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ORIGINAL RESEARCH PAPER

Non-radioactive and colorimetric quantification of monocyte adhesion to endothelial cells in early atherogenesis

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Abstract Monocyte adhesion to vascular endothelium is an initial step in atherogenesis. To quantify this, we incubated monocytes with cultured endothelial cells, and quantified the adhered live monocytes using a colorimetric assay. Endothelium activated with lipopolysaccharide attracted monocytes in a dose-dependent manner and the adhesion was attenuated with post-treatments with L-ascorbic acid (53%), α- (40%) and γ-tocopherol (39%), resveratrol (39%), and *Lithospermum erythrorhizon* root extract (45%). This non-radioactive, colorimetric assay may be useful for screening anti-atherogenic compounds in early atherogenesis.

Keywords Atherosclerosis · Endothelium/monocyte binding · Anti-atherogenic

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Introduction

Atherosclerosis progresses with the accumulation of cholesterol-rich lipoproteins in the arteries. The adhesion of circulating monocytes to the endothelium and the subsequent differentiation of monocytes into macrophages, with the uptake of oxidized low-density lipoproteins (LDL) by macrophages, are the earliest steps in the formation of atherosclerotic lesions (Ross 1993; Libby 2002). Cellular adhesion molecules such as ICAM-1 supports monocyte binding to, and their interaction with, endothelial cells and might play a crucial role in this complex process (Gräfe et al. 1997). Therefore, it is possible that attenuating monocyte adhesion to endothelium could delay the progression of atherosclerosis. Consequently, an assay of endothelium/monocyte binding in the formation of atherosclerotic lesions might be used to screen anti-atherogenic compounds (Usha et al. 2002; Woollard et al. 2002).

The techniques that have been used to measure endothelium/monocyte binding include counting paraformaldehyde-fixed monocytes on endothelium (Ferrero et al. 1998), quantifying chromium-labeled leukocytes adherent to target endothelial cells (Warren et al. 1996), and ELISA-based detection of cell-surface antigens (Takahashi et al. 1996). However, these methods have the disadvantages of low sensitivity, high price, and experimental inconvenience involving the handling of

radioactive materials. Consequently, an improved method for screening anti-atherogenic activity is needed. Therefore, we developed a rapid and convenient assay using the endothelium/monocyte interaction and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test to screen for anti-atherogenic activity.

Materials and methods

Endothelial cell cultures

Calf pulmonary arterial endothelial cells (CPAE) was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea) and maintained in RPMI 1640 supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0.5% gentamicin.

Monocyte cultures

Monocytic leukemia cell line THP-1 was obtained from KCLB and cultured in RPMI 1640 containing 10% (v/v) heat-inactivated FBS, 1% penicillin/streptomycin, and 0.5% gentamicin. The cells were maintained at a density between 5×10^4 and 8×10^5 viable cells/ml by adding fresh medium or replacing the medium.

Endothelium-monocyte binding assay for screening anti-atherogenic activity

Confluent monolayers of CPAE, cultured in six-well culture plates, were stimulated with LPS or TNF-α for 4 h, and then treated with resveratrol, gromwell (*Lithospermum erythrorhizon*) extract, α-tocopherol, γ-tocopherol, or vitamin C for 2 h. Then, 1,775,000 THP-1 cells in 1 ml RPMI 1640 medium containing 10% (v/v) FBS, 1% penicillin/streptomycin, and 0.5% gentamicin were added to each well and incubated for 30 min at 37°C under a 5% CO_2 atmosphere. After 30 min, non-bound cells were removed by gentle washing with RPMI 1640. Next, MTT reagent at 1 mg/ml in the culture media was added to each well, and then incubated for 2 h at 37°C under a 5% CO_2 atmosphere. After 2 h, 1 ml of the lysis buffer described below was added to each well and incubated for 20 h at 37°C under a 5% CO_2 atmosphere in the dark. The absorbance at 570 nm was measured. The endothelium/monocyte binding was calculated by subtracting the absorbance of CPAE without THP-1 cell binding from the absorbance when THP-1 cells were bound to CPAE.

Lysis buffer for the endothelium/monocyte binding assay

SDS was dissolved in water with moderate heating and stirring to give 40% (v/v). Then, an equal volume of *N*,*N*-dimethyl formaldehyde was added to the 40% (v/v), SDS solution, mixed for 30 min with stirring at room temperature and then filtered through filter paper and the pH adjusted to 4.7 by adding an equal volume of 80% (v/v), acetic acid and 1 M HCl (Hansen et al. 1989).

RT-PCR analysis

Total RNA from CPAE was extracted using Trizol reagent and quantified at 260 nm. The RNA was stored at –80°C until analysis, and the yield was analyzed within 1 month. Single-strand cDNA was synthesized from 2 μg mRNA using a cDNA cycle kit and oligo dT. The reaction was performed in a 19 μl final volume for 90 min at 42°C and subsequently for 15 min at 70°C. The resulting cDNA templates were subject to PCR amplification. The PCR was performed in a 20 μl final volume containing cDNA, 10 × PCR buffer, 2.5 mM dNTPs, 10 mM forward and reverse primers, DNA polymerase, and sterile water. The primers for bovine ICAM-1 (GenBank accession #U09134) were 5′-AACTCTTCCAGAACACCTCG-3′ (forward) and 5′-AGCACCAGGTAGACCTTAGC-3′ (reverse), and the product size was 169 bp. The primers for β_2-microglobulin (GenBank accession #X69084) were 5′-TGGCCTTGGTCCTTCTC-3′ (forward) and 5′-AGCACCAGGTAGACCTTAGC-3′ (reverse), and the product size was 358 bp. To amplify IACM-1 and β_2-microglobulin, 30 PCR cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C were used (Kampen and Mallard 2001a, b). The PCR products (10 μl) were electrophoresed on 1.2% agarose gels containing ethidium bromide in a

constant 110-V field. The relative band densities were determined using UN-SCAN-IT software (Silk Scientific, Orem, UT, USA).

Statistical analysis

All the data are expressed as means ± S.D. Two groups were compared using Student's *t*-test. Differences at $P < 0.05$ or $P < 0.01$ were considered statistically significant.

Results and discussion

Using RT-PCR, we confirmed that angiotensin-converting enzyme (ACE) was expressed in all of the CPAE endothelial cells which is a basic feature of CPAE endothelial cells (data not shown, Kobayashi et al. 2001). Furthermore, we chose CPAE for this assay because the growth and handling of CPAE is rapid and convenient compared with other endothelial cell lines.

CPAE grown in the absence or presence of LPS or TNF-α for 4 h was incubated with THP-1 cells for 30 min at 37°C. As shown in Fig. 1, LPS and TNF-α stimulation enhanced the binding of THP-1 cells to CPAE, and increasing the numbers of THP-1 cells induced further endothelium/monocyte binding. Furthermore, 1–5 μg LPS/ml induced slightly more endothelium/monocyte binding than did 1 pg TNF-α/ml.

CPAE was stimulated with 5 μg LPS/ml for 4 h, treated with 100 nM resveratrol for 2 h and then incubated with various concentrations of THP-1 cells. Except for the endothelium/monocyte binding in 700,000 THP-1 cells, resveratrol had no effect on the basal binding of monocytes to non-activated endothelium. Usha et al. (2002) suggested that resveratrol suppressed monocyte binding to endothelium by impairing the activation of endothelium, and monocyte binding in non-activated endothelium did not change. However, our data show a tendency for the binding to decrease, compared with the treatment with 5 μg LPS/ml (Fig. 2).

A further study was performed to screen for anti-atherogenic activity using resveratrol, gromwell extract, γ-tocopherol, α-tocopherol,

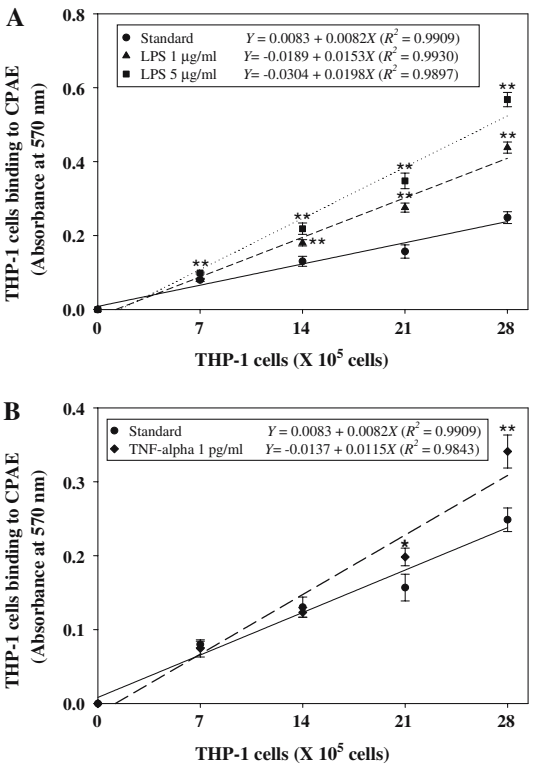

Fig. 1 The binding of THP-1 cells to CPAE in the absence or presence of LPS or TNF-α. CPAE was treated with LPS (**A**) or TNF-α (**B**) for 4 h and then incubated with various numbers of THP-1 cells for 30 min
*, ** Significantly different number of THP-1 cells from the standard (no treatment), $*P < 0.05$ and $**P < 0.01$. Data are expressed as the means ± S.D. for three independent experiments

and vitamin C. CPAE was stimulated with 5 μg LPS/ml or 1 pg TNF-α/ml for 4 h and then treated with 100 nM resveratrol, 0.1 μg gromwell extract/ml, 25 μM γ-tocopherol, 100 μM α-tocopherol, or 100 μM vitamin C for 2 h, and then incubated with 2,800,000 THP-1 cells. Figure 2 shows relative values compared with the absorbance of the control (no treatment). LPS significantly increased the binding of THP-1 cells to CPAE compared with the control, whereas resveratrol, gromwell extract, γ-tocopherol, α-tocopherol, and vitamin C markedly inhibited monocyte binding to endothelium on LPS stimulus (Fig. 3A). TNF-α slightly induced the binding of THP-1 to CPAE compared with the control, while resveratrol, gromwell extract, and

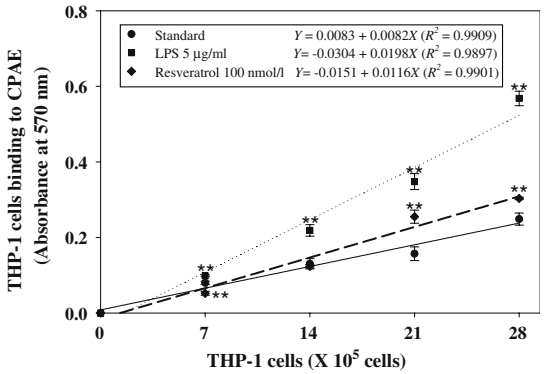

Fig. 2 Binding of THP-1 cells to CPAE in the absence or presence of LPS or resveratrol. CPAE was treated with LPS for 4 h or resveratrol for 2 h, and then incubated with various numbers of THP-1 cells for 30 min
*, ** Significantly different number of THP-1 cells from the standard (no treatment), $^{*}P < 0.05$ and $^{**}P < 0.01$. Data are expressed as the means ± S.D. for three independent experiments

γ-tocopherol treatment suppressed the monocyte binding to activated endothelium (Fig. 3B).

The MTT test is widely used for analyzing cytotoxicity, cell viability and the proliferation of living cells (Vellonen et al. 2004). In this study, measuring live cells using the MTT test was identical to detecting THP-1 cells binding to CPAE. The absorbance of the THP-1 cells is determined from the difference between the absorbance of CPAE alone and that of CPAE with THP-1 cells bound to it.

The expression of the cell adhesion molecules regulating the endothelium/monocyte binding interaction is affected by stimulating factors and the treatment time (Kampen and Mallard 2001a, b). Anti-atherogenic materials have different effects via many pathways depending on the class of the adhesion molecule and stimulating factors (Zapolska-Downar et al. 2001; Cominacini et al. 1997).

Figure 4 shows the relative expression of ICAM-1 mRNA compared with the control (no treatment). The expression of ICAM-1 on CPAE was increased after exposure to 5 μg LPS/ml for 4 h, while the gromwell extract and γ-tocopherol significantly suppressed the expression of ICAM-1 and TNF-α did not affect the expression of ICAM-1. Although the gromwell extract inhibited the expression of ICAM-1 after exposure to

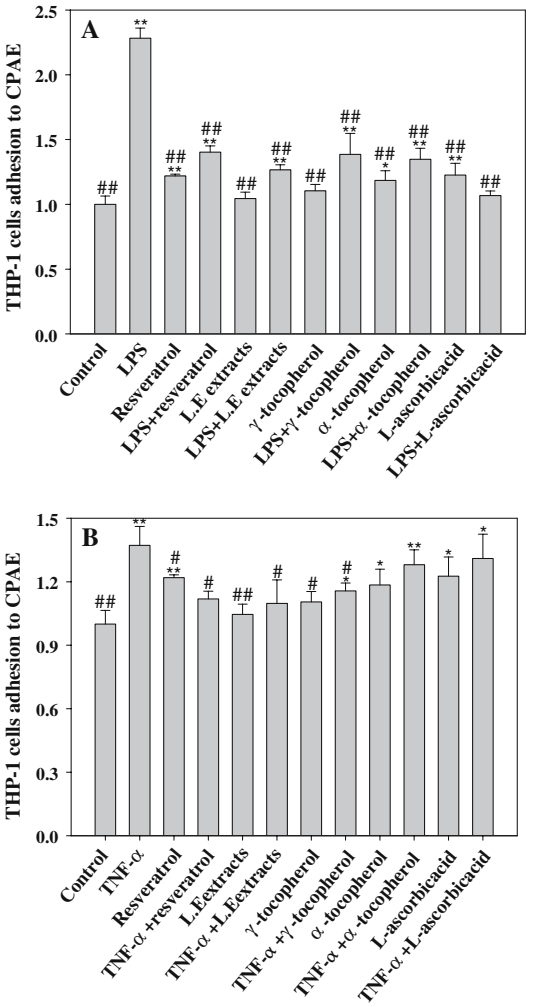

Fig. 3 Binding of THP-1 cells to CPAE treated with anti-atherogenic materials in the absence or presence of LPS or TNF-α stimulus. CPAE was stimulated with 5 μg LPS/ml or 1 pg TNF-α/ml for 4 h and then treated with anti-atherogenic materials for 2 h, and subsequently incubated with 2,800,000 THP-1 cells for 30 min. (**A**) Control, no treatment; LPS, 5 μg/ml; resveratrol, 100 nM; LE, *Lithospermun erythorhizon* extract, 0.1 μg; γ-tocopherol/ml, 25 μM; α-tocopherol, 100 μM; vitamin C, 100 μM. (**B**) CPAE was stimulated with 1 pg TNF-α/ml instead of LPS in (**A**)
*, ** Significantly different from control (no treatment), $^{*}P < 0.05$ and $^{**}P < 0.01$. $^{\#,\#\#}$ Significantly different from LPS or TNF-α, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$. Data are expressed as the means ± S.D. for three independent experiments

TNF-α, the effect was not significant. The effect of TNF-α on the endothelium/monocyte interaction was more prominent than on ICAM-1 expression.

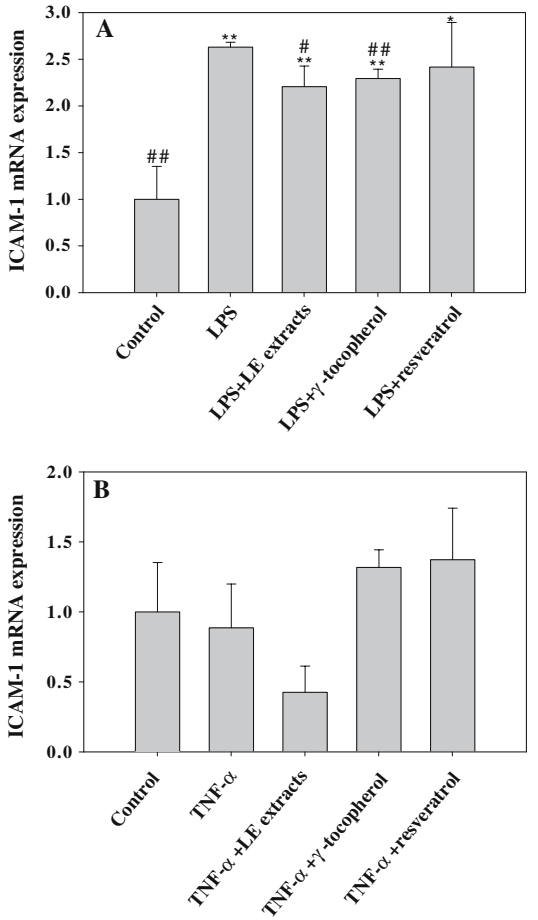

Fig. 4 ICAM-1 mRNA expression in CPAE treated with anti-atherogenic materials in the presence of LPS or TNF-α stimulus. CPAE was stimulated with 5 μg LPS/ml or 1 pg TNF-α/ml for 4 h and then treated with anti-atherogenic materials for 2 h. a, Control (no treatment); LPS (5 μg/ml); LE, *Lithospermun erythorhizon* extract (0.1 μg/ml); γ-tocopherol (25 μM); resveratrol (100 μM); TNF-α (1 pg/ml); LE, *Lithospermun erythorhizon* extract
*, ** Significantly different from control (no treatment), $^{*}P < 0.05$ and $^{**}P < 0.01$. $^{\#,\ \#\#}$ Significantly different from LPS or TNF-α, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$. Data are expressed as the means ± S.D. for three independent experiments. The ICAM-1 mRNA level in each sample was normalized using β_2-microglobulin as a reference

These data suggest that LPS is a more effective short-term endothelial stimulant than is TNF-α. Kampen and Mallard (2001a, b) suggested that the amount of bovine ICAM-1 mRNA expression varies significantly over time and the expression kinetics differ according to the stimulus. They showed that the surface expression of bovine ICAM-1 in LPS-stimulated bovine aortic endothelial cells (BAECs) peaked at 3 h, while that of TNF-α -stimulated BAECs peaked at 12–18 h. Therefore, prolonged treatment with LPS, TNF-α, and anti-atherogenic materials gives rise to more definitive results. However, it is thought that treatment with an anti-atherogenic material for 2 h after LPS stimulus for 4 h is sufficient for screening for anti-atherogenic activity. Therefore, our assay can be used for the rapid and convenient screening of anti-atherogenic activity by measuring endothelium/monocyte binding regulated by cell adhesion molecules.

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