Manassantin A and B Isolated from *Saururus chinensis* Inhibit TNF-α-Induced Cell Adhesion Molecule Expression of Human Umbilical Vein Endothelial Cells

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Leukocyte adhesion to the vascular endothelium is a critical initiating step in inflammation and atherosclerosis. We have herein studied the effect of manassantin A (1) and B (2), dineolignans, on interaction of THP-1 monocytic cells and human umbilical vein endothelial cells (HUVEC) and expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin in HUVEC. When HUVEC were pretreated with 1 and 2 followed by stimulation with TNF-α, adhesion of THP-1 cells to HUVEC decreased in dose-dependent manner with IC50 values of 5 ng/mL and 7 ng/mL, respectively, without cytotoxicity. Also, 1 and 2 inhibited TNF-α-induced up-regulation of ICAM-1, VCAM-1 and E-selectin. The present findings suggest that 1 and 2 prevent monocyte adhesion to HUVEC through the inhibition of ICAM-1, VCAM-1 and E-selectin expression stimulated by TNF-α, and may imply their usefulness for the prevention of atherosclerosis relevant to endothelial activation.

**Key words:** Manassantin A/B, Cell adhesion molecules, TNF-α, Monocyte, Human umbilical vein endothelial cells, Atherosclerosis

INTRODUCTION

Accumulating evidence suggests that inflammation plays a major role in the development of atherosclerosis. It is recognized that inflammation stimulates cell adhesion molecule (CAM) expression at the site of atherosclerosis (Ross, 1999). Atherosclerotic plaques develop from complex multicellular processes in which the recruitment of circulating monocytes to focal areas of the arterial subendothelium is an early event. Initially, monocytes adhere to activated endothelium displaying inducible adhesive glycoproteins or CAMs, before migrating across the endothelial layer to the intima where they differentiate into macrophages and sequester cholesterol to form characteristic foam cells. Localized endothelial cell adhesion molecule up-regulation, a pre-requisite for monocyte migration, is a dynamic process which is sensitive to inflammatory cytokines, shear stress and oxidative insults (Imhof and Dunon, 1995). Levels of certain CAMs are elevated in human atherosclerotic tissue with vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin, in particular, being indicators of inflammation and early atherosclerosis (Van der Wal et al., 1992).

Tumor necrosis factor-α (TNF-α) is one of the major inflammatory cytokines that mediates systemic inflammation and immune responses (Pfeffer et al., 1993). A major site of action of TNF-α for these effects is the vascular endothelium (Pober and Cotran, 1990), where it induces inflammatory responses by enhancing adhesion molecule expression and secretion of inflammatory mediators (Springer, 1994; Modur et al., 1996).

Manassantin A (1) and B (2), dineolignans, were previously isolated from *Saururus chinensis* (Rao and Alvarez, 1983) and are known to have a variety of biological activities, such as murine neuroleptic (Rao et al., 1987), anti-plasmodial (Kraft et al., 2002), anti-inflammatory (Hwang et al., 2003), and human ACAT inhibitory activities (Lee et al., 2004). We previously...
reported that 1 and 2 inhibited phorbol 12-myristate 13-acetate (PMA)-induced ICAM-1 expression of HL-60 cells (Rho et al., 2003). In this study, therefore, we investigated whether 1 and 2 affect to the interaction of monocyte and human umbilical vein endothelial cells (HUVEC) and TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin in HUVEC.

MATERIALS AND METHODS

Materials

Compounds 1 and 2 were isolated from MeOH extract of Saururus chinensis Baill. (Saururaceae) root as reported previously (Rho et al., 2003) (Fig. 1). 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy-methyl ester (BCECF-AM), 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), and fluorescein isothiocyanate (FITC)-conjugated antibodies were obtained from Sigma Chemical Co. (St. Louis, MO, USA). CAM monoclonal antibodies and recombinant human TNF-α were provided from R&D system (Minneapolis, MN, USA). Endothelial cell basal medium-2 (EBM-2) Bullet kit was purchased from Clonetics (San Diago, CA, USA) and all other tissue culture reagents were obtained from GIBCO-BRL (Gaithersburg, MD, USA).

Cell culture

Human acute monocytic leukemia THP-1 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA), cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), and maintained at a cellular density of 2 x 10^5 to 10^6 cells/mL, as described previously (Tsuchiya et al., 1980). HUVEC were purchased from Clonetics (San Diego, CA, USA) and were grown on gelatin-coated culture dishes or 24-well plates in EBM-2, and used for the experiments within the first 3 to 4 passages. The cells were supplemented at 37°C in a humidified atmosphere of 5% CO₂.

Fluorescent labeling of THP-1 cells

THP-1 cells were fluorescently labeled with BCECF-AM for quantitative adhesion assay (Vaporciyan et al., 1993). In brief, the fluorescence labeling of THP-1 cells was done by including cells (2 x 10^5 cells/mL) with 5 μM of BCECF-AM in RPMI-1640 medium for 30 min at 37°C and 5% CO₂. After loading of BCECF-AM, cells were washed three times with 1% FBS in phosphate buffered saline (PBS) to remove excess dye. Cells were then resuspended in EBM-2 at a density of 5 x 10^5 cells/mL.

Adhesion assay

HUVEC (5 x 10^5 cells/well) grown to confluence in a 24-well plate were pretreated with various concentrations of 1 and 2 at 37°C for 24 h and stimulated with 10 ng/mL of TNF-α for 8 h prior to the adhesion assay. BCECF-AM-labeled THP-1 cells (2.5 x 10^5 cells/well) were co-incubated with HUVEC for 60 min at 37°C. After incubation, non-adherent cells were removed by washing each well three times with 1% FBS in PBS. The attached cells were dissolved in 50 mM of Tris-HCl (pH 7.6) containing 0.1% sodium dodecyl sulfate (SDS). The fluorescence intensity of each well was measured using a fluorescence multi-well plate reader (Wallac 1420, Germany) at excitation and emission wave lengths of 485 and 530 nm, respectively.

Flow cytometry analysis

HUVEC (1 x 10^6 cells) were cultured to confluence in a culture dishes and treated with test samples at 37°C for 12 h. After incubation, 10 ng/mL of TNF-α was added and incubated for 4 h (E-selectin), 6 h (VCAM-1) and 12 h (ICAM-1), respectively. Following steps were performed at cold condition. The cells were harvested with a 1x trypsin/EDTA and wash once with PBS. After washing, cells were incubated with a blocking solution (2% FBS in PBS) for 1 hour. The cells were harvested and stained with 0.5 μg/mL anti-human ICAM-1, VCAM-1, E-selectin mAbs for 1 hour. After washing twice with PBS, primary antibody binding was detected with FITC-conjugated anti-mouse IgG (1:50 dilution in blocking solution) incubated for 1 hour in the dark condition. After washing three times with PBS, the cells were fixed (1% p-formaldehyde in PBS) for 30 minutes as a single-cell suspension (confirmed by phase-contrast microscopy). The fluorescence and light scattering properties (forward scatter and side scatter) of the cells were determined by using a FACSscan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA). Cells with FITC-conjugated antibodies were excited with a 488 nm argon ion laser, and emission was recorded at 525 nm. In each sample, at least 20,000 gated viable cells were examined.

Measurement of cell viability

Cell viability was assessed by morphology and by

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Fig. 1. The chemical structures of manassantin A (1) and B (2) isolated from roots of Saururus chinensis Baill. (Saururaceae).