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Role of Anti-Endothelial Cell Antibody in the Development of Coronary Arterial Lesions in Kawasaki Disease

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ABSTRACT

Background and Objectives : Anti-endothelial cell antibodies (AECA) are found in the sera of many patients with Kawasaki disease (KD). In this study, the pathogenic role of AECA in the development of coronary arterial lesions of KD was investigated. **Subjects and Methods :** Serum IgM-AECA concentrations were measured in 22 KD patients. Cultured human coronary artery endothelial cells (HCAEC) were incubated with either acute or convalescent phase sera, and their expressions of intercellular adhesion molecule-1 (ICAM-1) assessed. IgM fractions of the sera were purified, and their ability to induce ICAM-1 mRNA and protein expressions evaluated. To address the signal transduction pathways involved in IgM-AECA-induced ICAM-1 expression, the blocking effect of four protein kinase inhibitors, PD98059, SB203580, dimethylaminopurine (DMAP) and parthenolide were measured. **Results :** IgM-AECA was present in 14 out of 22 (64%) acute KD sera. ICAM-1 expression of HCAEC incubated with acute KD sera (117.1 ± 46.7) and AECA-positive acute KD sera (143.3 ± 37.5) were significantly higher than those of the convalescent KD sera (88.9 ± 14.4, $p<0.05$) or AECA-negative acute KD sera (71.2 ± 11.8, $p<0.05$), respectively. IgM-AECA from KD patients significantly induced ICAM-1 protein and mRNA expression. The upregulation of ICAM-1 expression was significantly inhibited by SB203580, DMAP and parthenolide, but not by PD98059. **Conclusion :** IgM-AECA was detected in the sera of about 2/3 of acute KD patients, which activated endothelial cells by upregulation of ICAM-1 expression, possibly via p38, JNK MAPK and NF-κB signal transduction pathways. Thus, IgM-AECA may play a pathogenic role in the development of coronary arterial lesions in KD patients. **(Korean Circulation J 2006;36:723-731)**

KEY WORDS : Kawasaki disease ; Coronary vessel anomalies ; Anti-endothelial cell antibody ; Intercellular adhesion molecule-1.

Introduction

Kawasaki disease (KD) is an acute childhood illness characterized by prolonged fever, diffuse mucosal inflammation, conjunctival injection, edema of the hands and feet, skin rash and non-suppurative lymphadenopathy.[1] Without appropriate treatment, many patients may develop carditis,[2] coronary abnormalities[3] and even acute myocardial infarction as late cardiac sequelae.[4] However, the pathogenesis of KD is still not completely understood.

The recruitment and adhesion of circulating polymorphonuclear cells (PMNs) to the vascular endothelium play a critical role in the inflammation response. This event is mediated through the expression of adhesive molecules onto the cell surface of endothelial cells (EC) and PMNs.[5] Although the molecular mechanism of adherence is not completely understood, in vitro studies have identified three molecules that contribute to cell adhesion: intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 and E-selectin. These adhesion molecules can be induced on the endothelial cell surface following stimulation. ICAM-1 is an inducible cell surface glycoprotein found on several cell types, which plays an important role in a number of inflammatory and immune responses. Upregulation of the expression of ICAM-1 on the surface of vascular EC enhances the targeted transmigration of PMNs into the extravascular space of inflammation.[6]

Several studies have demonstrated the presence of anti-endothelial cell antibodies (AECA) in the sera of the KD patients.[7] Although both IgG- and IgM-AECA can induce cytolysis, IgM-AECA is more effective.[8] Ka-

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neko et al.[9] demonstrated that significant cytolysis of cultured human umbilical vein endothelial cells (HUVEC) was induced by the IgM-rich fractions of the sera of KD patients, but not by the IgM-poor fractions. This finding suggested the cytotoxicity of the sera of KD patients was mainly caused by IgM AECA. The correlation between the levels of AECA and the disease activity, as well as the decline in AECA titres following treatment, suggest that AECA may be important in the development of autoimmune and vasculitic diseases, as well as in KD.[10] Despite some conflicting data concerning the ability of AECA to affect resting vs. pre-stimulated cells, several in vitro studies have revealed that KD sera induced activation or damage of EC.[8][9][11]

The expressions of ICAM-1 and other genes appear to be highly regulated by a number of mitogen-activated protein kinases (MAPKs) as well as nuclear factor-κB (NF-κB). In mammalian cells, three MAPK modules have been well characterized; ERK1/2, which preferentially regulates cell growth and differentiation, c-Jun N-terminal kinase (JNK), and the p38 cascades, which function mainly in response to stress, such as inflammation and apoptosis.[12] Depending on the cell type, these MAPKs can be independently and simultaneously activated by extracellular agonists. Following activation, MAPKs can induce nuclear transcription factors, which regulate gene expression and may promote the expression of adhesion molecules.[13]

In this study, the levels of IgM-AECA in the sera of KD patients were measured, and whether sera containing IgM-AECA could induce the expression of ICAM-1 in cultured human coronary artery endothelial cells (HCAEC) determined. Then, in order to directly assess the role of AECA, the ICAM-1 expressions of HCAEC, both before and after treatment with IgM-AECA isolated from the KD, were compared. In addition, the involvement of MAPK was addressed using specific protein kinase inhibitors.

Subjects and Methods

Patients

Twenty two patients meeting the diagnostic criteria for KD[1] were enrolled. All patients received intravenous gamma-globulin and aspirin after the diagnosis of KD. Thirteen age-matched consecutive patients with trivial congenital heart diseases were recruited as controls. No patient had signs of infection or congestive heart failure and no previous history of KD, and none were being treated with any drug.

Blood samples

Paired blood samples were collected from all KD patients. Acute and convalescent phase samples, between the third and eighth day of illness before any treatment, and more than 4 weeks later, when the C-reactive protein concentration had declined to below 0.3 mg/dL, respectively, were taken. Sera and plasmas were separated from the blood samples by centrifugation, and stored at -70℃ prior to use.

Concentrations of plasma tumor necrosis factor-α (TNF-α) measured by ELISA

The plasma TNF-α concentration was determined with a Human TNF-α/TNFSF1A immunoassay kit (Quantikine HS, R&D System Inc, Minneapolis, MN, USA), according to the manufacture's instructions. Briefly, 50 μL Assay Diluent HD1-11 was added to each well, and 200 μL of standard or samples then incubated in duplicate wells for 3 hours at room temperature. After six washes with wash buffer, 200 μL TNF-α HS conjugate was added to each well and incubated for 2 hours at room temperature. After six washes, 50 μL of substrate solution was added to each well, incubated for a further 1 hour at room temperature, and 50 μL amplifier solution then added and incubated for 30 minutes at room temperature. The chromogenic reaction was stopped by the addition of 50 μL stop solution within 30 minutes. The absorbance was read spectrophotometrically at 490 nm using a microplate reader (VERSA max, Molecular Devices, CA, USA). Wavelength correction was performed at either 650 or 690 nm.

Concentration of IgM-AECA in the sera measured by ELISA

The concentration of AECA in the sera was measured using a cyto-ELISA method employing unfixed HCAEC. The cells were seeded in 96-well microtitre plates, and allowed to grow to confluence over a 48 hour period, at a concentration of 4×10^4 cells per well. Cells were washed twice with phosphate-buffered saline (PBS). A total of 100 μL of serum samples, diluted 1 : 50 in Hanks balanced salt solution (HBSS) with divalent cations (Irvine Scientific, Santa Ana, CA, USA) and 1% bovine serum albumin (SIGMA, St. Louis, MO, USA), were added to each well and incubated for 60 minutes at 37℃. Cells were washed a further twice and incubated for 60 minutes at 37℃ with the secondary antibody, peroxidase-conjugated goat anti-human IgM (SIGMA, St. Louis, MO, USA), diluted 1 : 1000 with HBSS containing divalent cations and 5% new-born calf serum (Gibco, Gaithersburg, MD, USA). After washing, the binding of the antibody was quantified colorimetrically, using tetramethylbenzidine (TMB, SIGMA, St. Louis, MO, USA). A 1 mg/mL stock solution of TMB in acetone was added to 10 mL of distilled water. 1 μL of 30% H_2O_2 was immediately added prior to use. The chromogenic reaction was stopped by the addition of 25 μL 4M H_2SO_4. The absorbance was read spectrophotometrically at 450 nm on an ELISA reader (VERSA max,

Molecular Devices, CA, USA). Serum was taken from a positive control patient with systemic lupus erythematosus, as well as from a negative control patient. The results were expressed as an ELISA ratio (ER), which was calculated as ER=100× (S-A)/(C-A); where S is the absorbance of the sample, and A and C the absorbance of the standard negative and positive controls. Values are the means of duplicate determinations.

Culture of HCAEC

HCAEC were purchased from Cambrex Bio Science Walkersville, Inc (Walkersville, MD, USA). Cells were cultured in EGM-2 (endothelial basal media-2 supplemented with 5% FBS, hEGF, hydrocortisone, GA-1000, VEGF, hFGF-B, R3-IGF-1, ascorbic acid; Cambrex Bio Science Walkersville, Inc, Walkersville, MD, USA) at 37℃ in a humidified 5% CO_2 atmosphere. When the cultures reach confluence (5 days), the cells were detached from the culture flasks using 0.025% trypsin/ 0.01% EDTA, neutralized with trypsin neutralizing solution, washed and then resuspended in complete medium. The culture medium was changed after 24 hours, and every other day thereafter. Cells were starved in serum-free EGM-2 for 24 hours prior to treatment with sera or purified IgM fractions. All experiments were performed with the cells kept in culture for between four and seven passages.

Flow cytometric analysis of ICAM-1 expression by HCAEC treated with sera

The sera were diluted 25% with culture medium, and sterilized using a 0.2 μm pore sized filter (PALL, MI, USA). Diluted sera were added to confluent monolayers of HCAEC and incubated for 24 hours. The negative controls used in the experiments included culture medium alone and control subjects. After incubation, the HCAEC monolayers were washed once with 0.5% BSA/PBS. The cells were then stained with fluorescein-conjugated antibody directed against ICAM-1 (CD54, R&D system Inc, Minneapolis, MN, USA) for 45 minutes at 4℃. After three washes with 0.5% BSA/PBS, the cells were resuspended in 500 μL of 0.5% BSA/PBS, and then enumerated in a flow cytometer (Becton Dickinson, Franklin, NJ, USA). The appropriate settings of the forward and side scatter gates were used to examine 20,000 cells per experiment. The number of fluorescent molecules per cell was indirectly measured as the mean fluorescence intensity of the cells analyzed in each test.

Immunoglobulin purification

The IgM fractions of the sera were purified by gel filtration using Ultrogel AcA 34 columns (SIGMA, St. Louis, MO, USA), and stored at 4℃ while in use, or at -20℃ when stored for long periods. The IgM fractions were sterilized using a 0.2 μm pore sized filter (PALL, MI, USA), with the protein concentration determined spectrophotometrically immediately prior to use.

The ICAM-1 expression of HCAEC measured by ELISA

The cells were seeded in 96-well microtitre plates, and allowed to grow to confluence over 48 hours, at a concentration of 4×10^4 cells per well. Cells were incubated with the purified IgM fractions (200 μg/mL at the final volume of 100 μL) of AECA-positive patients (AECA-positive IgM) or AECA-negative patients (AECA-negative IgM) for 24 hours. A positive control was stimulated by 20 ng/mL TNF-α (SIGMA, St, Louis, MO, USA), and two negative controls were incubated with medium alone and with IgM fraction from control patients, respectively. Parallel experiments were performed with the inhibitors added prior to the application of the AECA-positive IgM. After incubation, the cells were washed twice with PBS, and incubated for 60 minutes at 37℃ with 100 μL/well monoclonal mouse anti-ICAM-1 antibody (R&D system Inc, Minneapolis, MN, USA), to a final dilution of 1 : 50. After two further washes, the cells were incubated with 100 μL peroxidase-conjugated goat anti-mouse IgG, diluted 1 : 200, for a further 60 minutes at 37℃. After two washes with PBS, the binding of the antibody was quantified colorimetrically by the addition of 100 μL TMB. The chromogenic reaction was stopped by the addition of 25 μL 4 M H_2SO_4, and the plates read spectrophotometrically at 450 nm on an ELISA reader (VERSA max, Molecular Devices, CA, USA).

Signal transduction inhibitors

Four different signal transduction inhibitors were purchased from SIGMA (St, Louis, MO, USA): PD 98059 [2-(2'-amino-3'-methoxyphenyl) oxanaphthalen-4-one] for specifically blocking MEK1, SB203580 [4-(4-fluorophenyl)-2-(4-ethylsulfinyl)-5-(4-pyridyl) imidazole] for blocking p38, DMAP (dimethylaminopurine) for blocking JNK and parthenolide for blocking NF-κB. Stock solutions of PD98059 (50 mM), SB203580 (10 mM) and parthenolide (10 mM) in dimethyl sulfoxide (DMSO) and DMAP (40 mM) in H_2O were stored at -20℃. All the inhibitors were diluted in EGM-2 prior to their addition to the cells. HCAEC were starved in serum-free EGM-2 for 24 hours. The inhibitors were added prior to the application of AECA-positive IgM [PD98059 (50 μM for 1 hour), SB203580 (10 μM for 1 hour), DMAP (1 mM for 15 minutes) and parthenolide (10 μM for 1 hour)].

Total RNA extraction and reverse transcriptionpolymerase chain reaction (RT-PCR)

After 4 hours of incubation with purified IgM fractions, HCAEC grown in 10-cm culture dishes were ha-

rvested. Total RNA was isolated from the cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the protocol of the manufacturer. The RNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260 and 280 nm.

First strand cDNA synthesis was performed using an Advantage RT-for-PCR Kit (Carpentaria, Palo Alto, CA, USA), in a final volume of 20 μL. 1 μg (12.5 μL) of total RNA and 1 μL of oligo-dT primer were heated at 70℃ for 2 minutes, and then quickly chilled on ice. The following components were then added: 4 μL 5× reaction buffer [final concentrations were 50 mM, Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$], 1 μL dNTP mix (10 mM, each final concentration 0.5 mM), 0.5 μL RNase inhibitor and 1 μL MMLV reverse transcriptase (200 units/μg RNA). The reaction was carried out at 42℃ for 60 minutes, and terminated by incubating at 94℃ for 5 minutes.

For the PCR reaction, 2 μL of the diluted cDNA, 2 μL 10×PCR buffer, 1 μL dNTP mix (10 mM each), 1.2 μL $MgCl_2$, 1 μL of ICAM-1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primer and 1 Taq Bead (1.25 u/bead) hot start polymerase (Promega, Madison, USA) were added, to a final volume of 20 μL.

The oligonucleotide primers for ICAM-1 and GAP DH were as follows:

ICAM-1: 5'-CGACTGGACGAGAGGGATTGT-3' (sense)
5'-ATTATGACTGCGGCTGCTACC-3' (anti-sense)
GAPDH: 5'-TCACCAGGGCTGCTTTTAACTC-3' (sense)
5'-GGTGAAGACGCCAGTGGACTC-3' (anti-sense)

The amplification profile included; 1 cycle of initial denaturation at 94℃ for 5 minutes, 35 cycles of denaturation at 94℃ for 30 seconds, primer annealing at 58℃ for 30 seconds and extension at 72℃ for 30 seconds, followed by 1 cycle of final extension at 72℃ for 5 minutes. The sizes of the PCR products were 290 and 257 bp for ICAM-1 and GAPDH, respectively. The expression of GAPDH was used as an internal control for the assay of a constitutively expressed gene.

Statistics

The results are expressed as the mean±SD. Statistical analysis was performed using the non-parametric Mann-Whitney U test for inter-group comparison, corrected using the Bonferroni method. Values of $p<0.05$ were considered statistically significant.

Results

Patients

Of the twenty-two patients 14 were male and 8 were female, with a mean age of 2.4±1.7 years (0.5-6 years) and duration of fever of 7.4±0.8 days. Conjunctivial injection was noted in 18 (82%), oral mucosal changes in 17 (77%), a rash in 21 (95%), extremity changes in 15 (68%) and cervical enlargement in 14 (64%). Two patients developed coronary aneurysms. The initial white blood cells (WBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and TNF-α in the plasma of acute stage were $14.6\pm8.2\times10^3/mm^3$, 8.9±6.9 mg/dL, 71.5±40.3 mm/1 hour and 4.4±1.6 pg/mL, respectively. Among the thirteen children in the control group, 7 and 6 were male and female, respectively, with a mean age of 2.8±1.8 years, admitted for the evaluation of noncyanotic congenital heart disease (5 with a small ventricular septal defect, 4 with a small atrial sepal defect and 4 with patent ductus arteriosus).

Plasma TNF-α concentrations

The concentration of TNF-α in the plasmas of the acute stage KD patients (4.4±1.6 pg/mL) was significantly higher than in those of the convalescent stage (2.5±1.4 pg/mL, $p<0.05$) or in the control subjects (1.9±0.5 pg/mL, $p<0.01$) (Fig. 1). However, the level was lower than the rhTNF-α concentration needed to up-regulate ICAM-1 (10 pg/mL).[14]

Serum IgM-AECA concentrations

The IgM-AECA titers were significantly higher in the sera of KD patients in the acute stage (0.85±0.64) than those in the convalescent stage (0.36±0.27, $p<0.01$) or in the sera of the control subjects (0.35±0.14, $p<0.01$). Using a cut-off point of 0.63, the mean+2SD in the sera of the control subjects, 14 of the 22 (64%) KD patients in the acute stage and 2 (9%) in the convalescent stage were positive for IgM-AECA. None of the sera of the control subjects were positive for IgM-AECA

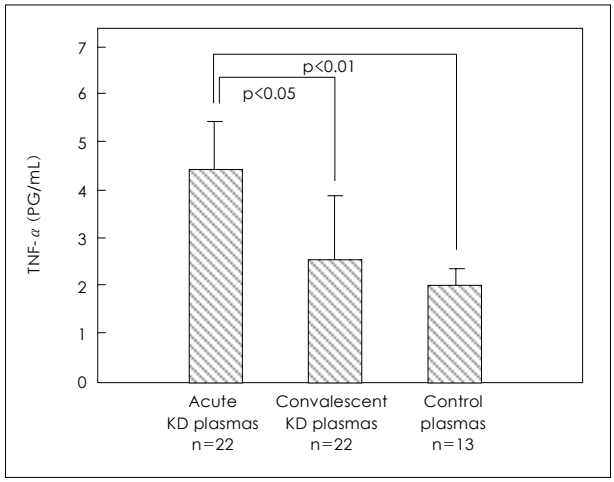

Fig. 1. Comparison of plasma TNF-α measured by ELISA. The concentration of TNF-α in the acute KD plasmas was significantly higher than those in the convalescent KD ($p<0.05$) or control plasmas ($p<0.01$). TNF-α: tumor necrosis factor-α, KD: Kawasaki disease.

(Fig. 2). The titers in the sera of those in the acute stage declined in the convalescent stage in all KD patients. Two KD patients with highest IgM-AECA levels developed coronary aneurysms, and their sera in the convalescent stage remained IgM-AECA positive.

The clinical and laboratory findings were compared between the sera of KD patients with positive IgM-AECA in the acute stage (n=14) and those with negative IgM-AECA (n=8) (Table 1). The initial serum CRP and ESR levels in the former group were significantly higher than those in the latter ($p<0.01$ and $p<0.05$, respectively).

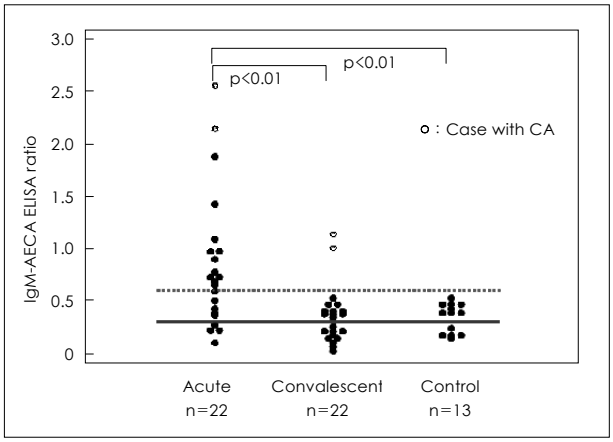

Fig. 2. Serum IgM-AECA levels measured by ELISA. The IgM-AECA titers were significantly higher in the acute KD sera than those in the convalescent KD or control sera ($p<0.01$, respectively). Using a cut-off point of 0.63, the mean±2SD in control sera, 14 of the 22 (64%) acute KD sera and 2 (9%) of the convalescent KD sera were positive for IgM-AECA. The lower solid line indicates the mean level of the control subjects, and the upper dotted line indicates the mean ±2SD of the control subjects. AECA: anti-endothelial cell antibody, KD: Kawasaki disease, CA: coronary aneurysm.

Table 1. Comparison of the clinical and laboratory findings of the KD patients according to the positivity of IgM-AECA in the acute sera

	AECA (+) KD patients	AECA (-) KD patients	p
No. of patients	14	8	
Age (years)	2.6± 1.6	2.2± 1.2	NS
Duration of fever (days)	7.8± 0.7	6.7± 0.5	NS
Conjunctivial injection	11 (79%)	7 (88%)	
Oral mucosal changes	11 (79%)	6 (75%)	
Rash	13 (93%)	8 (100%)	
Extremity changes	10 (71%)	5 (63%)	
Cervical enlargement	10 (71%)	4 (50%)	
Coronary aneurysm	2 (14%)	0 (0%)	
Initial WBC ($\times 10^3/mm^3$)	13.7± 4.2	16.2± 4.8	
Initial CRP (mg/dL)	10.8± 6.0	5.5± 1.4	<0.01
Initial ESR (mm/1 hr)	78.4±22.3	59.5±19.7	<0.05
TNF-α* (pg/mL)	4.5± 1.6	4.1± 1.3	NS
IgM-AECA	1.1± 0.6	0.3± 0.1	<0.01

*: in the plasma of the acute stage. KD: Kawasaki disease, AECA: anti-endothelial cell antibody, WBC: white blood cell, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, TNF-α: tumor necrosis factor-α, NS: no significance

Effects of the KD sera on the ICAM-1 expression of cultured HCAEC

The ICAM-1 expression of HCAEC treated with the sera of KD patients in the acute stage was significantly higher than that of HCAEC treated with the sera of those in the convalescent stage (117.1±46.7 vs. 88.9±14.4, $p<0.05$) or the sera of the control subjects (117.1±46.7 vs. 65.2±9.5, $p<0.01$) (Fig. 3A). The IgM-AECA-positive KD sera of the acute stage significantly induced the ICAM-1 expression of HCAEC compared to the IgM-AECA-negative KD sera of the acute stage (143.3±37.5 vs. 71.2±11.8, $p<0.01$) or the sera of the control subjects(143.3±37.5 vs. 65.2±9.5, $p<0.01$). There was no significant difference between the effect of

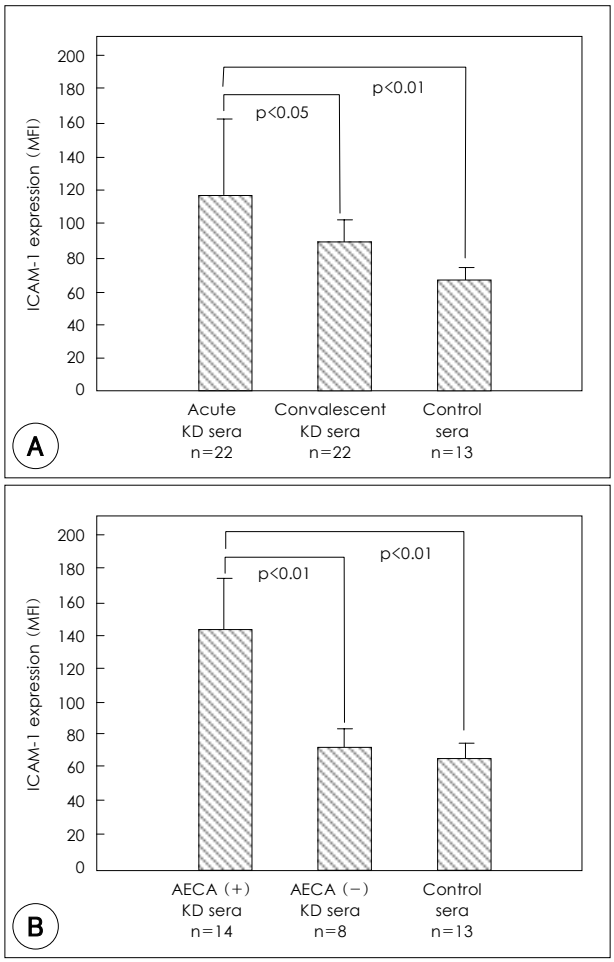

Fig. 3. Effects of the KD sera on the ICAM-1 expression of HCAEC. HCAEC were incubated for 24 hours with 1 : 4 diluted sera from KD patients, with/without ACEA or control subjects, and were stained with a fluorescent monoclonal antibody against human ICAM-1 prior to flow cytometry. Expression was indicated as the mean fluorescence intensity (MFI). A: the ICAM-1 expression of HCAEC treated with the acute KD sera was significantly higher than those for HCAEC treated with the convalescent KD or control subjects. B: the IgM-AECA-positive acute KD sera significantly induced the ICAM-1 expression of HCAEC higher than the IgM-AECA-negative acute KD or control sera. KD: Kawasaki disease, ICAM-1: intercellular adhesion molecule-1, HCAEC: human coronary artery endothelial cells, AECA: anti-endothelial cell antibody.

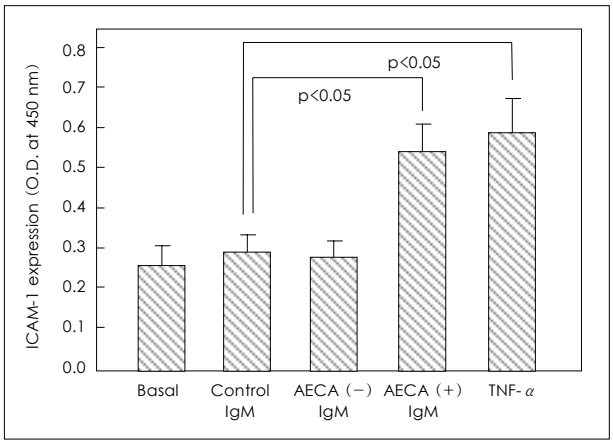

Fig. 4. Effects of the IgM-AECA on the ICAM-1 expression of HCAEC. HCAEC were incubated for 24 hours with 200 μg/mL of IgM fractions purified from KD sera, with/without AECA or control sera. The ICAM-1 expression was measured by ELISA, with the results obtained from eight independent experiments. Basal, unstimulated cells; TNF-α, a positive control treated with 20 ng/mL of TNF-α. AECA: anti-endothelial cell antibody, ICAM-1: intercellular adhesion molecule-1, HCAEC: human coronary artery endothelial cells, KD: Kawasaki disease, TNF-α: tumor necrosis factor-α.

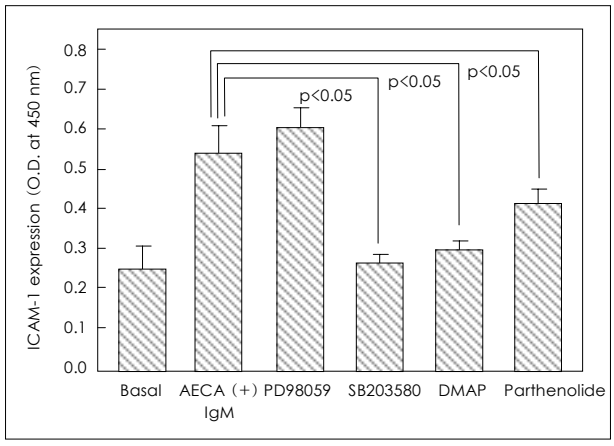

Fig. 5. Effects of four different signal transduction inhibitors on the IgM-AECA-induced ICAM-1 protein expression of HCAEC. Cells were pretreated with PD98059 (50 μM for 1 hour), SB203580 (10 μM for 1 hour), DMAP dimethylaminopurine (DMAP, 1 mM for 15 minutes) or parthenolide (10 μM for 1 hour) prior to incubation with AECA-positive IgM (200 μg/mL) for 24 hours. The ICAM-1 expression was measured by ELISA, with the results obtained from eight independent experiments. Basal, unstimulated cells. AECA: anti-endothelial cell antibody, ICAM-1: intercellular adhesion molecule-1, HCAEC: human coronary artery endothelial cells.

AECA-negative sera of the acute stage and that of the control subjects (Fig. 3B).

Effects of IgM-AECA and signal transduction inhibitors on the ICAM-1 protein expression of HCAEC

The ICAM-1 expression of cultured HCAEC, incubated for 24 hours with 200 μg/mL of the AECA-positive IgM, was significantly higher than that of HC AEC incubated with AECA-negative IgM (0.54±0.07 vs. 0.27±0.05, p<0.05) or the control IgM (0.54±0.07 vs. 0.29±0.05, p<0.05) (Fig. 4).

Pretreatment with SB203580 (a P38 inhibitor) prior to exposure to AECA-positive IgM inhibited the ICAM-1 expression of HCAEC by 93% (0.27±0.02, p<0.05). Pretreatment of DMAP (a JNK inhibitor) decreased the ICAM-1 expression by 83% (0.30±0.03, p<0.05), and that of parthenolide (an NF-κB inhibitor) reduced the ICAM-1 expression by 41% (0.41±0.04, p<0.05). In contrast, pretreatment with PD98059 (an ERK1/2 inhibitor) did not reduce the IgM-AECA-induced ICAM-1 expression of HCAEC (0.61±0.05, p>0.05) (Fig. 5).

Effects of IgM-AECA and signal transduction inhibitors on the ICAM-1 mRNA expression of HCAEC

The ICAM-1 mRNA expression of HCAEC incubated for 4 hours, with 200 μg/mL of the AECA-positive IgM, was significantly higher than those of HCAEC incubated with the AECA-negative IgM (p<0.01) or the control IgM (p<0.01) (Fig. 6).

Pretreatment of the cells with SB203580, DMAP or parthenolide prior to exposure to AECA-positive IgM significantly inhibited the ICAM-1 mRNA expression (p<0.01 in all three comparisons). However, pretreatment with PD98059 had no effect on the IgM-AECA-induced ICAM-1 mRNA expression of HCAEC (Fig. 7). These results suggested that IgM-AECA induces the ICAM-1 expression of HCAEC via p38 and JNK MAPK pathways and activation of NF-κB.

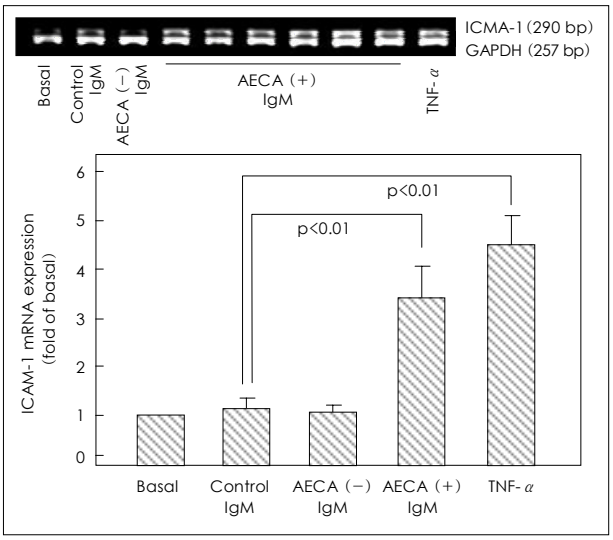

Fig. 6. IgM-AECA-induced ICAM-1 mRNA expression of HCAEC. Cells were incubated with 200 μg/mL of AECA-positive IgM, AE CA-negative IgM or control IgM. The isolated RNA samples were analyzed by reverse transcription-polymerase chain reaction (RT-PCR), using primers specific for ICAM-1 and GAPDH. Densitometric evaluation of ethidium bromide stained gels was performed. The density of the ICAM-1 PCR products were corrected by that of the corresponding GAPDH PCR products, and then expressed as the fold values of unstimulated cells. Data represent the mean±SD of six experiments. Basal, unstimulated cells; TNF-α, a positive control treated with 20 ng/mL of TNF-α. AECA: anti-endothelial cell antibody, ICAM-1: intercellular adhesion molecule-1, HCAEC: human coronary artery endothelial cells, TNF-α: tumor necrosis factor-α.

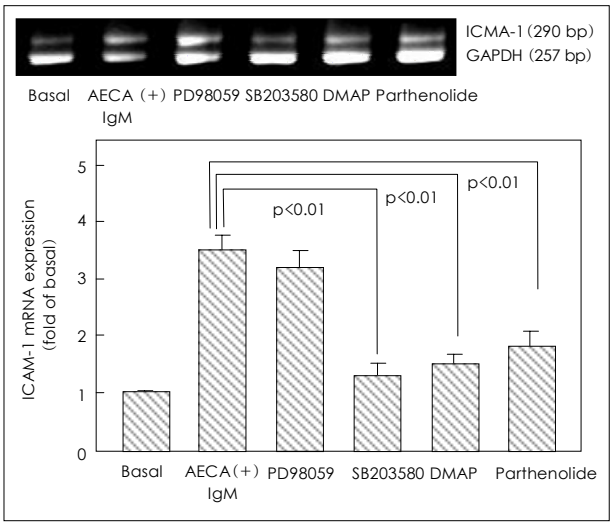

Fig. 7. Effects of four different signal transduction inhibitors on the IgM-AECA-induced ICAM-1 mRNA expression of HCAEC. Cells were pretreated with PD98059 (50 μM for 1 hour), SB203580 (10 μM for 1 hour), dimethylaminopurine (DMAP, 1 mM for 15 minutes) or parthenolide (10 μM for 1 hour) prior to incubation with 200 μg/mL of AECA-positive IgM for 4 hours. The isolated RNA samples were analyzed by RT-PCR, using primers specific for ICAM-1 and GAPDH, with the amount of ICAM-1 mRNA determined as described in Fig. 6. Basal, unstimulated cells. AECA: anti-endothelial cell antibody, ICAM-1: intercellular adhesion molecule-1, HCAEC: human coronary artery endothelial cells.

Discussion

Although the pathogenesis of KD is still not completely understood, several pieces of clinical and laboratory evidence suggest a central role of EC dysfunction in the development of the disease and its cardiac complications. The cause of the EC dysfunction is unclear, but either a profound disturbances of immunoregulation associated with abnormal apoptosis of neutrophils or mononuclear cells, endothelial tissue infiltration by inflammatory cells, or circulating immune complexes and diverse autoantibody have been proposed as possible immunologic causes. AECA is one of the pathogenic autoantibody candidates.

AECA were originally identified in the sera of patients with rheumatic diseases, due to their ability to react with rodent EC.[15] The largest and most consistent groups of diseases in which AECA have been demonstrated are the systemic autoimmune and vasculitic diseases.[16] The majority of these conditions share some degree of vascular injury, which initially led to the suspicion that AECA are merely epi-phenomena to the disruption of the vessel wall integrity.[17] However, the correlation between AECA serum levels and disease activity, as well as the decline in their titers after treatment that led to a halt in disease progression, are indirect evidence for the significance of AECA.[10]

Several studies have demonstrated the presence of increased AECA titers in 26 to 72% of the patients with KD.[7] The correlation between the levels of AECA and the disease activity, as well as the decline in AECA titers after treatment, are also evident in KD.[18] Indeed, several in vitro studies have reported the sera of patients with KD induced activation or damage of EC. However, the actual role of AECA in the development of KD remains debatable, as conflicting data exist concerning the ability of AECA to activate or damage EC, or even the frequency of AECA in KD patients.

In this study, about 2/3 of the KD patients had abnormally high levels of IgM-AECA in their acute sera, and the serum levels of IgM-AECA declined in the convalescent phase in all patients. The two patients with the highest AECA developed coronary aneurysms. These findings suggest the serum IgM-AECA levels may be related to the disease activity, and high serum levels may predispose patients to the development of coronary lesions.

This study has also documented that acute KD sera induced the activation of HCAEC, which was assessed by the degree of ICAM-1 expression in the cells. IgM-AECA may be the very substance associated with this action of the sera. Previous studies have shown that ICAM-1 is involved in the pathogenesis of coronary artery lesions associated with KD. During the acute phase, KD patients with coronary artery lesions had a higher soluble ICAM-1 concentration in the sera than KD patients without coronary artery lesions.[19] An immunohistological study revealed that the EC of coronary arteries and skin of KD patients expressed ICAM-1 as well as E-selectin.[20] The pathogenetic consequences of an increased expression of ICAM-1 on the surface of EC in KD may be deduced as follows. ICAM-1 is constitutively present on the surface of EC, and its expression is augmented by several stimulating factors. ICAM-1 has an important role in the migration of leukocytes to sites of inflammation, enabling firm adhesion and diapedesis of leukocytes via the interaction with α m β 2 and α L β 2.[21] The expression of ICAM-1 induced by sera from KD patients is likely to promote adhesion and transendothelial migration of leukocytes, resulting in the progression of vasculitis in KD. Indeed, the histopathological findings in KD include; EC injury and the infiltration of neutrophils, monocytes and lymphocytes into the walls of small and medium-sized blood vessels.[22]

In this study, the plasma concentrations of TNF-α in the KD patients were measured to ascertain whether this cytokine induces activation or damage of EC. The mean concentration of TNF-α in the plasma of acute KD patients was 4.4 pg/mL, which was far less than the concentration of rhTNF-α needed to induce upregulation of ICAM-1, i.e. 10 pg/mL.[14] Thus, it is suggested that the induction of ICAM-1 expression of HCAEC is mainly caused by IgM-AECA, but not by TNF-α, al-

though the plasma TNF-α levels are increased in KD patients.

Conflicting data have been presented on the necessity of EC preactivation in other reports. Namely, AECA lyses cytokine activated cells only in some studies,[8] while other studies have demonstrated that AECA from KD patients also influences resting cells.[9)11)] In this study, IgM-AECA was able to induce ICAM-1 expression of HCAEC in the resting state, and co-stimulation with IgM-AECA and 10 pg/mL of TNF-α resulted in no further increase of the ICAM-1 expression of HCAEC (data not shown).

In this study, the signal transduction pathways involved in the regulation of ICAM-1 gene expression induced by IgM-AECA were addressed using several specific inhibitors. SB203580 is a specific inhibitor of p38,[23] and the kinase directly upstream to p38 MAPK. SB20 3580 attenuated IgM-AECA-induced ICAM-1 mRNA and the protein expression of HCAEC almost completely, indicating the p38 MAPK signal transduction pathway is important to the upregulation of ICAM-1 expression in vascular EC.[24] Recently, DMAP, a protein kinase inhibitor, has been identified as an useful reagent, which inhibits JNK MAPK by increasing the expression of mitogen-activated protein phosphatase 2.[25] Pretreatment of HCAECs with DMAP decreased the IgM-AECA-induced ICAM-1 mRNA in addition to the protein expression, suggesting the involvement of JNK kinase as well as p38 MAPK. It has been well established that inflammatory responses following exposure to various stimuli are highly dependent on the activation of NF-κB transcription factor, which plays an important role in the regulation of several gene expressions.[26)27)] The sequestration of NF-κB in the cytoplasm, and IκB phosphorylation leading to proteasomal degradation of IκB-α, result in activation and translocation of NF-κB into the nucleus, which is essential in the expression of several genes, such as ICAM-1.[27] In this study, the expression of ICAM-1 induced by IgM-AECA was partially abolished by the specific NF-κB inhibitor, parthenolide, indicating that activation of NF-κB, at least partially, is involved in the AECA-induced expression of ICAM-1. This result is consistent with the report that AECA reacts with endothelial membrane antigen and induces a pro-inflammatory endothelial phenotype through the activation of NF-κB.[26] Thus, the activations of p38 MAPK and JNK, as well as NF-κB, appear to be involved in the IgM-AECA-induced expression of ICAM-1 of HCAEC. However, it remains unclear how the activations of p38 MAPK and JNK are associated with ICAM-1 gene expression. In EC, p38 and JNK MAPK can be activated and rapidly translocated to the nucleus, where they phosphorylate and activate the transcription factors AP-1, Ets, C/EBP and NF-κB, etc. The bindings sites of these factors exist in the ICAM-1 promoter, which suggests that the upregulation of ICAM-1 expression of EC may be mediated by p38 and JNK MAPK activating these transcription factors.[28)29)]

In summary, about 2/3 of patients with KD had IgM-AECA in their acute sera, and the titers of IgM-AECA decreased in the convalescent phase. Patients with higher AECA titers were prone to develop coronary aneurysms. Acute KD sera and purified IgM-AECA were able to induce upregulation of the ICAM-1 expression of HCAEC in vitro. JNK, p38 MAPK and NF-κB signaling pathways may be involved in the upregulation of the ICAM-1 expression of HCAEC induced by IgM-AECA. Thus, the IgM-AECA in KD patients may play an important role in the development of coronary artery lesions. Further studies will be required to investigate the signal transduction pathways involved in the regulation of ICAM-1 gene expression induced by IgM-AECA.

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