function of the free radicals inhibitor (Fig. 4).

DISCUSSION

Epidemiological studies have revealed an associations between the consumption of antioxidants-rich foods or beverages and the prevention of various diseases. Possible plant nutrients providing this protection include antioxidants and dietary fiber. Recently, many phytochemicals have been found to have diverse properties, including antioxidant, antiallergic, anti-inflammtory, antiviral, and anti-carcinogenic (Middleton, 1998; Eastwood, 1999; Fotsis *et al.*, 1997). Although alginate, allicin and ascorbic acid possess anti-inflammatory properties, very little is known regarding their effects on the induction of the cell adhesion molecules by TNF-α. In this study, these compounds blocked the expression of the leukocyte adhesion molecules, ICAM-1, induced by TNF-α. Therefore, these compounds exhibit anti-inflammatory effects on the expression of the adhesion proteins induced by TNF-α. These compounds also inhibited NO and H_2O_2 production in the TNF-α-induced HUVECs.

TNF-α, which participates in the inflammatory response, induces the expression of the adhesion molecules when added to the endothelial cells in the culture (Sherman *et al.*, 1991; Brach *et al.*, 1993; Bevilacqua *et al.*, 1989). Nitric oxide is a biologically active gas that is synthesized by a variety of cells, including vascular endothelium cells, from the guanido group of L-arginine. This reactive nitrogen molecule is invoked as a mediator of the vascular phenomena such as arteriolar dilation, platelet aggregation, and platelet-leukocyte adhesion (Moncada, 1992). It has been suggested that NO can inhibit the LPS-induced ICAM-1 expression (Spiecker *et al.*, 1998). Hence, NO could inhibit the expression of the adhesion molecules. These results showed that TNF-α induces NO production and the three compounds partially inhibit the release of NO.

Intracellular reactive oxygen intermediates (ROIs) play an important role in inflammation, and are involved in signaling the upregulation of leukocyte-endothelial cells, particularly ICAM-1 on the endothelial cells by TNF-α (Bauerle *et al.*, 1994). This study showed that three compounds partially prevent the intracellular increase in the H_2O_2 concentration induced by TNF-α. It is likely that this effect is related to the antioxidant activities of these three compounds, and their protective effects correlates

with their direct radical scavenging ability. In addition, it was shown that the intracellular free radicals function as secondary messengers for signal transduction (Hensley *et al*., 2000). Therefore, these results suggest that the three compounds from the dietary sources decrease ICAM-1 expression level by modulating the production of the reactive oxygen/nitrogen components, which may block the expression of this adhesion molecule through an intracellular mechanism. However, this pharmacological intervention does not prove the role of the specific mediators. In addition, the results did not totally rule out the possibility that these compounds directly inhibit the expression of ICAM-1 induced by TNF-α, because the three compounds partially differentially inhibited the production of the free radicals at the various concentrations

Recently, the signaling pathway involved in TNF-α-induced ICAM-1 expression in the human epithelial or endothelial cells has been reported. Previous studies reported that TNF-α activated the phosphatidylcholine-specific phospholipase C (PC-PLC) to induce the activation of protein kinase C, the protein tyrosine kinase, and the NF-κB site of promoter, followed by the initiation of ICAM-1 expression (Chen *et al*., 2001; Johnson *et al*., 1996). Therefore, it is conceivable that alginate, allicin and ascorbic acid modulate intracellular signaling including these signaling pathways. A recent study showed that allicin blocked the expression of ICAM-1 induced by γ-irradiation by inactivating p38 MAP kinase in the HUVECs (unpublished data). We currently investigate the molecular basis for the regulatory signaling modulated by the three compounds in this system.

During severe injury, infection, or ischemia and reperfusion damage, a spillover of the chemoactivators in the systemic circulation results in the cellular activation. This then leads to the release of injurious agents that damage the host tissues. These inflammatory mediators can alter the functional integrity of the vascular system, which may be due to the upregulation of the expression level of the cell adhesion molecules. Therefore, various strategies, such as monoclonal antibodies against adhesion molecules, soluble receptors, soluble counter-receptors, peptides derived from the adhesion molecules to prevent receptor-ligand interactions, and antisense oligonucleotides have been employed to inhibit the cell adhesion molecules (Weiser *et al*. 1997). Glucorticoid, bensothiophene-carboxamide and vitamin A inhibit cytokine-or irradiation-induced cell adhesion molecule expression (Brostjan *et al*., 1997; Cobb *et al*. 1996; Redlich *et al*., 1998). This study demonstrated that alginate, allicin and ascorbic acid effectively blocked the expression of leukocyte adhesion molecule, ICAM-1. Overall phytochemicals from dietary sources may serve as a potential therapeutic tool against a variety of diseases including inflammation. Further studies are needed to clarify how this modulation occurs and to what extent it occurs *in vivo*.

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