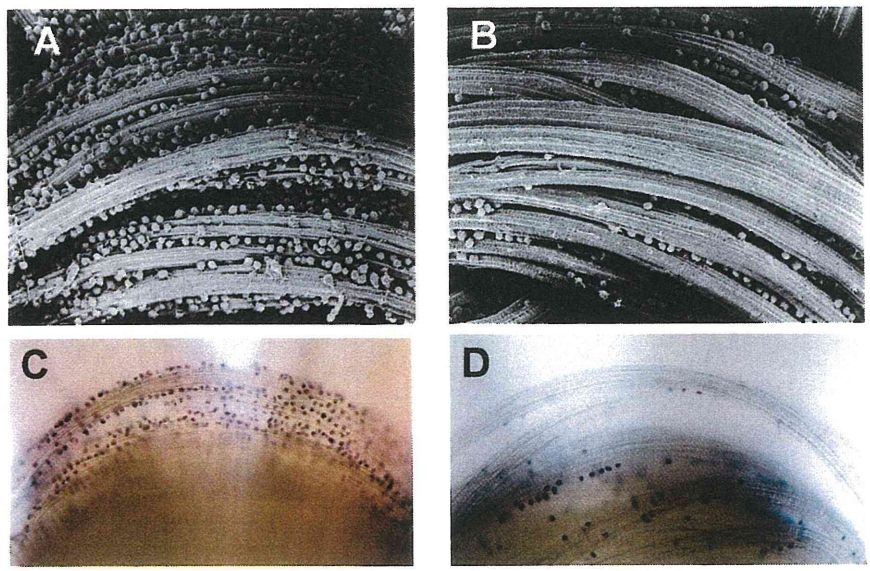

Fig. 1 Analysis of cellular components in the PMX columns. Scanning electron micrographs of the filter (A, B) and light micrographs of the hematoxylin-stained filter (C, D) show the cellular components in the PMX column before (A, C) and after the cells were collected (B, D). Numerous leukocytes were trapped on the filter, and a major portion of these cells were easily collected from the filter.

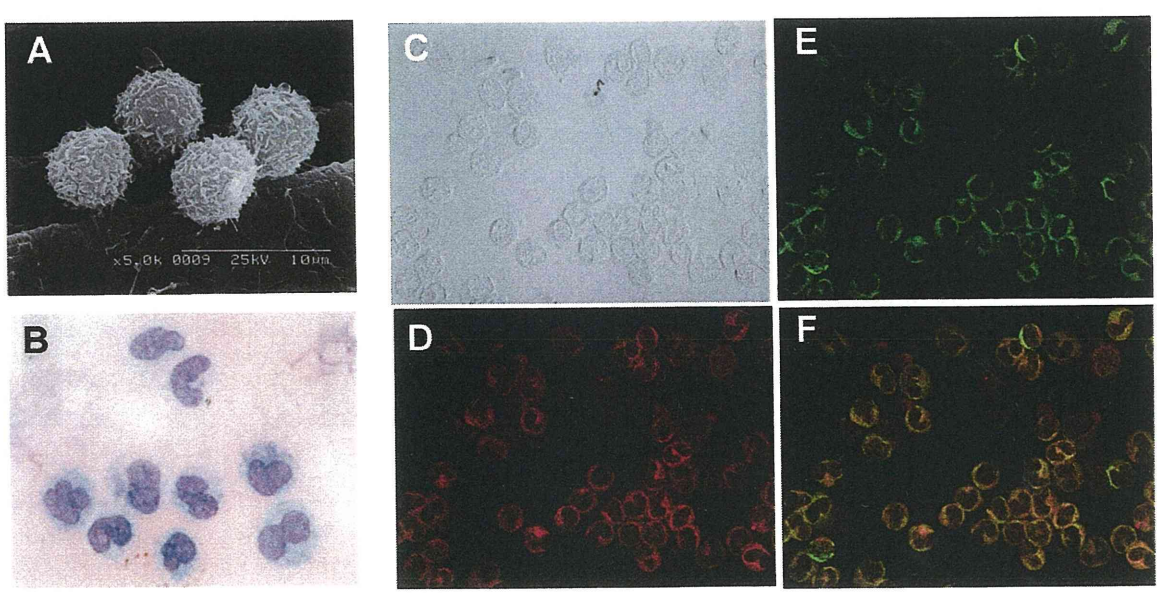

Fig. 2 Identification of leukocyte population. (A) Image of scanning electron microscopy of leukocytes on PMX filter. Smeared leukocytes from the column stained with May-Grunwald-Giemsa presented the typical monocyte appearance (B). Smeared samples (phase contrast image (C)) were immunostained with PE-conjugated anti-CDI4 (D) and FITC-conjugated anti-CD68 (E). Merged view (F). The specimen was from the patient identified as case 2.

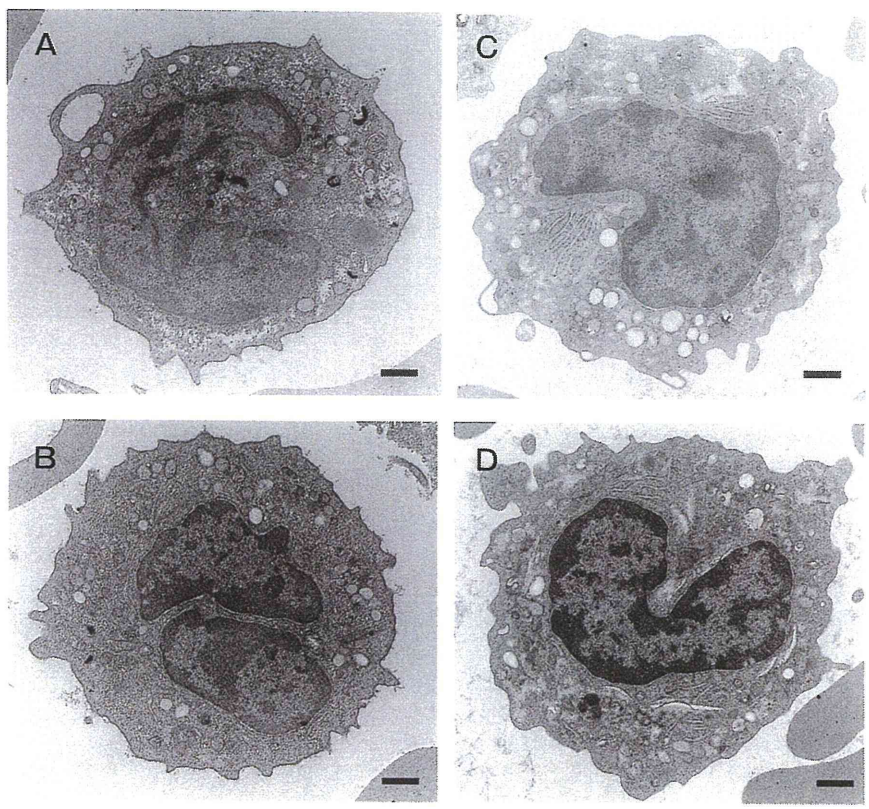

Fig. 3 Transmission electron micrograph of monocytes detached from the filter. The recovered leukocytes were fixed for transmission electron micrography. As shown in Fig. 2, most of the cells were monocytes. Four representative 4 cells are shown. There were vacuoles observed in the cytosol however, apoptotic changes such as condensation of nuclei were not present. The specimen was from the patient identified as case 2. Scale bars equal 1 μm.

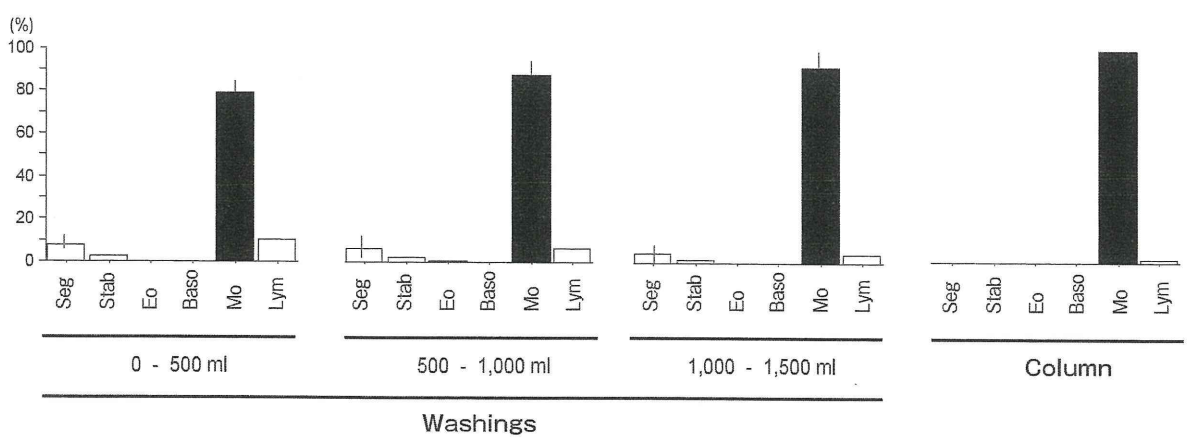

Fig. 4 Classification of leukocyte populations from washings and polymixin B-immobilized column. Smeared samples from each 500 ml washing and polymixin B-immobilized filter were used for the classification of leukocytes by May-Grunwald-Giemsa staining. The results are expressed as percentages of total cells (means ± SEM of 4 patients). Seg, segmented neutrophil; Stab, stab neutrophil; Eo, eosinophil; Baso, basophil; Mo, monocytes; Lym, lymphocyte.

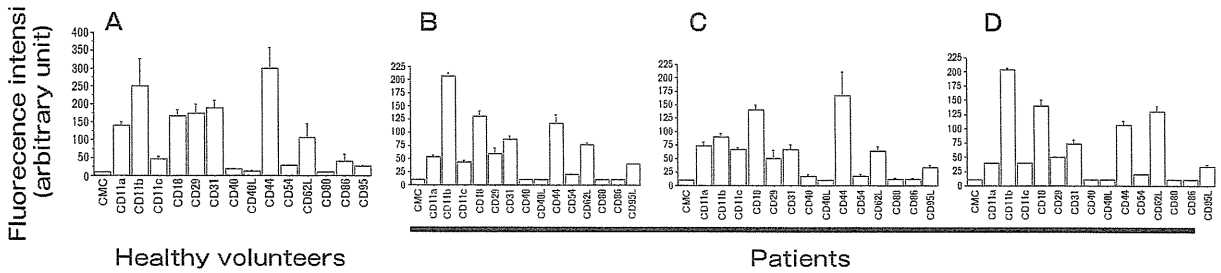

Fig. 5 FACS analysis of adhesion molecule expression on monocytes from healthy volunteers and patients. The peripheral blood was collected from 4 healthy volunteers (A) and patients before (B) and after (D) PMX treatment. PBMC was prepared from the peripheral blood. A diverse range of adhesion molecules on CD14-positive monocytes was analyzed by FACS. In C, the data on monocytes collected from PMX columns are shown. The data are the means ± SEM of 4 individuals.

monocytes and the beneficial effects of PMX treatment in septic shock.

References

1. Riedemann NC, Guo RF and Ward PA: Novel strategies for the treatment of sepsis. Nat Med (2003) 9: 517-524.
2. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, Steingrub JS, Garber GE, Helterbrand JD, Ely EW and Fisher CJ Jr; Recombinant human protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study group: Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med (2001) 344: 699-709.
3. Shoji H: Extracorporeal endotoxin removal for the treatment of sepsis: endotoxin adsorption cartridge (Toraymyxin). Ther Apher Dial (2003) 7: 108-114.
4. Suzuki H, Nemoto H, Nakamoto H, Okada H, Sugahara S, Kanno Y and Moriwaki K: Continuous hemodiafiltration with polymyxin-B immobilized fiber is effective in patients with sepsis syndrome and acute renal failure. Ther Apher (2002) 6: 234-240.
5. Takahashi HK, Yoshida A, Iwagaki H, Yoshino T, Itoh H, Morichika T, Yokoyama M, Akagi T, Tanaka N, Mori S and Nishibori M: Histamine regulation of interleukin-18-initiating cytokine cascade is associated with down-regulation of intercellular adhesion molecule-1 expression in human peripheral blood mononuclear cells. J Pharmacol Exp Ther (2002) 300: 227-235.

0022-3565/09/3301-89–98$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

JPET 330:89–98, 2009

Vol. 330, No. 1
150581/3485881
Printed in U.S.A.

Advanced Glycation End Products Subspecies-Selectively Induce Adhesion Molecule Expression and Cytokine Production in Human Peripheral Blood Mononuclear Cells

Hideo Kohka Takahashi, Shuji Mori, Hidenori Wake, Keyue Liu, Tadashi Yoshino, Katsuhisa Ohashi, Noriaki Tanaka, Kenichi Shikata, Hirofumi Makino, and Masahiro Nishibori

Departments of Pharmacology (H.K.T., H.W., K.L., M.N.), Pathology (T.Y.), Gastroenterological Surgery, Transplant, and Surgical Oncology (K.O., N.T.), and Medicine and Clinical Science (K.S., H.M.), Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; and Department of Pharmacy, Shujitsu University, Okayama, Japan (S.M.)

Received January 7, 2009; accepted April 17, 2009

ABSTRACT

Advanced glycation end products (AGEs) are proteins or lipids that become glycated after exposure to diverse reducing sugars. Accumulation of AGEs induces diabetes complications. Microinflammation is a common major mechanism in the pathogenesis of diabetic vascular complications. Activation of monocytes/macrophages and T cells plays roles in the pathogenesis of atherosclerosis. The activation of T cells requires the enhanced expression of adhesion molecules on monocytes. AGEs activate monocytes by engaging the receptor for AGE (RAGE); however, little is known about the profile of agonist activity of diverse AGE moieties on monocytes. We investigated the effect of four distinct AGE subtypes (AGE-modified bovine serum albumin; AGE-2, AGE-3, AGE-4, and AGE-5) at concentrations ranging from 0.1 to 100 μg/ml on the expression of intercellular adhesion molecule-1, B7.1, B7.2, and CD40 on monocytes and its impact on the production of interferon-γ and tumor necrosis factor-α in human peripheral blood mononuclear cells. Among the AGEs examined, AGE-2 and AGE-3 selectively induced adhesion molecule expression and cytokine production. Antagonism experiments using antibodies against adhesion molecules demonstrated that cell-to-cell interaction between monocytes and T/natural killer cells was involved in AGE-2- and AGE-3-induced cytokine production. AGE-2 and AGE-3 up-regulated the expression of RAGE on monocytes. The effects of AGE-2 and AGE-3 were inhibited by nuclear factor-κB and p38 mitogen-activated protein kinase inhibitors. These results indicated that AGE-2 and AGE-3 activated monocytes via RAGE, leading to the up-regulation of adhesion molecule expression and cytokine production.

Advanced glycation end products (AGEs) are a heterogeneous class of compounds and modifications of proteins or lipids that become nonenzymatically glycated and oxidized after contact with aldose sugars (Schmidt et al., 1994). Accumulation of AGEs in disorders such as diabetes, renal failure, Alzheimer's disease, and natural aging has suggested their potential contribution to the pathogenesis of complications that typify these conditions (Brownlee, 1995; Takedo et al., 1996). Direct immunochemical evidence for the existence of six distinct AGE structures, including AGE-1, AGE-2, AGE-3, AGE-4, AGE-5, and AGE-6, was provided from the analysis of AGEs within modified proteins and peptides (Takeuchi and Yamagishi, 2004). Recently, it was demonstrated that toxic AGE structures, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3), have diverse biological activities on vascular endothelial cells, vascular smooth muscle cells, mesangial cells, Schwann cells, malignant melanoma cells, and cortical neurons (Oka-

This work was supported in part by the Japan Society for the Promotion of Science [Grants 18590509, 20590539, 17659159, 19659061]; and by the Takeda Science Foundation.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.109.150581.

ABBREVIATIONS: AGE, advanced glycation end product; RAGE, Receptor for advanced glycation end product; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; sRAGE, soluble form of receptor for advanced glycation end product; ICAM, intercellular adhesion molecule; IFN, interferon; TNF, tumor necrosis factor; PBMC, peripheral blood mononuclear cells; Ab, antibody; SN50, H_2N-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Val-Gln-Arg-Lys-Arg-Gln-Lys-Leu-Met-Pro-OH; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; IL, interleukin.

moto et al., 2002). AGEs have also been suggested to have profound effects on inflammatory and immune cells (Figarola et al., 2007); however, it is still not clear which AGE subtypes play a role in the modulation of immune response.

Receptor for AGE (RAGE), a member of the immunoglobulin superfamily, was first described as a cell surface receptor for AGEs (Neeper et al., 1992). It is reported that AGEs ligate cell surface RAGE on the vascular endothelium, mononuclear phagocytes, vascular smooth muscle, and neurons to activate cell signaling pathways such as p44/p42 mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) (Yan et al., 1994), leading to redirect cellular function in a manner linked to the expression of inflammatory and prothrombotic genes important in the pathogenesis of chronic disorders such as diabetic microvascular disease and amyloidosis (Schmidt et al., 1994; Miyata et al., 1996; Park et al., 1998). Interestingly, AGEs up-regulate RAGE expression in various tissues, facilitating the AGEs-RAGE response by forming a positive feedback loop (Yamagishi and Imaizumi, 2005). Conversely, it is suggested that interruption of the interaction of AGEs with RAGE in vivo, by the administration of soluble form of RAGE (sRAGE), an extracellular ligand-binding domain of RAGE, reversed vascular hyperpermeability and suppressed accelerated atherosclerotic lesion development in diabetic rodents (Miyata et al., 1996; Park et al., 1998). Thus, ligation of AGEs with RAGE might play an important role in the development of various diabetic complications, including atherosclerosis.

Microinflammation is a common major mechanism in the pathogenesis of diabetic vascular complications. It is reported that diabetes has greater macrophage and T-cell infiltration in atherosclerotic plaques (Burke et al., 2004). Macrophages are recruited by abnormal endothelium over developing atherosclerotic plaques (Boyle, 2005). In addition, recruitment of monocytes/macrophages and T cells in diabetic glomeruli during the early stage of diabetes is considered to be involved in the progression of diabetic nephropathy (Sugimoto et al., 1997). Monocyte/macrophage activation, adhesion, and migration are key events in the pathogenesis of atherosclerosis (Figarola et al., 2007). Activated T cells induce the progression of inflammatory atherosclerotic plaques (Stoll and Bendszus, 2006). AGEs are implicated in the pathogenesis of atherosclerotic vascular disease of diabetic etiology (Stitt et al., 1997). The stimulation of RAGE is reported to induce plaque rupture in diabetic patients (Cuccurullo et al., 2006).

It is known that the enhanced expression of adhesion molecules, including intercellular adhesion molecule (ICAM)-1, B7.1, B7.2, and CD40, on monocytes results in the activation of T cells (Durie et al., 1994; Ranger et al., 1996; Camacho et al., 2001). We also found that cell-to-cell interactions mediated by the engagement between ICAM-1, B7.1, B7.2, and CD40, respectively, on monocytes and their ligands, lymphocyte function-associated antigen-1, CD28, and CD40 ligand, on T cells were involved in T-cell activation, leading to induce the production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α in human peripheral blood mononuclear cells (PBMC) (Takahashi et al., 2003). However, little is known about the effect of AGE subtypes on adhesion molecule expression as well as cytokine production. Therefore, we examined the effect of AGE-2, AGE-3, AGE-4, and AGE-5 on the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes and the production of IFN-γ and TNF-α in PBMC to better understand the profile of each AGE in monocyte activation. Moreover, we analyzed changes in the expression of RAGE on monocytes induced by AGEs, the involvement of adhesion molecules in the cytokine-producing action of AGEs, and the relevant signal pathways triggered by AGEs.

Materials and Methods

Reagents and Drugs. Recombinant human IFN-γ, TNF-α, anti-IFN-γ Ab and anti-TNF-α Ab were purchased from BD Biosciences (San Jose, CA). SN50 and SB203580 were purchased from Calbiochem (San Diego, CA). AGE-modified bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) was prepared as described previously (Takeuchi et al., 2000). In brief, each protein was incubated under sterile conditions with glyceraldehyde-3-phosphate (AGE-2) (Sigma-Aldrich), glycolaldehyde (AGE-3) (Sigma-Aldrich), methylglyoxal (AGE-4) (Sigma-Aldrich), or glyoxal (AGE-5) (Tokyo Kasei, Tokyo, Japan) in 0.2 M phosphate buffer, pH 7.4, at 37°C for 7 days. BSA was incubated under the same conditions. AGE-BSA and BSA were dialyzed for 2 days at 4°C. The endotoxin concentration of AGEs at 100 μg/ml described above was measured at SRL (Okayama, Japan), and it was found to be 1.2 pg/ml. AGE-specific fluorescence was measured at 450 nm after excitation at 390 nm with a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). sRAGE was prepared as described previously (Goren et al., 2007). In brief, sRAGE was cloned into the *Escherichia coli* expression vector pASK-IBA32; some modifications to the pASK-IBA32 polylinker region were made for cloning purposes. sRAGE, from amino acids 23 to 340 (Neeper et al., 1992), was amplified by polymerase chain reaction using the following oligonucleotides (MWG Biotech, High Point, NC); the underlined bases designate restriction sites: 5′-CTGACCTATG CGGCCGCTGC TCAAAACATCACAGC-3′ and 5′-GACTGAATTC ATCAGTGAT-GATGGTGATGG TGAGTTCCCA GCCCTGATCC-3′. Anti-human RAGE rabbit polyclonal Ab was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Isolation of PBMC, Monocytes, and T Cells. Normal human PBMC were obtained from ten healthy volunteers after acquiring Institutional Review Board approval (Okayama University Institutional Review Board no. 106). Samples of 20 to 50 ml of peripheral blood were withdrawn from a forearm vein, after which the PBMC were prepared, and monocytes isolated from PBMC were separated by counterflow centrifugal elutriation as described previously (Takahashi et al., 2003). T cells were then enriched from PBMC by passing them through a nylon wool column to a purity of 85% T cells as determined by flow cytometry with FITC-conjugated anti-CD3 antibody. PBMC, monocytes, and T cells were then suspended at a final concentration of 1×10^6 cells/ml in the medium as described previously (Takahashi et al., 2003).

Measuring AGEs-RAGE Binding Using Microtiter Plate in Vitro. Ninety six-well flat-bottomed microtiter plates were coated with BSA-AGEs at increasing concentrations from 0.3125 to 20 μg/ml and were incubated at 4°C for 16 h with gentle shaking on an orbital microplate shaker. After three times washings with washing buffer (10 mM Tris-buffered saline containing 0.05% Tween 20, pH 7.5), plates were blocked with 10% BSA at 4°C for 16 h. The plates were then incubated with His-tagged sRAGE at 0.83 μg/ml in 10 mM Tris-buffered saline at 4°C for 16 h. After three times washings, nickel-nitrilotriacetic acid-horseradish peroxidase conjugate (QIAGEN, Osaka, Japan) was added to the well, and the incubation was performed at room temperature for 1 h. The reaction was developed by the addition of 0.15% H_2O_2 and 2.5 mM 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) (Tokyo Kasei Kogyo Co., Ltd., Tokyo Japan) in 0.2 M citrate buffer, pH 4.0.

Flow Cytometric Analysis for Adhesion Molecule Expression. For flow cytometric analysis, FITC-conjugated mouse IgG1 monoclonal (m) Ab against ICAM-1/CD54 and phycoerythrin-conju-

gated anti-CD14 mAb were purchased from Dako Denmark A/S (Glostrup, Denmark). FITC-conjugated mouse IgG1 mAb against B7.1 was purchased from Immunotech (Marseille, France), FITC-conjugated mouse IgG1 mAb against B7.2 and CD40 was from BD Biosciences Pharmingen (San Diego, CA), and FITC-conjugated IgG1 isotype-matched control was obtained from Sigma-Aldrich. Changes in the expression of human leukocyte antigens, ICAM-1, B7.1, B7.2, and CD40, on monocytes were examined by multicolor flow cytometry using a combination of anti-CD14 Ab with anti-ICAM-1, anti-B7.1, anti-B7.2, or anti-CD40 Ab. In addition, to analyze changes in the expression of human RAGE on monocytes by multicolor flow cytometry using a combination of phycoerythrin-conjugated anti-human CD14 mouse IgG (Dako Denmark A/S) and anti-human RAGE rabbit IgG (Santa Cruz Biotechnology, Inc.) followed by FITC-conjugated anti-rabbit IgG goat IgG (Dako Denmark A/S) was performed. PBMC at 1×10^6 cells/ml were incubated for 24 h. Cultured cells at 5×10^5 cells/ml were prepared for flow cytometric analysis as described previously (Takahashi et al., 2003). The cells were analyzed with a FACSCalibur (BD Biosciences). Data were processed using the Cell Quest program (BD Biosciences).

Cytokine Production. PBMC at 1×10^6 cells/ml were used for analyzing IFN-γ and TNF-α production. After culturing for 24 h at 37°C in a 5% CO_2, air mixture, the cell-free supernatant was assayed for IFN-γ and TNF-α protein by enzyme-linked immunosorbent assay (ELISA) using the multiple Abs sandwich principle (R&D Systems, Minneapolis, MN). The detection limit of ELISA for IFN-γ and TNF-α was 10 pg/ml.

Immunofluorescence Staining. For double immunofluorescence staining, PBMC at 1×10^6 cells/ml were incubated for 24 h in the presence or absence of AGE-2 AGE-3 and BSA at 100 μg/ml. The cultured cells were fixed in 1% paraformaldehyde and stained with anti-human CD14 mouse IgG (Dako Denmark A/S) followed by Alexa 555-conjugated anti-mouse IgG rabbit IgG (Millipore Bioscience Research Reagents, Temecula, CA) and anti-human RAGE rabbit IgG (Santa Cruz Biotechnology, Inc.) followed by Alexa Fluor 488-conjugated anti-rabbit IgG goat IgG (Millipore Bioscience Research Reagents), respectively. The stained cells were mounted for viewing by fluorescent confocal microscopy (Biozero BZ8000; Keyence, Osaka, Japan).

Western Blot Analysis. The effect of AGE-2 and AGE-3 on the expression of RAGE was determined by Western blot analysis. Anti-human RAGE rabbit IgG was obtained by immunization of rabbit with recombinant human sRAGE. The lysates of human monocytes or A549 cells, a human pulmonary epithelial cell line, were electrophoresed on SDS-polyacrylamide gel electrophoresis gel. The fractionated proteins were transferred to nitrocellulose membrane. The blotting of β-actin was used as a loading control.

Statistical Analysis. Statistical significance was evaluated using analysis of variance followed by Dunnett's test. A probability value of less than 0.05 was considered to indicate statistical significance. The results are expressed as the means ± S.E.M. of triplicate findings from five donors.

Results

AGE Subtype Binding to sRAGE. To evaluate the binding of AGE subtypes to RAGE, we established the in vitro assay by using the immobilized AGE subspecies and the His-tagged sRAGE protein (Fig. 1). AGE-2 and AGE-3 showed relatively high-affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed a moderate affinity (8 times less potent than AGE-2 and AGE-3) for sRAGE. We confirmed that the immobilization efficiency of each AGE was the same when detected by anti-BSA Ab (data not shown).

Time Course Effects of AGEs on the Expression of ICAM-1, B7.1, B7.2, and CD40 on Monocytes and the Production of IFN-γ and TNF-α in PBMC. To determine

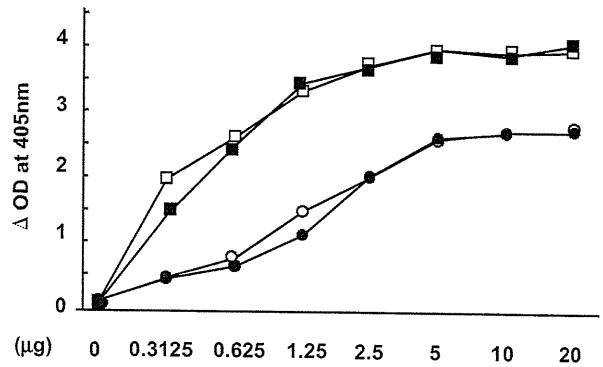

Fig. 1. AGE subtype binding to sRAGE. Ninety six-well flat-bottomed microtiter plates were coated with BSA-AGEs at increasing concentrations from 0.3125 to 20 μg/ml. After His-tagged sRAGE binding at 4°C for 16 h, the bound sRAGE was detected by nickel-nitrilotriacetic acid-horseradish peroxidase. Open squares (□) filled squares (■), open circles (○), or filled circles (●) represent the binding of AGE-2, AGE-3, AGE-4, and AGE-5 with sRAGE, respectively. The results are expressed as the means ± S.E.M. of triplicate determinations. When an error bar was within a symbol, the bar was omitted.

the proper incubation time, we examined the kinetics as shown in Figs. 2 and 3. In the absence of AGEs and BSA, the expression of ICAM-1, B7.1, B7.2, and CD40 moderately increased at 16 h and thereafter up to 24 and 48 h (Fig. 2A). AGE-2 and AGE-3 at 100 μg/ml significantly enhanced the expression of ICAM-1, B7.1, B7.2, and CD40 at 16 h and thereafter up to 24 and 48 h (Fig. 2, B, C, and G), whereas AGE-4, AGE-5, and BSA at 100 μg/ml had no effect at all (Fig. 2, D–F). In the absence of AGEs and BSA, the production of IFN-γ and TNF-α was under the detection limit during the incubation period (Fig. 3A). AGE-2 and AGE-3 at 100 μg/ml also time-dependently induced the production of IFN-γ and TNF-α in PBMC (Fig. 3, B and C). Neither AGE-4, AGE-5, nor BSA induced cytokine production (Fig. 3, D–F).

Dose-Response Relationship of AGEs on the Expression of ICAM-1, B7.1, B7.2, and CD40 on Monocytes, and the Production of IFN-γ and TNF-α in PBMC. The level of glyceraldehyde-derived AGE (AGE-2) is reported to be 17 μg/ml in the serum of patient with diabetes (Enomoto et al., 2006; Nakamura et al., 2007). It is reported that AGEs at the concentrations ranging from 50 to 200 μg/ml remarkably induce human monocyte adhesion to bovine retinal endothelial cells (Mamputu et al., 2004). AGEs at 200 μg/ml induce the expression of CD40, CD80, and CD86 and the production of IFN-γ in dendritic cells (Ge et al., 2005). Therefore, the effects of AGE-2, AGE-3, AGE-4, AGE-5, and BSA at concentrations ranging from 100 ng/ml to 100 μg/ml on the expression of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ and TNF-α were determined (Figs. 4 and 5). AGE-2 and AGE-3 concentration-dependently induced adhesion molecule expression with similar potency (Fig. 4, A and B), whereas AGE-4, AGE-5, and BSA showed no effect (Fig. 4, C–E). Consistent with the up-regulation of adhesion molecules, AGE-2 and AGE-3 concentration-dependently induced the production of IFN-γ and TNF-α (Fig. 5, A and B). The levels of IFN-γ and TNF-α production induced by AGE-2 at 100 μg/ml were 820 and 800 pg/ml, and those by AGE-3 at 100 μg/ml were 805 and 810 pg/ml, respectively. When we assumed that the effects of AGE-2 and AGE-3 were maximal at the concentration of 100 μg/ml, the ED_{50} values of AGE-2 for the induction of ICAM-1 expression and TNF-α produc-

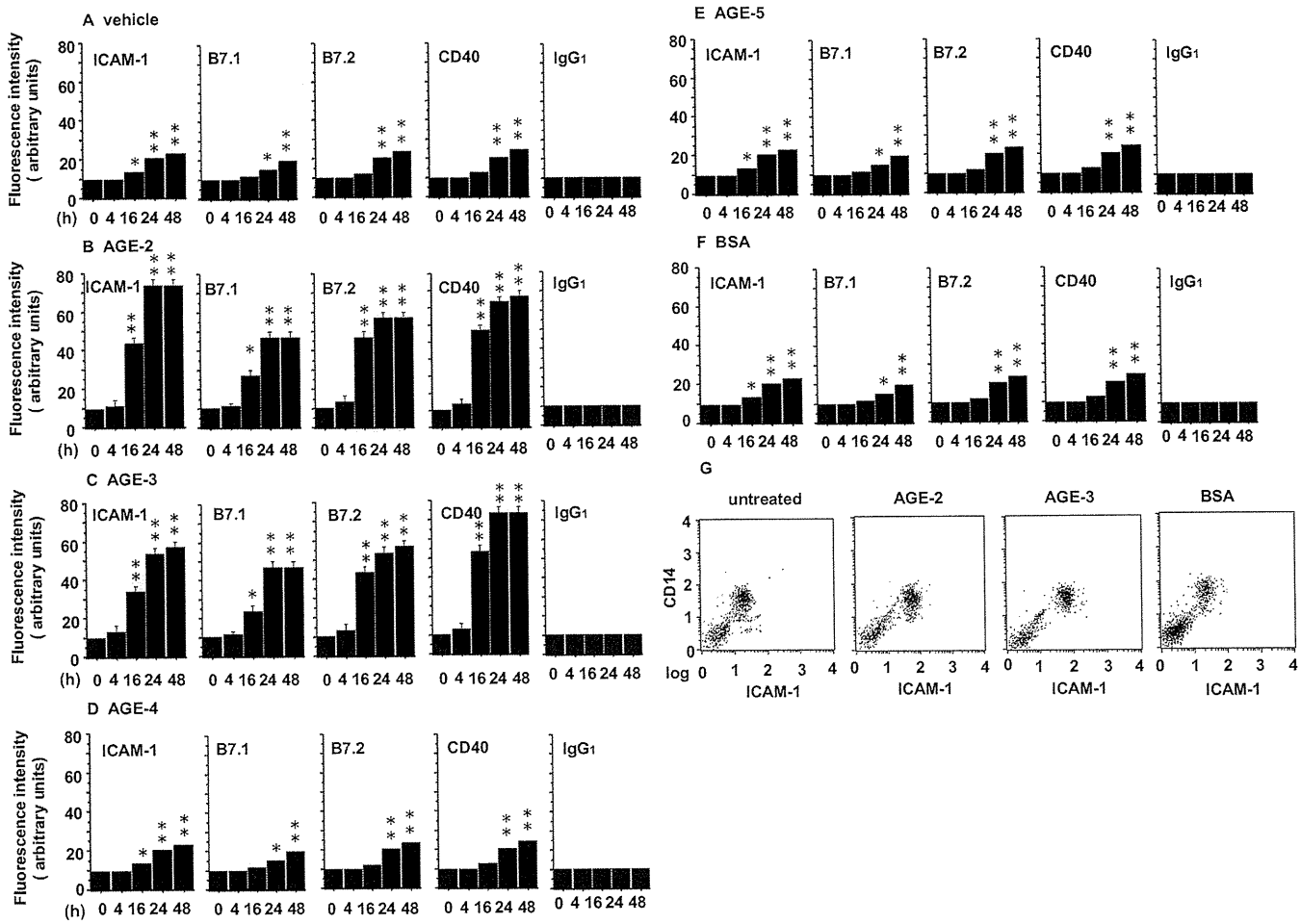

Fig. 2. Time course effects of AGEs on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes. PBMC at 1×10^6 cells/ml were incubated with vehicle (A and G), AGE-2 (B and G), AGE-3 (C and G), AGE-4 (D), AGE-5 (E), or BSA (F and G) at 100 μg/ml for the indicated periods. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. FITC-conjugated IgG1 was used as an isotype-matched control Ab. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. *, $p < 0.05$ and **, $p < 0.01$ compared with the value for 0 h.

tion were calculated to be 3 and 5 μg/ml, and those of AGE-3 were 10 and 5 μg/ml, respectively. However, AGE-4, AGE-5, and BSA had no effect on cytokine production (Fig. 5, C–E).

Involvement of IFN-γ and TNF-α in the Effect of AGE-2 and AGE-3 on Adhesion Molecule Expression. We examined the involvement of IFN-γ and TNF-α in the

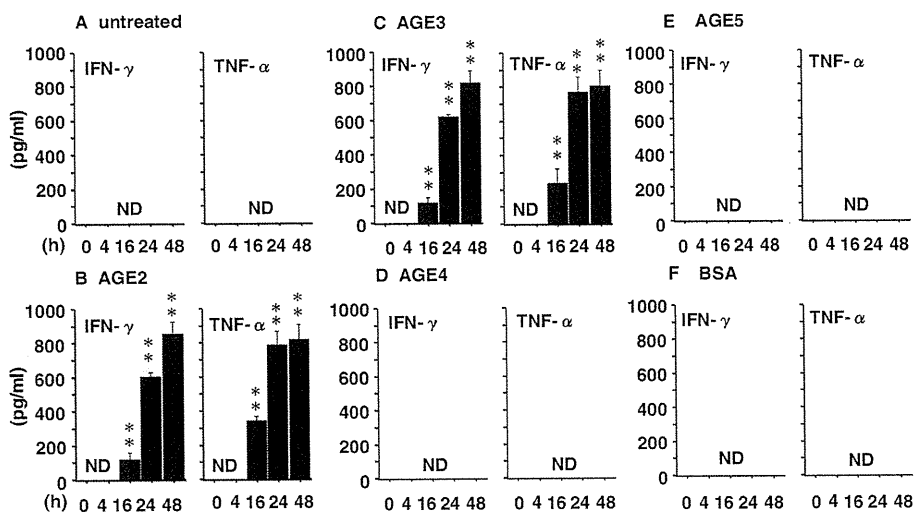

Fig. 3. The time-course effects of AGEs on the production of IFN-γ and TNF-α in PBMC. PBMC at 1×10^6 cells/ml were incubated with vehicle (A), AGE-2 (B), AGE-3 (C), AGE-4 (D), AGE-5 (E), or BSA (F) at 100 μg/ml for the indicated periods. The production of IFN-γ and TNF-α in PBMC was determined by ELISA. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the value for 0 h.

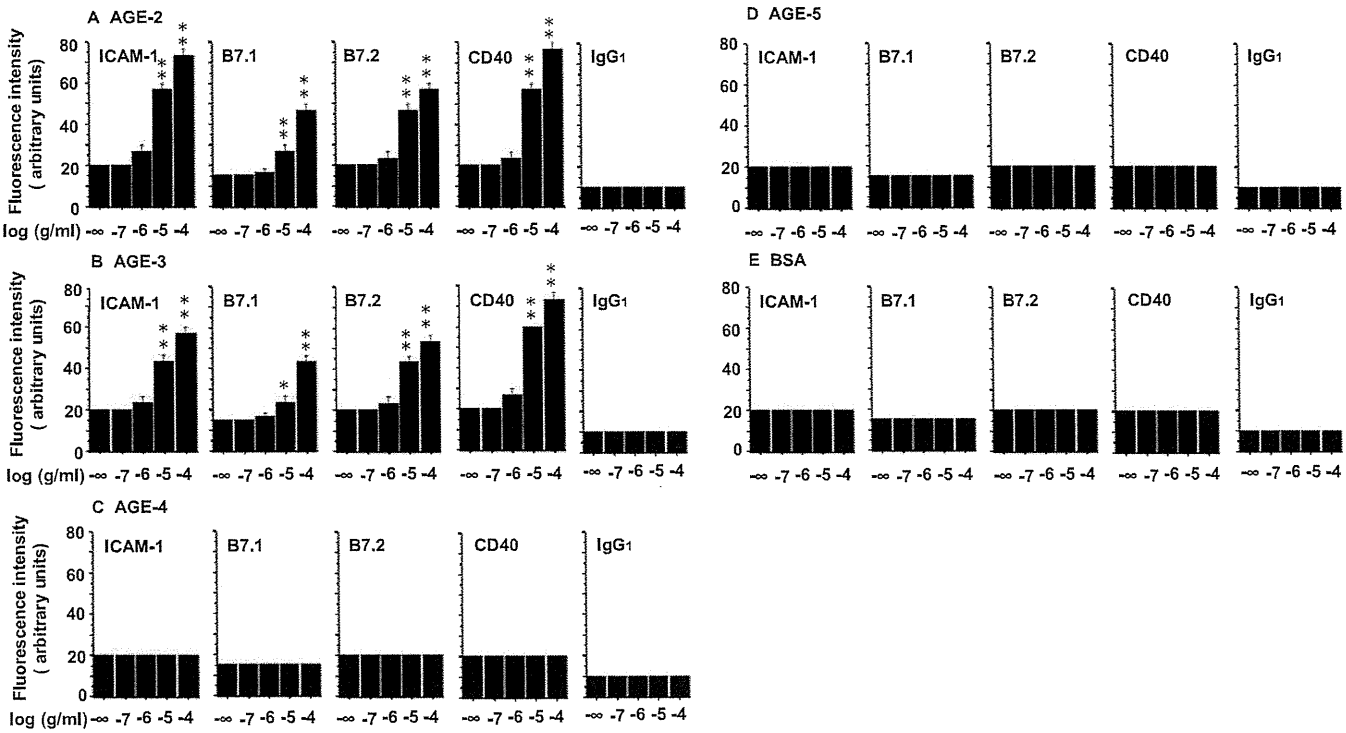

Fig. 4. Effect of AGEs on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes. PBMC at 1×10^6 cells/ml were incubated with AGE-2 (A), AGE-3 (B), AGE-4 (C), AGE-5 (D), or BSA (E) at increasing concentrations from 0.1 to 100 μg/ml for 24 h. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. FITC-conjugated IgG1 was used as an isotype-matched control Ab. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. *, $p < 0.05$ and **, $p < 0.01$ compared with the value for medium alone.

AGE-2 and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes (Fig. 6). IFN-γ and TNF-α at 10 ng/ml remarkably increased the expression of all adhesion molecules examined, and the expression reached a maximum level at 100 ng/ml. Moreover, the effects of cytokines and AGEs were additive. IFN-γ and TNF-α at 10 and 100 ng/ml enhanced the actions of AGE-2 and AGE-3, but those at 1 ng/ml had no effect (Fig. 6, A and B). Alternatively, anti-IFN-γ and anti-TNF-α Abs up to 100 ng/ml, which blocked the effect of exogenous IFN-γ and TNF-α at 100 ng/ml on adhesion molecule expression (data not shown), had no effect on the actions of AGE-2 and AGE-3 at 100 μg/ml (Fig. 6, C and D).

Involvement of Adhesion Molecule in the Effect of AGE-2 and AGE-3 on Cytokine Production. We examined the involvement of ICAM-1, B7.1, B7.2, and CD40 in the AGE-2- and AGE-3-induced production of IFN-γ and TNF-α in PBMC. As shown in Fig. 7, A and C, AGE-2 and AGE-3 concentration-dependently induced the production of IFN-γ and TNF-α in monocytes isolated from PBMC, exhibiting 20% of the amount obtained in PBMC, as shown in Fig. 5, B and C. AGE-2 and AGE-3 had no effect on the production of

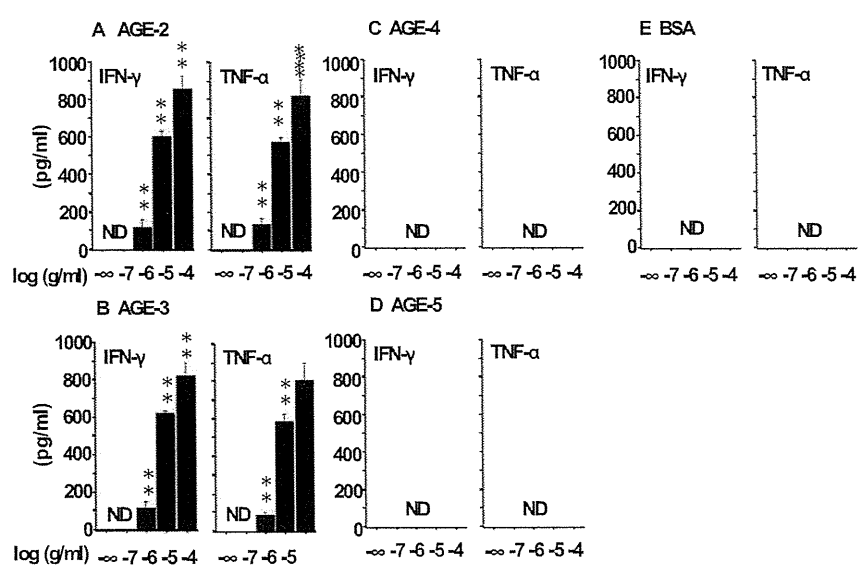

Fig. 5. Effect of AGEs on the production of IFN-γ and TNF-α in PBMC. PBMC at 1×10^6 cells/ml were incubated with AGE-2 (A), AGE-3 (B), AGE-4 (C), AGE-5 (D), or BSA (E) at increasing concentrations from 0.1 to 100 μg/ml for 24 h. The production of IFN-γ and TNF-α in PBMC was determined by ELISA. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the value for medium alone.

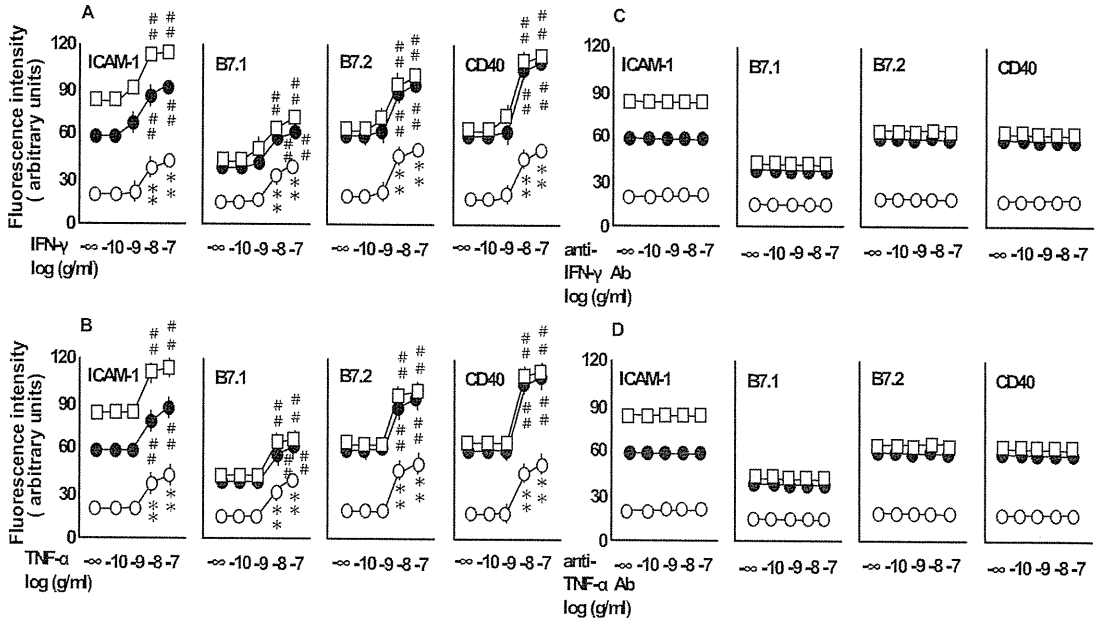

Fig. 6. Involvement of IFN-γ and TNF-α in the effect of AGE-2 and AGE-3 on adhesion molecule expression. PBMC at 1×10^6 cells/ml were incubated with IFN-γ (A), TNF-α (B), anti-IFN-γ (C), and anti-TNF-α (D) Abs at increasing concentrations from 0.1 to 100 ng/ml in the absence (○) or presence of AGE-2 (□) and AGE-3 (●) at 100 μg/ml for 24 h, and the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the value for medium alone. ##, $p < 0.01$ compared with the value for AGE alone. When an error bar was within a symbol, the bar was omitted.

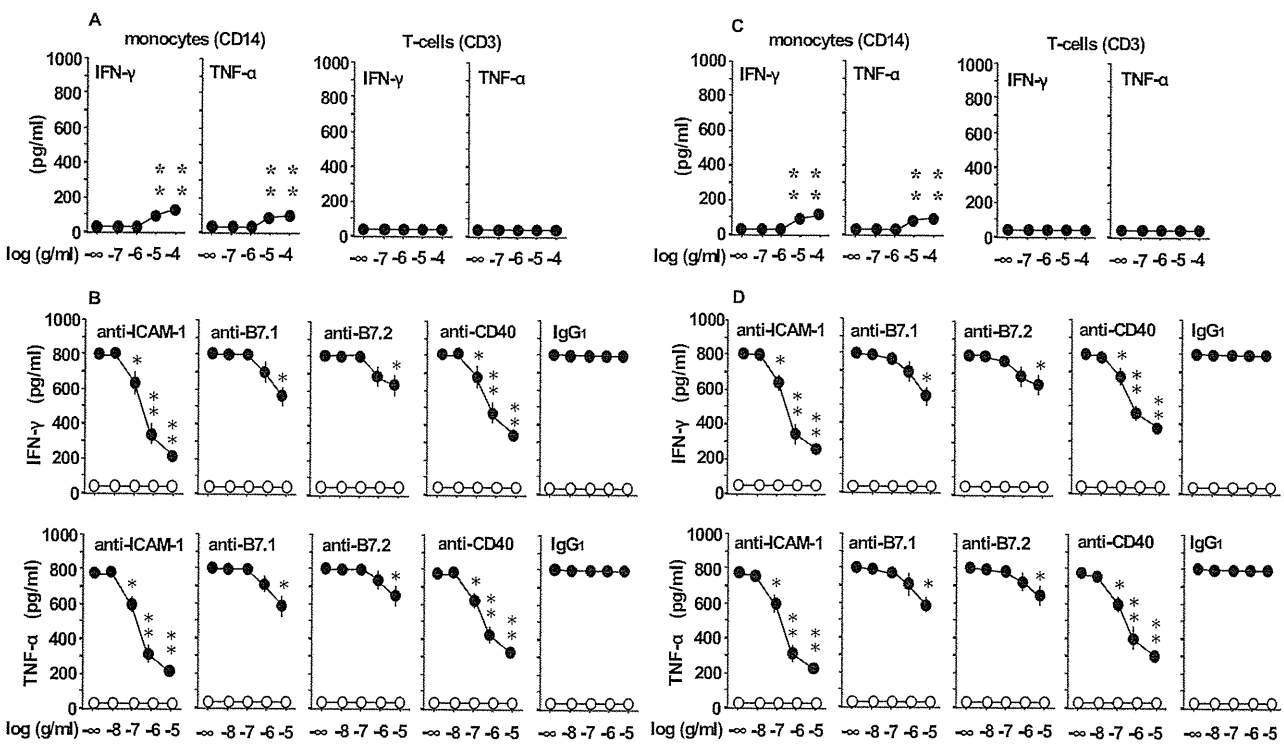

Fig. 7. Involvement of adhesion molecules in the effect of AGE-2 or AGE-3 on cytokine production. A and C, monocytes and T cells isolated from PBMC at 1×10^6 cells/ml were incubated with AGE-2 (A) or AGE-3 (C) at increasing concentrations from 0.1 to 100 μg/ml, and the production of IFN-γ and TNF-α was determined by ELISA. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the value for medium alone. B and D, PBMC at 1×10^6 cells/ml were incubated with anti-ICAM-1, anti-B7.1, anti-B7.2, and anti-CD40 Abs at increasing concentrations from 0.1 to 100 ng/ml in the absence (○) or presence (●) of AGE-2 (B) or AGE-3 (D) at 100 μg/ml, and the production of IFN-γ and TNF-α in PBMC was determined by ELISA. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. *, $p < 0.05$ and **, $p < 0.01$ compared with the value for AGE-2 or AGE-3 alone. If an error bar is within a symbol, the bar is omitted.

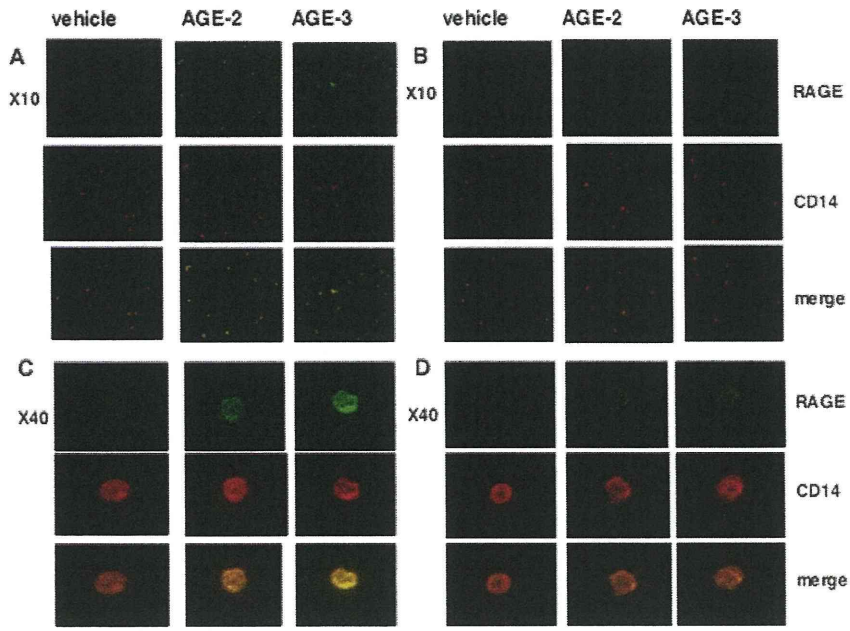

Fig. 8. Expression of RAGE on monocytes. The expression of RAGE on monocytes in the presence or absence of AGE1, AGE-2, AGE-3, AGE-4, AGE-5, and BSA at 100 μg/ml was analyzed by fluorescent microscopy. Staining with anti-RAGE rabbit IgG followed by Alexa 488-labeled goat anti-rabbit IgG as shown at 10× original magnification (A) and 40× original magnification (C). To block the engagement of anti-RAGE Ab with RAGE, anti-RAGE Ab was mixed with sRAGE for 24 h before use. The specificity of staining was confirmed by the absorption of primary Ab with sRAGE (B and D).

IFN-γ and TNF-α in T cells isolated from PBMC (Fig. 7, A and C). As shown in Fig. 7, B and D, anti-ICAM-1, anti-B7.1, anti-B7.2, and anti-CD40 Abs concentration-dependently inhibited the AGE-2- and AGE-3-induced production of IFN-γ and TNF-α in PBMC. The maximal inhibitory effect obtained by each Ab at 10 μg/ml was varied, and the rank order for the inhibition was as follows: ICAM-1 > CD40 > B7.1 > B7.2.

Expression of RAGE on Monocytes. The expression of RAGE on monocytes was determined by immunocytochemical staining (Fig. 8, A–D) and flow cytometry (Fig. 9A) using anti-RAGE Ab. AGE-2 and AGE-3 remarkably enhanced the expression of RAGE 24 h after stimulation, but AGE-4 and AGE-5 had no effect (data not shown). To confirm the binding specificity of anti-RAGE Ab to RAGE, we used a combination of anti-RAGE Ab with sRAGE for flow cytometry and immunocytochemical staining in the presence or absence of AGE-2 and AGE-3 (Figs. 8, B and D, and 9A). The detection of RAGE in the presence or absence of AGE-2 and AGE-3 was inhibited completely by the addition of sRAGE, indicating that anti-RAGE Ab recognized the extracellular domain of RAGE. As shown in Fig. 9B, Western blot analysis showed that RAGE is highly expressed on A549 cells, a human pulmonary epithelial cell line, as reported previously (Nakano et al., 2006). We confirmed that AGE-2 and AGE-3 at 100 μg/ml

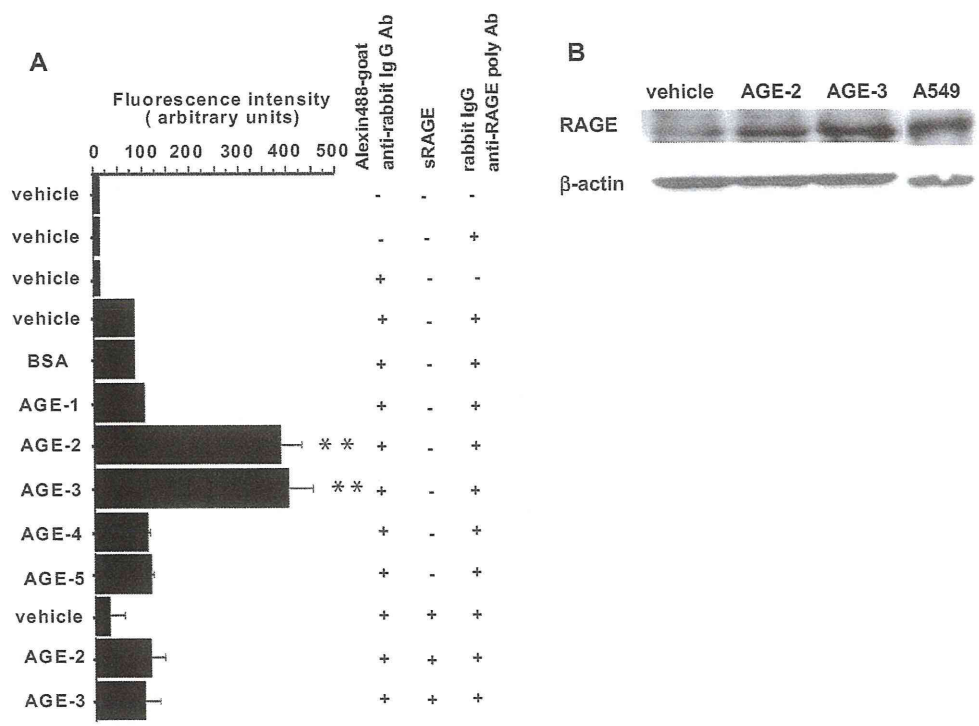

Fig. 9. Expression of RAGE on monocytes. A, expression of RAGE on monocytes in the presence or absence of AGE-1, AGE-2, AGE-3, AGE-4, AGE-5, and BSA at 100 μg/ml was examined by flow cytometry. The results of flow cytometry obtained under different conditions were quantified. To block the engagement of anti-RAGE Ab with RAGE, anti-RAGE Ab was mixed with sRAGE for 24 h before use. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the corresponding value for medium alone. B, protein levels in the presence or absence of AGE-2 or AGE-3 at 100 μg/ml were assessed by Western blot. Monocytes and A549 cells were cultured for 24 h at 37°C. RAGE levels were normalized to the level of β-actin.

induced the up-regulation of protein on human monocytes 24 h after the start of stimulation (Fig. 9B).

Involvement of NF-κB and MAPK in the Actions of AGE-2 and AGE-3. We examined the involvement of NF-κB and p38 MAPK activation in AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes and production of TNF-α and IFN-γ in PBMC (Fig. 10). A NF-κB inhibitor, SN50, and a p38 MAPK inhibitor, SB203580, reduced the AGE-2- and AGE-3-enhanced adhesion molecule expression as well as cytokine production. Even in the absence of AGE-2 and AGE-3, the SN50 and SB203580 inhibitors concentration-dependently inhibited adhesion expression, but neither had any effect on basal cytokine production.

Discussion

The diabetic state produces micro- and macrovascular lesions via various metabolic derangements, leading to crucial complications, including acquired blindness, end-stage renal failure, and neuropathies. In diabetic patients, the infiltration of macrophages and T cells into atherosclerotic plaques seems to be facilitated (Burke et al., 2004). Monocytes or macrophages, major components of atherosclerotic lesions and in contact with circulating plasma, play important roles in the hypercoagulable state and the progression of vascular injury, which develop during the progression of diabetes mellitus (Radoff et al., 1990). Macrophages are activated by atherosclerotic risk factors, including oxidized low-density

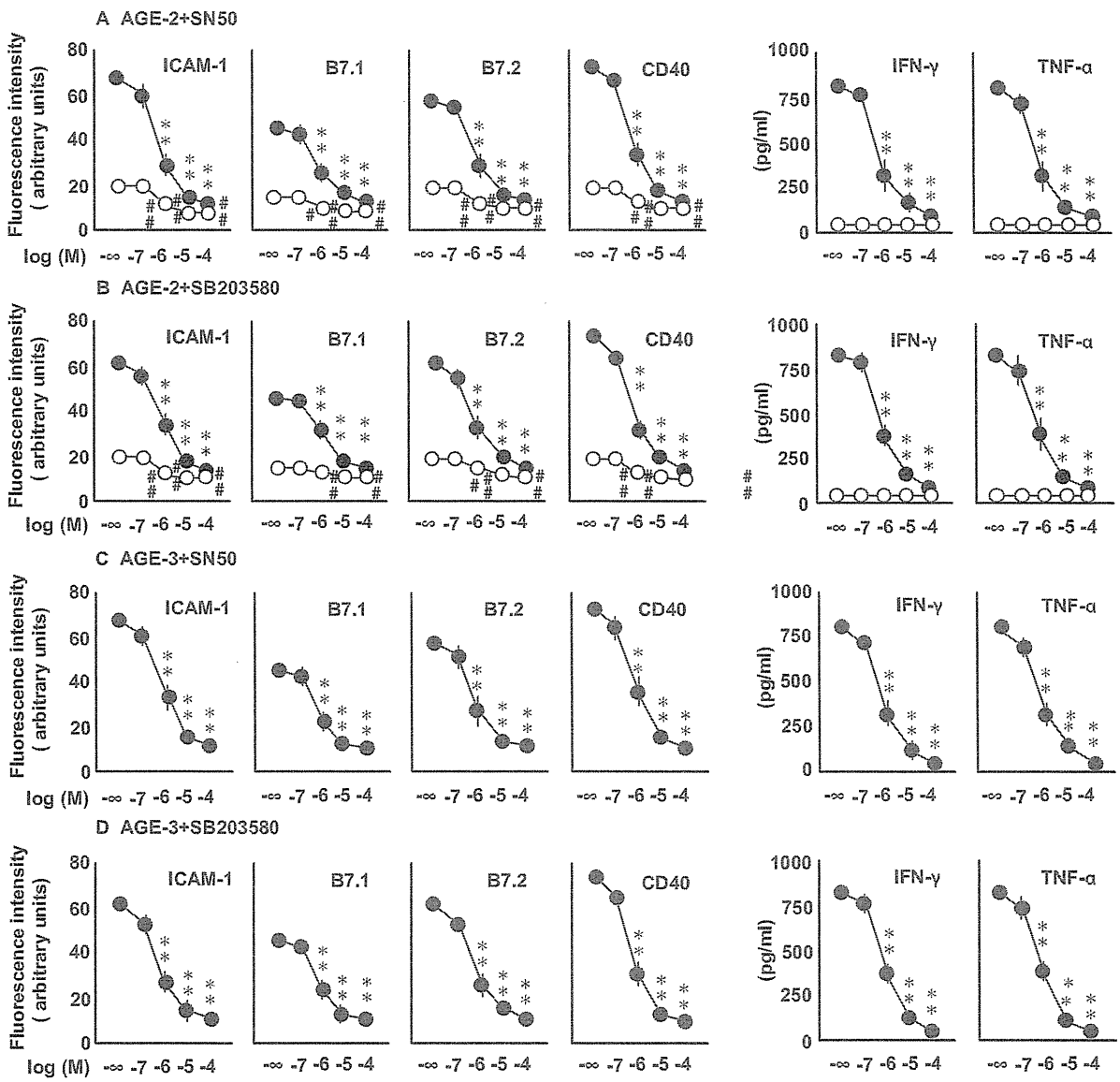

Fig. 10. Involvement of NF-κB and MAPK in the enhanced expression of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ and TNF-α by AGE-2 and AGE-3. PBMC at 1×10^6 cells/ml were incubated with AGE-2 (A and B) or AGE-3 (C and D) at 100 μg/ml in the presence of the NF-κB activation inhibitor SN50 and the p38 MAPK inhibitor SB203580 at increasing concentrations from 0.1 to 100 μM. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry, and the production of IFN-γ and TNF-α in PBMC was determined by ELISA. Open circles (○) represent the results in the absence of AGE, and filled circles (●) represent those in the presence of AGE. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the value for AGE alone. #, $p < 0.05$ and ##, $p < 0.01$ compared with the value for medium alone. If an error bar is within a symbol, the bar is omitted.

lipoprotein and AGEs, leading to death of cells and degradation of the extracellular matrix (Figarola et al., 2007). AGEs are involved in the cause of foam cell formation via the increased expression of oxidized low-density lipoprotein receptors in accelerated atherosclerotic lesions of patients with diabetes (Iwashima et al., 2000). NO by macrophages up-regulates the expression of Fas on vascular smooth muscle cells, priming them for apoptosis. The phenotypes of advanced diabetic nephropathy were prevented by administering an AGE inhibitor, (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide, in RAGE-transgenic mice crossbred with transgenic mice carrying human cDNA for inducible NO synthase under the control of the insulin promoter (Yamamoto et al., 2001). We therefore suggested that the activation of T cells by the enhancement of adhesion molecule expression on monocytes might result in the development of diabetic microangiopathy.

AGEs develop diabetic microangiopathy through the stimulation of RAGE (Stern et al., 2002). AGEs and RAGE are also detected in atherosclerotic plaque of diabetic patients (Cuccurullo et al., 2006). Takeuchi and Makita (2001) provided direct immunological evidence for the existence of four distinct AGE classes (AGE-2–5) among the AGE-modified proteins and peptides found in the serum of diabetic patients on hemodialysis. Antibodies, which cross-reacted with BSA modified by AGE-2, AGE-3, AGE-4, and AGE-5, recognized non–*N*-carboxymethyllysine AGE-2, AGE-3, AGE-4, and AGE-5 in serum samples obtained from type 2 diabetic patients, respectively. Among various subtypes of AGE, it has been shown that AGE-2 and AGE-3 are the main structures of AGEs that are detectable in the serum of diabetic patients (Takeuchi and Yamagishi, 2004). It is reported that AGE-2 induces diabetic microangiopathy (Takeuchi et al., 2000). Therefore, it has been suggested that blockade of the engagement of AGEs and RAGE is a target for the treatment of diabetic microangiopathy. However, little is known about the effect of AGE subtypes on the engagement of monocytes and T cells.

Several reports have shown a positive correlation between serum levels of TNF-α and circulating AGE levels. TNF-α promotes macrophage-induced apoptosis of human vascular smooth muscle cells (Boyle et al., 2003). Goren et al. (2007) reported that anti-TNF-α therapy, widely used in chronic inflammatory diseases in humans, improves healing of diabetes skin ulcers by targeting activated TNF-α-expressing macrophage subsets. Although it is reported that AGEs induce the production of TNF-α in monocytes (Boyle et al., 2001), little is known about the mechanism of enhancement of TNF-α production in monocytes. In the present study, we examined, for the first time, the affinity of AGE-2, AGE-3, AGE-4, and AGE-5 for RAGE by using in vitro assay system (Fig. 1). AGE-2 and AGE-3 have higher affinity for RAGE compared with AGE-4 and AGE-5. As shown in Figs. 2 to 5, we examined the effect of AGE subtypes on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, and the production of IFN-γ and TNF-α in PBMC. AGE-2 and AGE-3, but not AGE-4 and AGE-5, increased the expression of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ and TNF-α. Consistent with high-affinity binding of AGE-2 and AGE-3 to RAGE in vitro, our results concerning the adhesion molecule expression and cytokine production may support the idea that AGE-2 and AGE-3 function as toxic AGEs and play a central role in the pathophysiological processes associated with AGE formation. As shown in Fig. 6, A and B, the actions of AGE-2 and AGE-3 might be independent of the endogenous IFN-γ and TNF-α. IL-18 and IL-12, as well as IFN-γ and TNF-α, are associated with accelerated atherosclerosis (Gerdes et al., 2002; Wen et al., 2006). Recently, we reported that IL-18 induced the production of IFN-γ and TNF-α in PBMC (Takahashi et al., 2003). We found that the levels of IL-18 and IL-12, monocyte-derived cytokines, were under the detection limit (10 pg/ml) in the presence of AGE-2 and AGE-3 (data not shown). Anti-IL-18 and anti-IL-12 Abs had no effect on the AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40 and production of IFN-γ and TNF-α (data not shown), indicating that the actions of AGE-2 and AGE-3 might be also independent of IL-18 and IL-12. As shown in Fig. 7, the enhanced production of IFN-γ and TNF-α by AGE-2 and AGE-3 may require the enhancement of cell-to-cell interaction between monocytes and T cells through the induction of plural adhesion molecule expression on monocytes.

As shown in Figs. 8 and 9A, we found, for the first time, the up-regulation of RAGE expression on monocytes by AGE-2 and AGE-3 using flow cytometry and immunocytochemistry. Moreover, we confirmed the up-regulation of RAGE protein induced by AGE-2 and AGE-3 using Western blot analysis (Fig. 9B). It is reported that the interaction of AGE-2 with RAGE alters intracellular signaling, gene expression, and the release of proinflammatory molecules in monocytes and macrophages (Sato et al., 2006). AGEs are reported to induce the activation of NF-κB in monocytes via RAGE (Hofmann et al., 1999). In endothelial cells, the activation of NF-κB induces the expression of cytokines, including IL-1 and transforming growth factor-β, and adhesion molecules, including ICAM-1 and vascular cell adhesion molecule-1 (Miyata et al., 1996). The interaction of AGEs and RAGE is reported to result in the up-regulation of RAGE through the activation of NF-κB in monocytes (Li and Schmidt, 1997). Consistent with this finding, pharmacological experiments using the inhibitors of NF-κB and p38 MAPK suggested that the activation of NF-κB and p38 MAPK might be in part involved in the effects of AGE-2 and AGE-3 on adhesion molecule expression and cytokine production (Fig. 10), although it is not excluded that other pathways may be involved in the actions of AGEs. Further studies on the signal pathway leading to the up-regulation of RAGE and adhesion molecule expression should be performed.

In conclusion, among AGEs, AGE-2 and AGE-3 selectively induced the expression of ICAM-1, B7.1, B7.2, and CD40, and the production of IFN-γ and TNF-α. Although adhesion molecule expression on monocytes was independent of endogenous cytokine production in the presence of AGE-2 and AGE-3, the induction of IFN-γ and TNF-α depended on the engagement of monocytes and T cells through the up-regulation of ICAM-1, B7.1, B7.2, and CD40. The actions of AGE-2 and AGE-3 might be through the stimulation and up-regulation of RAGE and the activation of NF-κB and p38 MAPK in monocytes. Together with these results, toxic AGE-dependent responses, including the enhancement of adhesion molecule expression on monocytes, may partially contribute to facilitation of the development of atherosclerotic plaques in diabetes.

Acknowledgments

We thank Miyuki Shiotani for technical assistance.

References

Boyle JJ (2005) Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. *Curr Vasc Pharmacol* **3:**63–68.

Boyle JJ, Bowyer DE, Weissberg PL, and Bennett MR (2001) Human blood-derived macrophages induce apoptosis in human plaque–derived vascular smooth muscle cells by Fas-ligand/Fas interactions. *Arterioscler Thromb Vasc Biol* **21:**1402–1407.

Boyle JJ, Weissberg PL, and Bennett MR (2003) Tumor necrosis factor-alpha promotes macrophage-induced vascular smooth muscle cell apoptosis by direct and autocrine mechanisms. *Arterioscler Thromb Vasc Biol* **23:**1553–1558.

Brownlee M (1995) Advanced protein glycosylation in diabetes and aging. *Annu Rev Med* **46:**223–234.

Burke AP, Kolodgie FD, Zieske A, Fowler DR, Weber DK, Varghese PJ, Farb A, and Virmani R (2004) Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. *Arterioscler Thromb Vasc Biol* **24:**1266–1271.

Camacho SA, Heath WR, Carbone FR, Sarvetnick N, LeBon A, Karlsson L, Peterson PA, and Webb SR (2001) A key role for ICAM-1 in generating effector cells mediating inflammatory responses. *Nat Immunol* **2:**523–529.

Cuccurullo C, Iezzi A, Fazia ML, De Cesare D, Di Francesco A, Muraro R, Bei R, Ucchino S, Spigonardo F, Chiarelli F, et al. (2006) Suppression of RAGE as a basis of simvastatin-dependent plaque stabilization in type 2 diabetes. *Arterioscler Thromb Vasc Biol* **26:**2716–2723.

Durie FH, Foy TM, Masters SR, Laman JD, and Noelle RJ (1994) The role of CD40 in the regulation of humoral and cell-mediated immunity. *Immunol Today* **15:** 406–411.

Enomoto M, Adachi H, Yamagishi S, Takeuchi M, Furuki K, Hino A, Hiratsuka A, Takajo Y, and Imaizumi T (2006) Positive association of serum levels of advanced glycation end products with thrombogenic markers in humans. *Metabolism* **55:** 912–917.

Figarola JL, Shanmugam N, Natarajan R, and Rahbar S (2007) Anti-inflammatory effects of the advanced glycation end product inhibitor LR-90 in human monocytes. *Diabetes* **56:**647–655.

Ge J, Jia Q, Liang C, Luo Y, Huang D, Sun A, Wang K, Zou Y, and Chen H (2005) Advanced glycosylation end products might promote atherosclerosis through inducing the immune maturation of dendritic cells. *Arterioscler Thromb Vasc Biol* **25:**2157–2163.

Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, and Schönbeck U (2002) Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. *J Exp Med* **195:**245–257.

Goren I, Müller E, Schiefelbein D, Christen U, Pfeilschifter J, Mühl H, and Frank S (2007) Systemic anti-TNFα treatment restores diabetes-impaired skin repair in ob/ob mice by inactivation of macrophages. *J Invest Dermatol* **127:**2259–2267.

Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, Avila C, Kambham N, Bierhaus A, Nawroth P, et al. (1999) RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* **97:**889–901.

Iwashima Y, Eto M, Hata A, Kaku K, Horiuchi S, Ushikubi F, and Sano H (2000) Advanced glycation end products-induced gene expression of scavenger receptors in cultured human monocyte-derived macrophages. *Biochem Biophys Res Commun* **277:**368–380.

Li J and Schmidt AM (1997) Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation endproducts. *J Biol Chem* **272:**16498–16506.

Miyata T, Hori O, Zhang J, Yan SD, Ferran L, Iida Y, and Schmidt AM (1996) The receptor for advanced glycation end products (RAGE) is a central mediator of the interaction of AGE-beta2 microglobulin with human mononuclear phagocytes via an oxidant-sensitive pathway. Implications for the pathogenesis of dialysis-related amyloidosis. *J Clin Invest* **98:**1088–1094.

Nakamura K, Yamagishi SI, Matsui T, Adachi H, Takeuchi M, and Imaizumi T (2007) Serum levels of soluble form of receptor for advanced glycation end products (sRAGE) are correlated with AGEs in both diabetic and non-diabetic subjects. *Clin Exp Med* **7:**188–190.

Nakano N, Fukuhara-Takaki K, Jono T, Nakajou K, Eto N, Horiuchi S, Takeya M, and Nagai R (2006) Association of advanced glycation end products with A549 cells, a human pulmonary epithelial cell line, is mediated by a receptor distinct from the scavenger receptor family and RAGE. *J Biochem* **139:**821–829.

Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, Pan YC, Elliston K, Stern D, and Shaw A (1992) Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem* **267:**14998–15004.

Okamoto T, Yamagishi S, Inagaki Y, Amano S, Koga K, Abe R, Takeuchi M, Ohno S, Yoshimura A, and Makita Z (2002) Angiogenesis induced by advanced glycation end products and its prevention by cerivastatin. *FASEB J* **16:**1928–1930.

Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS, Stern D, and Schmidt AM (1998) Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med* **4:**1025–1031.

Radoff S, Cerami A, and Vlassara H (1990) Isolation of surface binding protein specific for advanced glycosylation end products from mouse macrophage-derived cell line RAW 264.7. *Diabetes* **39:**1510–1518.

Ranger AM, Das MP, Kuchroo VK, and Glimcher LH (1996) B7-2 (CD86) is essential for the development of IL-4-producing T cells. *Int Immunol* **8:**1549–1560.

Sato T, Iwaki M, Shimogaito N, Wu X, Yamagishi S, and Takeuchi M (2006) TAGE (toxic AGEs) theory in diabetic complications. *Curr Mol Med* **6:**351–358.

Schmidt AM, Hasu M, Popov D, Zhang JH, Chen J, Yan SD, Brett J, Cao R, Kuwabara K, and Costache G (1994) Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins. *Proc Natl Acad Sci U S A* **91:**8807–8811.

Stern DM, Yan SD, Yan SF, and Schmidt AM (2002) Receptor for advanced glycation endproducts (RAGE) and the complications of diabetes. *Ageing Res Rev* **1:**1–15.

Stitt AW, He C, Friedman S, Scher L, Rossi P, Ong L, Founds H, Li YM, Bucala R, and Vlassara H (1997) Elevated AGE-modified ApoB in sera of euglycemic, normolipidemic patients with atherosclerosis: relationship to tissue AGEs. *Mol Med* **3:**617–627.

Stoll G and Bendszus M (2006) Inflammation and atherosclerosis: novel insights into plaque formation and destabilization. *Stroke* **37:**1923–1932.

Sugimoto H, Shikata K, Hirata K, Akiyama K, Matsuda M, Kushiro M, Shikata Y, Miyatake N, Miyasaka M, and Makino H (1997) Increased expression of intercellular adhesion molecule-1 (ICAM-1) in diabetic rat glomeruli: glomerular hyperfiltration is a potential mechanism of ICAM-1 upregulation. *Diabetes* **46:**2075–2081.

Takahashi HK, Iwagaki H, Tamura R, Xue D, Sano M, Mori S, Yoshino T, Tanaka N, and Nishibori M (2003) Unique regulation profile of prostaglandin E1 on adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells. *J Pharmacol Exp Ther* **307:**1188–1195.

Takedo A, Yasuda T, Miyata T, Mizuno K, Li M, Yoneyama S, Horie K, Maeda K, and Sobue G (1996) Immunohistochemical study of advanced glycation end products in aging and Alzheimer's disease brain. *Neurosci Lett* **221:**17–20.

Takeuchi M, Makita Z, Bucala R, Suzuki T, Koike T, and Kameda Y (2000) Immunological evidence that non-carboxymethyllysine advanced glycation end-products are produced from short chain sugars and dicarbonyl compounds in vivo. *Mol Med* **6:**114–125.

Takeuchi M and Makita Z (2001) Alternative routes for the formation of immunochemically distinct advanced glycation end-products in vivo. *Curr Mol Med* **1:**305–315.

Takeuchi M and Yamagishi S (2004) TAGE (toxic AGEs) hypothesis in various chronic diseases. *Med Hypotheses* **63:**449–452.

Yamagishi S and Imaizumi T (2005) Diabetic vascular complications: pathophysiology, biochemical basis and potential therapeutic strategy. *Curr Pharm Des* **11:** 2279–2299.

Yamamoto Y, Kato I, Doi T, Yonekura H, Ohashi S, Takeuchi M, Watanabe T, Yamagishi S, Sakurai S, Takasawa S, et al. (2001) Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. *J Clin Invest* **108:** 261–268.

Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, Pinsky D, and Stern D (1994) Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* **269:**9889–9897.

Wen Y, Gu J, Li SL, Reddy MA, Natarajan R, and Nadler JL (2006) Elevated glucose and diabetes promote interleukin-12 cytokine gene expression in mouse macrophages. *Endocrinology* **147:**2518–2525.

Address correspondence to: Dr. Masahiro Nishibori, Department of Pharmacology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. E-mail: mbori@md.okayama-u.ac.jp

0022-3565/09/3303-826–833$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

JPET 330:826–833, 2009

Vol. 330, No. 3
155960/3510190
Printed in U.S.A.

Histamine Inhibits Advanced Glycation End Products-Induced Adhesion Molecule Expression on Human Monocytes

Hidenori Wake, Hideo Kohka Takahashi, Shuji Mori, Keyue Liu, Tadashi Yoshino, and Masahiro Nishibori

Departments of Pharmacology (H.W., H.K.T., K.L., M.N.) and Pathology (T.Y.), Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan; and Department of Pharmacy, Shujitsu University, Okayama, Japan (S.M.)

Received May 11, 2009; accepted June 26, 2009

ABSTRACT

Advanced glycation end products (AGEs) are modifications of proteins/lipids that become nonenzymatically glycated after contact with aldose sugars. Among various subtypes of AGEs, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3) are suggested to play roles in inflammation in diabetic patients. Because the engagement of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2, and CD40 on monocytes with their ligands on T cells plays roles in cytokine production, we examined the effects of AGE-2 and AGE-3 on the expression of adhesion molecules and cytokine production in human peripheral blood mononuclear cells (PBMC) and their modulation by histamine in the present study. AGE-2 and AGE-3 induced the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes and the production of interferon-γ in PBMC. Histamine concentration-dependently inhibited the action of AGE-2 and AGE-3. The effects of histamine were antagonized by an H2 receptor antagonist, famotidine, and mimicked by H2/H4 receptor agonists dimaprit and 4-methylhistamine. Histamine induced cAMP production in the presence and absence of AGE-2 and AGE-3. The effects of histamine were reversed by a protein kinase A (PKA) inhibitor, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline (H89), and mimicked by a dibutyryl cAMP and an adenylate cyclase activator, forskolin. These results as a whole indicated that histamine inhibited the AGE-2- and AGE-3-induced adhesion molecule expression and cytokine production via H2 receptors and the cAMP/PKA pathway.

AGEs are products of the nonenzymatic glycation/oxidation of proteins/lipids that accumulate during natural aging and are also greatly augmented in disorders such as diabetes, renal failure, and Alzheimer's disease (Schmidt et al., 1994; Brownlee, 1995; Takedo et al., 1996). AGEs are also implicated in the pathogenesis of atherosclerotic vascular disease of diabetic etiology (Takeuchi and Yamagishi, 2004). Direct immunochemical evidence for the existence of four distinct AGE structures, including AGE-2, AGE-3, AGE-4, and AGE-5, was identified, within the AGE-modified proteins and peptides (Takeuchi and Yamagishi, 2004). Among various subtypes of AGE, toxic AGE structures, AGE-2 and AGE-3, are the main structures of AGEs that are detectable in the serum of diabetic patients (Takeuchi and Yamagishi, 2004). AGE-2 is reported to induce diabetic microangiopathy (Takeuchi et al., 2000). AGE-2 and AGE-3 also have diverse biological activities on vascular wall cells, mesangial cells, Schwann cells, malignant melanoma cells, and cortical neurons (Okamoto et al., 2002; Yamagishi and Imaizumi, 2005). AGEs and the receptor for AGEs (RAGE) are detected in atherosclerotic plaque of diabetic patients (Cuccurullo et al., 2006). The stimulation of RAGE induces plaque rupture in diabetic patients. In vitro work has shown the involvement of RAGE, leading to oxidative stress and vascular damage, particularly in atherosclerosis (Vlassara and Palace, 2002) and in diabetes (Ruderman et al., 1992).

Microinflammation is a common major mechanism in the

This work was supported in part by grants from the Japan Society for the Promotion of Science [Grants 18590509, 20590539, 17659159, 19659061, 21659141, 21390071, 215905694]; the Scientific Research from Ministry of Health, Labour and Welfare of Japan; and the Takeda Science Foundation.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.109.155960.

ABBREVIATIONS: AGE, advanced glycation end product; RAGE, receptor for advanced glycation end product(s); IFN, interferon; PBMC, peripheral blood mononuclear cell(s); ICAM, intercellular adhesion molecule; H, histamine; PKA, protein kinase A; IL, interleukin; 4-MH, 4-methylhistamine dihydrochloride; BSA, bovine serum albumin; dbcAMP, dibutyryl cAMP; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; Ab, antibody; ELISA, enzyme-linked immunosorbent assay; sRAGE, soluble advanced glycosylation end product(s); LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; SN50, H-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Val-Gln-Arg-Lys-Arg-Gln-Lys-Leu-Met-Pro-OH; HDC, histidine decarboxylase.

pathogenesis of diabetic vascular complications. It is reported that diabetes has greater infiltration of macrophages and T cells in atherosclerotic plaques (Burke et al., 2004). Activation of monocytes/macrophages and T cells induces the progression of inflammatory atherosclerotic plaques (Stoll and Bendszus, 2006). It has been found that the enhancement of ICAM-1, B7.1, B7.2, and CD40 expression on monocytes results in the activation of T cells (Durie et al., 1994; Ranger et al., 1996; Camacho et al., 2001). Therefore, the blockade of engagement of adhesion molecules by antibodies against ICAM-1, B7.1, B7.2, and CD40 reduced the production of IFN-γ by PBMC (Morichika et al., 2003; Takahashi et al., 2003). In a previous study, we found that AGE-2 and AGE-3 induced the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes and production of IFN-γ in human PBMC, but AGE-4 and AGE-5 had no effect (Takahashi et al., 2009). The effect of AGE-2 and AGE-3 on the production of IFN-γ was dependent on cell-to-cell interaction via engagement between ICAM-1, B7.1, B7.2, and CD40 on monocytes and their ligands on T cells, and the stimulation of RAGE on monocytes was involved in the actions of AGE-2 and AGE-3 (Takahashi et al., 2009).

Histamine has immunoregulatory properties because it modulates cytotoxic T-cell activity (Khan et al., 1989), natural killer cell activity (Hellstrand et al., 1994), and cytokine production in PBMC (Dohlsten et al., 1987; Elenkov et al., 1998; van der Pouw Kraan et al., 1998). Histamine exerts its effects through the stimulation of H1, H2, H3, and H4 receptors (Elenkov et al., 1998; van der Pouw Kraan et al., 1998). In general, immunoregulatory effects of histamine depend on the stimulation of H2 receptors (Elenkov et al., 1998; van der Pouw Kraan et al., 1998; Hough, 2001). H2 receptor stimulation is coupled with the activation of adenylate cyclase and the cAMP/PKA pathway in monocytes (Shayo et al., 1997). However, little is known about the effect of histamine on the AGEs-induced activation of monocytes. In the present study, we examined the effect of histamine on the expressions of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ induced by AGE-2 and AGE-3.

Materials and Methods

Reagents and Drugs. Recombinant human IL-18 was purchased from Medical and Biological Laboratories (Nagoya, Japan). Histamine dihydrochloride was purchased from Nacalai Tesque (Kyoto, Japan). Dimaprit dihydrochloride and 4-methylhistamine dihydrochloride (4-MH) were gifts from Drs. W. A. M. Duncan and D. J. Durant (The Research Institute, Smith Kline and French Laboratories, Welwyn Garden City, Hertfordshire, UK). *d*-Chlorpheniramine maleate, ranitidine, and famotidine were provided by Yoshitomi Pharmaceutical Co. Ltd. (Tokyo, Japan), Glaxo Japan (Tokyo, Japan), and Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan), respectively. Thioperamide hydrochloride was provided by Eisai Co. Ltd. (Tokyo, Japan). AGE-modified bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) was prepared as described previously (Takeuchi et al., 2000; Takahashi et al., 2009). In brief, each protein was incubated under sterile conditions with glyceraldehyde-3-phosphate (AGE-2) (Sigma-Aldrich) or glycolaldehyde (AGE-3) (Sigma-Aldrich) in 0.2 M phosphate buffer, pH 7.4, at 37°C for 7 days. AGE-BSA was dialyzed for 2 days at 4°C. The endotoxin concentration of AGEs at 100 μg/ml described above was measured at SRL (Okayama, Japan) and was found to be 1.2 pg/ml. AGE-specific fluorescence was measured at 450 nm after excitation at 390 nm with a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). db-cAMP and forskolin were purchased from Wako Pure Chemicals (Tokyo, Japan). H89 was purchased from Sigma-Aldrich. For flow cytometric analysis, fluorescein isothiocyanate (FITC)-conjugated mouse IgG1 monoclonal antibody (mAb) against ICAM-1/CD54 and phycoerythrin-conjugated anti-CD14 mAb were purchased from Dako Denmark A/S (Glostrup, Denmark). FITC-conjugated mouse IgG1 mAb against B7.2 and CD40 were purchased from BD Biosciences Pharmingen (San Diego, CA), and FITC-conjugated IgG1, as an isotype-matched control, was obtained from Sigma-Aldrich.

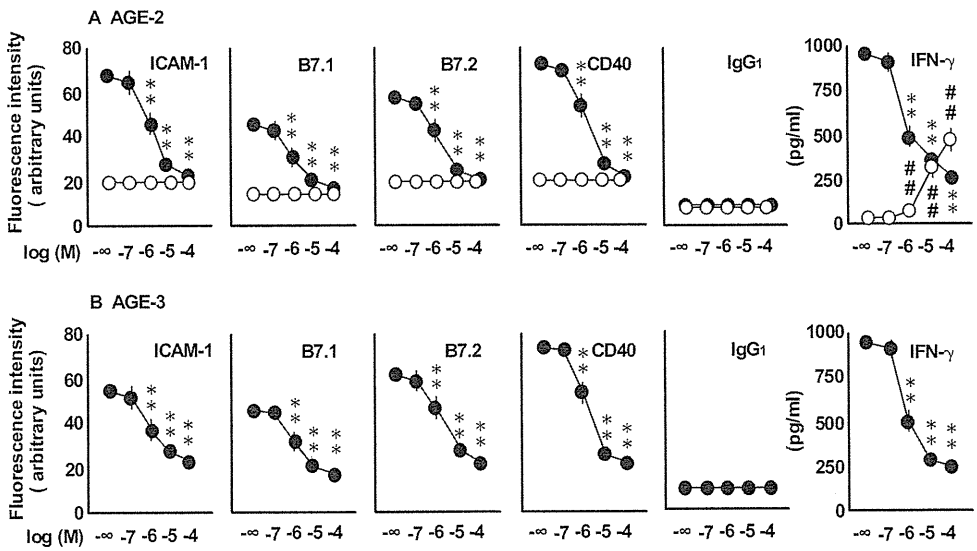

Fig. 1. Effects of histamine on the AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes and production of IFN-γ in PBMC. PBMC at 1×10^6 cells/ml were incubated with AGE-2 (A) and AGE-3 (B) at 100 mg/ml and histamine at increasing concentrations from 0.1 to 100 μM for 24 h. The expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes were determined by flow cytometry. FITC-conjugated IgG1 was used as an isotype-matched control Ab. IFN-γ concentration in conditioned media was determined by ELISA. Filled circles (●) represent the effect of histamine on the adhesion molecule expression and cytokine production in the presence of AGE-2 and AGE-3. Open circles (○) represent the effect of histamine in the absence of AGE-2 and AGE-3. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $P < 0.01$ compared with the value for AGE-2 and AGE-3. ##, $P < 0.01$ compared with the value for medium alone. When an error bar was within a symbol, the bar was omitted.

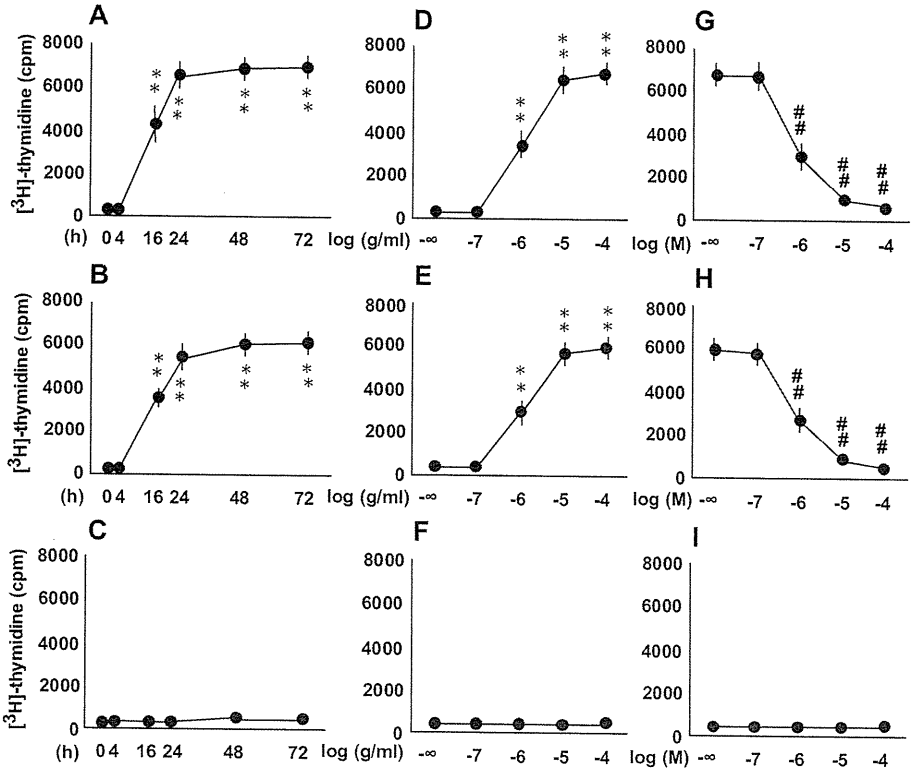

Fig. 2. Effects of histamine on the AGE-2- and AGE-3-induced lymphocyte proliferation. PBMC at 2×10^6 cells/ml were incubated with AGE-2 (A), AGE-3 (B), or BSA (C) at 100 μg/ml for the indicated periods, and the lymphocyte proliferation was determined by [^{3}H]thymidine uptake as described under *Materials and Methods*. The concentration-response relationships for the effects of AGE-2 (D), AGE-3 (E), or BSA (F) on the lymphocyte proliferation were determined at 24 h. The effects of increasing concentrations of histamine on 100 μg/ml AGE-2- (G), AGE-3- (H), or BSA (I)-induced lymphocyte proliferation were determined at 48 h. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $P < 0.01$ compared with the value for 0 h (A–C) or 0 μg/ml (D–F). ##, $P < 0.01$ compared with the value in the presence of AGE-2 or AGE-3 alone.

Isolation of PBMC and Monocytes. Normal human PBMC were obtained from 10 healthy volunteers after acquiring institutional review board approval (Okayama University Institutional Review Board 106). Twenty to 50 ml of peripheral blood was withdrawn from a forearm vein, and PBMC were prepared from buffy coat as described previously (Takahashi et al., 2003). When monocytes were isolated from PBMC, counterflow centrifugal elutriation was used for separation as described previously (Takahashi et al., 2003). The PBMC and monocytes were then suspended at a final concentration of 1×10^6 cells/ml in the medium as described previously (Takahashi et al., 2003).

Flow Cytometric Analysis. Changes in the expression of human leukocyte antigens ICAM-1, B7.1, B7.2, and CD40 on monocytes were examined by multicolor flow cytometry using a combination of anti-CD14 Ab with anti-ICAM-1, anti-B7.1, anti-B7.2, or anti-CD40 Ab. PBMC at 1×10^6 cells/ml were incubated for 24 h. Cultured cells at 5×10^5 cells/ml were prepared for flow cytometric analysis as described previously (Takahashi et al., 2003) and analyzed with an FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The data were processed using the CellQuest program (BD Biosciences).

Cytokine Assay. PBMC at 1×10^6 cells/ml were used to analyze IFN-γ production. After culturing for 24 h at 37°C in a 5% CO_2/air mixture, cell-free supernatant was assayed for IFN-γ protein by enzyme-linked immunosorbent assay (ELISA) using the multiple Abs sandwich principle (R&D Systems, Minneapolis, MN). The detection limit of ELISA for IFN-γ was 10 pg/ml.

Proliferation Assay. PBMC were treated with various conditions. Cultures were incubated for 24 h, during which they were pulsed with [^{3}H]thymidine (3 Ci/well) for the final 16 h. Cells were then divided into 96-well microplates, 200 μl/well, resulting in 1 μCi of [^{3}H]thymidine per well and harvested by the Micro-Mate 196 cell harvester (PerkinElmer Life and Analytical Sciences, Boston, MA). Thymidine incorporation was measured by a beta-counter (Matrix 9600; PerkinElmer Life and Analytical Sciences).

Measurement of cAMP Production in Monocytes. Monocytes at 1×10^6 cells/ml were incubated at 37°C in a 5% CO_2/air mixture under different conditions. When the effects of histamine receptor antagonists were examined, the antagonists were added to the media 30 min before histamine addition. AGEs and histamine were simultaneously added to the media. After 24 h, cells at 2×10^5 cells/200 μl/well were supplemented with trichloroacetic acid to a final concentration of 5% and 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase, at 100 μM and frozen at −80°C. Frozen samples were subsequently sonicated and assayed for cAMP using a cAMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions, for which no acetylation procedures were performed. The results are expressed as the means ± S.E.M. for five donors.

Statistical Examination. Statistical significance was evaluated using analysis of variance followed by Dunnett's test. A probability value of less than 0.05 was considered to indicate statistical significance. The results are expressed as the means ± S.E.M. of triplicate findings from five donors.

Results

Effects of Histamine on AGE-2- and AGE-3-Induced Expressions of ICAM-1, B7.1, B7.2, and CD40 on Monocytes; Production of IFN-γ; and Lymphocyte Proliferation in PBMC. In a previous study, to evaluate the binding of AGE subtypes to RAGE, we established an in vitro assay by using immobilized AGE subspecies and His-tagged sRAGE protein (Takahashi et al., 2009). AGE-2 and AGE-3 showed relatively high-affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed moderate affinity for sRAGE. The proper incubation time and concentration of AGEs were determined according to Takahashi et al. (2009). AGE-2 and AGE-3 at 100 μg/ml significantly induced the expressions of ICAM-1, B7.1, B7.2, and CD40 and production of IFN-γ at 16 h and thereafter up to 24 and 48 h. As shown in Fig. 1, we observed the effects of histamine at concentrations ranging from 0.1 to 100 μM on the expression of ICAM-1, B7.1, B7.2,

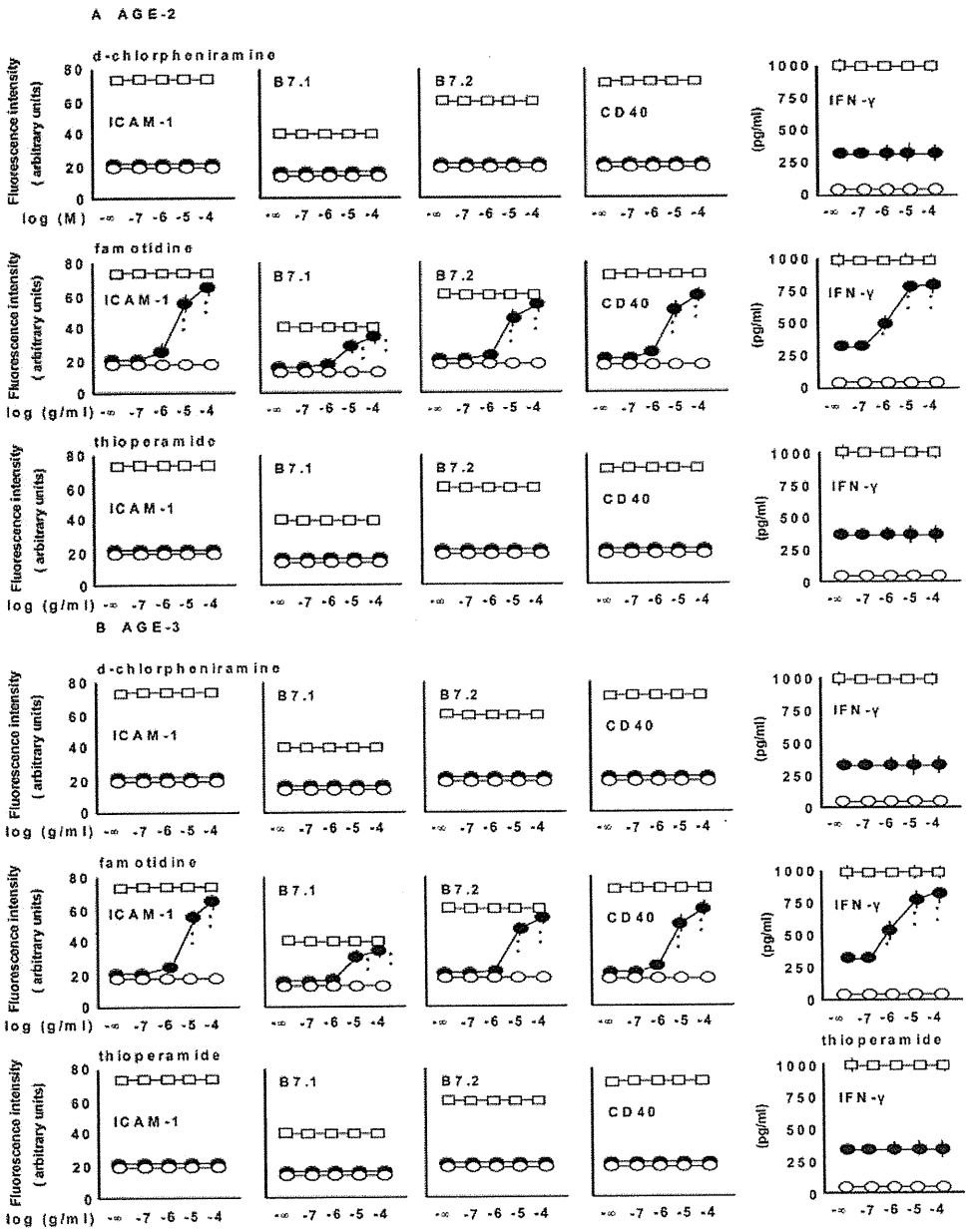

Fig. 3. Effects of histamine receptor antagonists on the histamine-induced inhibition of expressions of ICAM-1, B7.1, B7.2, and CD40 and production of IFN-γ. PBMC at 1×10^6 cells/ml were incubated with different classes of histamine receptor antagonists, including *d*-chlorpheniramine (H1 antagonist), famotidine (H2 antagonist), and thioperamide (H3/4 antagonist), at increasing concentrations from 0.1 to 100 μM in the presence or absence of AGE-2 (A) and AGE-3 (B) at 100 μg/ml. The expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes were determined by flow cytometry. In addition, IFN-γ concentration was determined by ELISA. Filled circles (●) represent the effect of antagonists on histamine-inhibited adhesion molecule expression and cytokine production in the presence of AGE-2 and AGE-3. Open squares (□) represent the effect of antagonists in the presence of AGE-2 and AGE-3 without histamine stimulation. Open circles (○) represent the effect of antagonists on adhesion molecule expression cytokine production in the absence of histamine, AGE-2, and AGE-3. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. *, $P < 0.05$; **, $P < 0.01$ compared with the value for histamine. When an error bar was within a symbol, the bar was omitted.

and CD40, the production of IFN-γ in the presence (100 μg/ml) or absence of AGE-2 and AGE-3 at 24 h. Moreover, AGE-2 and AGE-3 significantly induced the lymphocyte proliferation at 16 h and thereafter up to 24, 48, and 72 h (Fig. 2). The effect of histamine on lymphocyte proliferation was determined at 24 h.

Histamine concentration-dependently inhibited the AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40; production of IFN-γ; and lymphocyte proliferation. The IC_{50} values for the inhibitory effect of histamine on the expressions of ICAM-1, B7.1, B7.2, and CD40, the production of IFN-γ, and the lymphocyte proliferation in the presence of AGE-2 were 1, 1, 1.5, 2, 0.8, and 0.8 μM, and those in the presence of AGE-3 were 1, 1, 1.7, 1.5, 0.8, and 0.8 μM, respectively. In the absence of AGE-2 and AGE-3, histamine induced the production of IFN-γ but had no effect on adhesion molecule expression and lymphocyte proliferation. The ED_{50} value for the effect of histamine alone on the production of IFN-γ was 4 μM.

Involvement of H2 Receptor in the Actions of Histamine. To determine the histamine receptor subtypes involved in the effects of histamine on the expressions of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ in the presence of AGE-2 and AGE-3, the effects of an H1 receptor antagonist *d*-chlorpheniramine; an H2 receptor antagonist, famotidine; and an H3/4 receptor antagonist, thioperamide, at concentrations ranging from 0.1 to 100 μM on adhesion molecule expression and cytokine production were examined in the presence of histamine at 100 μM (Fig. 3). Famotidine concentration-dependently inhibited the action of histamine, but *d*-chlorpheniramine and thioperamide had no effect. Another H2 receptor antagonist, ranitidine, exerted a substantially similar effect to famotidine (data not shown). As shown in Fig. 4, the effects of H2/H4 receptor agonists

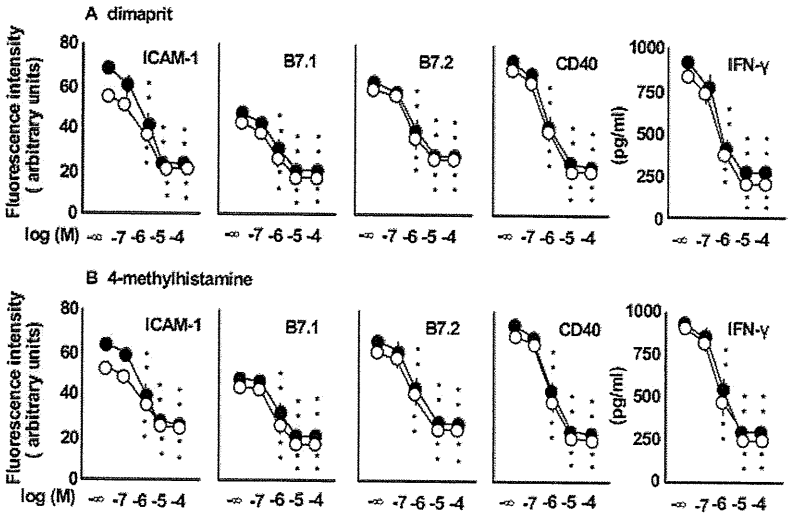

Fig. 4. Effects of histamine receptor agonists on the AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 and production of IFN-γ. PBMC at 1×10^6 cells/ml were incubated with histamine H2/H4 receptor agonists dimaprit (A) and 4-MH (B) at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 at 100 μg/ml for 24 h. The expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes were determined by flow cytometry, and IFN-γ concentration in the conditioned media was determined by ELISA. Filled circles (●) represent the effect of agonists on AGE-2-induced responses, and open circles (○) represent AGE-3-induced responses. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $P < 0.01$ compared with the value for AGE-2 or AGE-3 alone. When an error bar was within a symbol, the bar was omitted.

dimaprit and 4-MH (Parsons et al., 1977), at concentrations ranging from 0.1 to 100 μM, were determined in the presence of AGE-2 and AGE-3 at 100 μg/ml. Both dimaprit and 4-MH inhibited the expressions of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ in a concentration-dependent manner. The potency and efficacy of two agonists were quite similar to those of histamine in each response. Moreover, we found that an H1 agonist, 2-(2-pyridyl)ethylamine dihydrochloride (Durant et al., 1975), and an H3 agonist, (*R*)-α-methylhistamine dihydrochloride (Arrang et al., 1987), had no effect on the adhesion molecule expression and cytokine production induced by AGE-2 and AGE-3 (data not shown).

Effects of Histamine on the Production of cAMP in Monocytes in the Presence or Absence of AGE-2 and AGE-3. The effects of histamine at 100 μM on the production of intracellular cAMP in monocytes isolated from PBMC in the presence (100 μg/ml) or absence of AGE-2 and AGE-3 were determined (Fig. 5). Histamine induced the production of cAMP in monocytes with a peak 30 min after stimulation. The presence of AGE-2 and AGE-3 did not influence the production of cAMP induced by histamine. The H2 receptor antagonist famotidine at 100 μM inhibited the effect of histamine on the production of cAMP (Fig. 5). In addition, the H2/H4 receptor agonist dimaprit at 100 μM induced the production of cAMP (Fig. 5).

Involvement of cAMP in the Action of Histamine. To investigate the involvement of the cAMP/PKA pathway in the action of histamine, the effects of a PKA inhibitor, H89, at concentrations ranging from 0.1 to 100 μM, on the action of histamine at 100 μM were determined (Fig. 6). In the absence of histamine, the PKA inhibitor had no effect on adhesion molecule expression and cytokine expression. H89 reversed the inhibitory effect of histamine on the expressions of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ in the presence of AGE-2 or AGE-3. As shown in Fig. 7, the effects of a membrane-permeable cAMP analog, dbcAMP, and an adenylate cyclase activator, forskolin, at concentrations ranging from 0.1 to 100 μM, on the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes and the production of IFN-γ in PBMC were examined. Both dbcAMP and forskolin inhibited the AGE-2- and AGE-3-induced adhesion molecule expression and cytokine production in a concentration-dependent manner.

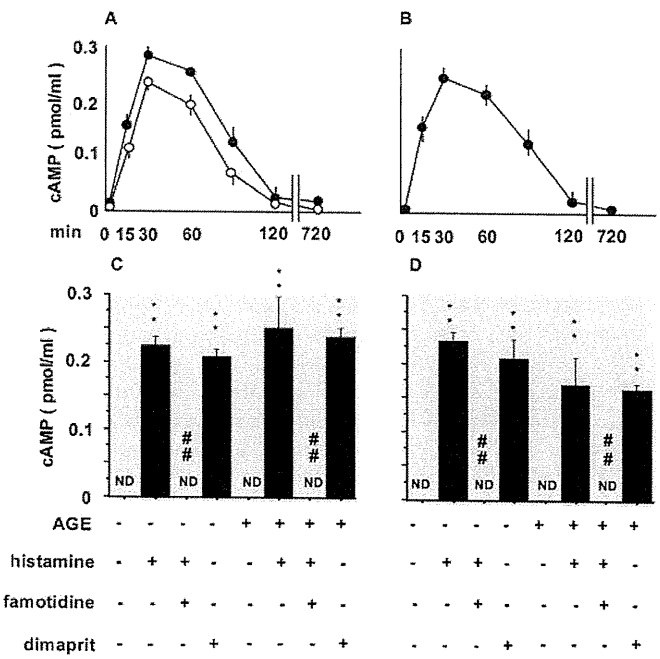

Fig. 5. Effects of histamine on the production of cAMP in monocytes in the presence or absence of AGE-2 and AGE-3. Monocytes at 1×10^6 cells/ml were incubated with histamine at 100 μM in the presence (filled circles; ●) and absence (open circles; ○) of AGE-2 (A) and AGE-3 (B) at 100 μg/ml, and time course changes in the levels of cAMP in monocytes were determined at the indicated time points. C, effects of histamine and dimaprit at 100 μM in combination with famotidine on the production of cAMP were determined in the presence or absence of AGE-2 at 100 μg/ml. D, effects of histamine and dimaprit at 100 μM in combination with famotidine on the production of cAMP were determined in the presence or absence of AGE-3 at 100 μg/ml. **, $P < 0.01$ compared with the corresponding value in the absence of histamine. ##, $P < 0.01$ compared with the corresponding value in the presence of histamine. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. When an error bar was within a symbol, the bar was omitted. ND, not detected.

Effect of Addition of IL-18 on Modulatory Effects of Histamine on AGE-2- and AGE-3-Induced ICAM-1 Expressions on Monocytes. The addition of increasing concentrations of IL-18 to the culture medium at the start of incubation antagonized the inhibitory effect of histamine at

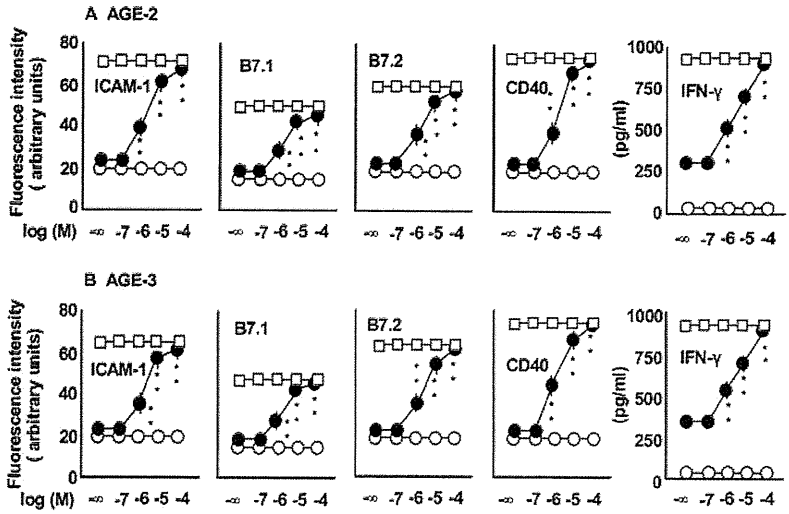

Fig. 6. Effects of PKA inhibitor on the histamine-induced expressions of ICAM-1, B7.1, B7.2, and CD40 and production of IFN-γ. The effects of a PKA inhibitor, H89, at increasing concentrations from 0.1 to 100 μM, on the 100 μM histamine-induced inhibition of expressions of ICAM-1, B7.1, B7.2, and CD40 and production of IFN-γ in the presence of AGE-2 (A) and AGE-3 (B) at 100 μg/ml were determined. Filled circles (●) represent the effect of H89 on the histamine-induced inhibition of responses in the presence of AGE-2 and AGE-3. Open squares (□) represent those in the presence of AGE-2 and AGE-3 without histamine stimulation. Open circles (○) represent the effect of H89 on the responses in the absence of both histamine and AGEs. The results are expressed as the means ± S.E.M. of triplicate findings from five donors. **, $P < 0.01$ compared with the value in the presence of histamine and AGEs. When an error bar was within a symbol, the bar was omitted.

100 μM on AGE-2- and AGE-3-induced ICAM-1 expression (Fig. 8).

Discussion

It is reported that the AGE-BSA enhances dendritic cell-induced stimulation of lymphocyte proliferation and cytokine production (Ge et al., 2005). In contrast, histamine inhibits lymphocyte proliferation and cytokine production via H2 receptors (Nakane et al., 2004). However, little is known about the effect of histamine on the actions of AGE-2 and AGE-3 in human PBMC. In the present study, we clearly demonstrated that histamine inhibited the AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on human monocytes; production of IFN-γ; and lymphocyte proliferation in PBMC (Figs. 1 and 2). The action of histamine was inhibited by the H2 antagonist famotidine but not the H1 antagonist *d*-chlorpheniramine and the H3/4 antagonist thioperamide (Fig. 3). The H2/H4 receptor agonists dimaprit and 4-MH mimicked the action of histamine (Fig. 4). Because the IC_{50} values of histamine and H2/H4 receptor agonists to prevent the up-regulation of adhesion molecule expression and cytokine production were consistent with the affinity of those agonists to typical H2 receptors (Johnson, 1982; Elenkov et al., 1998; Kohka et al., 2000; Takahashi et al., 2002; Morichika et al., 2003), it was concluded that the inhibitory effect of histamine was mediated by the stimulation of H2 receptors but not H1, H3, and H4 receptors.

As shown in Fig. 5, histamine induced the production of cAMP in monocytes via H2 receptor irrespective of the presence of AGE-2 or AGE-3. The findings that the PKA inhibitor H89 inhibited the action of histamine (Fig. 6) and that the cAMP analog dbcAMP and the adenylate cyclase activator forskolin mimicked the effect of histamine (Fig. 7) strongly suggested the involvement of the cAMP/PKA pathway in the action of histamine.

We observed a similar pattern of inhibitory effects of histamine on lipopolysaccharide (LPS)- and IL-18-induced activation of monocytes in human PBMC via H2 receptors (Takahashi et al., 2002; Morichika et al., 2003). IL-18 is reported to induce the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes (Takahashi et al., 2002, 2003). Although AGE-2 and AGE-3 do not induce production of IL-18 in PBMC (Takahashi et al., 2009), histamine induces production of IL-18 via H2 receptor and the cAMP/PKA pathway in monocytes (Takahashi et al., 2006). Thus, there may be a common pathway triggered by LPS, IL-18, and AGEs that

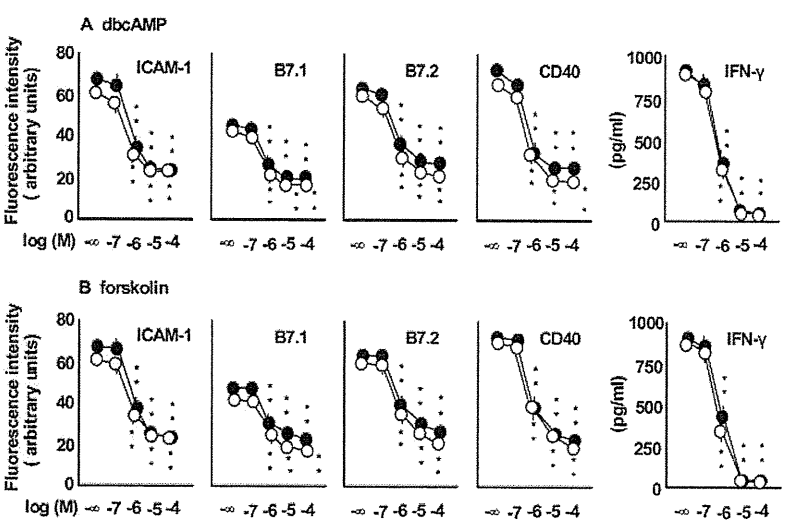

Fig. 7. Effects of forskolin and dbcAMP on AGEs-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes and production of IFN-γ in PBMC. PBMC at 1×10^6/ml were incubated with a cAMP analog, dbcAMP (A), and an adenylate cyclase activator, forskolin (B), at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 at 100 μg/ml for 24 h. Filled circles (●) represent the effects of dbcAMP or forskolin on AGE-2-induced responses, and open circles (○) represent the effects of dbcAMP or forskolin on the AGE-3-induced responses. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $P < 0.01$ compared with the value for AGE-2 or AGE-3 alone. When an error bar was within a symbol, the bar was omitted.

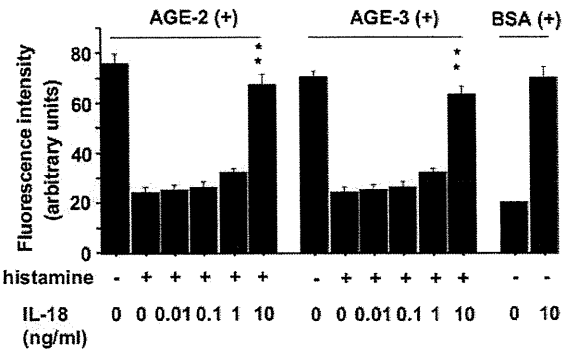

Fig. 8. Effect of IL-18 on the modulatory effects of histamine on AGE-2- and AGE-3-induced ICAM-1 expression on monocytes. PBMC at 1×10^6 cells/ml were incubated with IL-18 at increasing concentrations from 0.01 to 10 ng/ml in the absence or presence of AGE-2 and AGE-3 at 100 μg/ml and histamine at 100 μM for 24 h. At the end of the culture, the expressions of ICAM-1 were determined by flow cytometry. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $P < 0.01$ compared with the value for histamine.

was regulated by the H2 receptors-cAMP/PKA system. Further work is necessary on this issue.

Although AGE-2 and AGE-3 do not induce production of IL-18 in PBMC (Takahashi et al., 2009), histamine induces production of IL-18 via H2 receptor and the cAMP/PKA pathway in monocytes (Takahashi et al., 2006). The amount of IL-18 production induced by histamine at 100 μM was 2.5 ng/ml. IL-18 is reported to induce the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes (Takahashi et al., 2002, 2003). As shown in Fig. 8, the requirement of relatively higher concentration of exogenous IL-18 for reversing the inhibitory effect of histamine on AGE-2- and AGE-3-induced action may reflect the IL-18 concentration needed for the functional antagonism of histamine action on ICAM-1 expression. The regulatory mechanisms for ICAM-1 expression in the presence of plural stimuli should be clarified.

In the previous study, we found that AGE-2 and AGE-3 have higher affinity for RAGE than AGE-4 and AGE-5 by using an in vitro binding assay (Takahashi et al., 2009). AGE-2 and AGE-3, but not AGE-4 and AGE-5, induced the up-regulation of their receptor RAGE expression on the cell surface of monocytes. A NF-κB activation inhibitor, SN50, inhibits AGE-2- and AGE-3-induced adhesion molecule expressions and cytokine production (Takahashi et al., 2009), and the interaction of AGE and RAGE enhances the expression of RAGE through activation of NF-κB in monocytes (Li and Schmidt, 1997). However, histamine had no effect on the expression of RAGE in the presence and absence of AGE-2 and AGE-3 (data not shown). Thus, it might be possible that the downstream pathways of NF-κB activation leading to up-regulation of adhesion molecules and RAGE are probably differentially regulated by the cAMP-PKA system.

Histidine decarboxylase (HDC), which produces histamine from L-histidine, is detected in monocytes/macrophages located in the arterial intima in human atherosclerotic lesions (Higuchi et al., 2001). In a rat model of streptozotocin-induced diabetes mellitus, histamine levels are elevated in various tissues, including the aorta (Gill et al., 1990). Moreover, vascular smooth muscle cells express HDC after injury to endothelial cells (Sasaguri et al., 2005). The resultant production of histamine may regulate vascular contraction directly or indirectly via nitric oxide production (Tanimoto et al., 2007). In addition to the control of vascular contraction, the modulatory effects of histamine on microinflammation in atherosclerotic intima through the regulation of monocytes/macrophages are shown in the present study. In fact, AGEs have been demonstrated in atherosclerotic plaque, where macrophages with up-regulated RAGE are present (Cuccurullo et al., 2006). Therefore, the regulatory action of histamine through the stimulation of H2 receptors may inhibit the macrophage-mediated events stimulated by AGE-2 and AGE-3, including the production of IFN-γ and adhesion molecule-dependent activation of T cells (Takahashi et al., 2009). Thus, locally produced histamine may exert inhibitory influence on the secretory response of macrophages and T-cell activation, leading to the reduction of atherosclerotic plaque formation. Such a possibility should be evaluated by an in vivo atherosclerotic model. It is reported that atherosclerotic lesions are reduced in HDC-knockout mice, compared with wild-type mice (Tanimoto et al., 2006). However, in HDC-knockout mice, the deficiency of granule formation and proteinases expression in mast cells is also observed (Ohtsu et al., 2002). Therefore, attention should be paid to obtaining a straightforward explanation for the phenotype of knockout mice.

It is reported that an H1 receptor antagonist, diphenhydramine, reduces the formation of intimal hyperplasia in a mouse model with endothelial injury; however, an H2 receptor antagonist, cimetidine, is ineffective (Miyazawa et al., 1998). Cimetidine inhibits neither the proliferation nor migration of mouse vascular smooth muscle cells stimulated by platelet-derived growth factor, whereas diphenhydramine significantly inhibited proliferation but did not inhibit migration (Miyazawa et al., 1998). Therefore, stimulation of H1 receptor may induce the proliferation of vascular smooth muscle cells, whereas H2 receptor stimulation may inhibit the activation of monocytes, leading to the prevention of atherosclerosis. Further study of the role of H1 and H2 receptor stimulation should be continued.

In conclusion, histamine inhibited the AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 and production of IFN-γ via H2 receptors and the cAMP/PKA pathway. Through the inhibition of toxic AGE-dependent responses in monocytes, the stimulation of H2 receptors may partially contribute to regulating the development of atherosclerotic plaques in diabetes.

Acknowledgments

We thank Miyuki Shiotani and Yukinari Isomoto for technical assistance.

References

Arrang JM, Garbarg M, Lancelot JC, Lecomte JM, Pollard H, Robba M, Schunack W, and Schwartz JC (1987) Highly potent and selective ligands for histamine H3-receptors. *Nature* **327**:117–123.

Brownlee M (1995) Advanced protein glycosylation in diabetes and aging. *Annu Rev Med* **46**:223–234.

Burke AP, Kolodgie FD, Zieske A, Fowler DR, Weber DK, Varghese PJ, Farb A, and Virmani R (2004) Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. *Arterioscler Thromb Vasc Biol* **24**:1266–1271.

Camacho SA, Heath WR, Carbone FR, Sarvetnick N, LeBon A, Karlsson L, Peterson PA, and Webb SR (2001) A key role for ICAM-1 in generating effector cells mediating inflammatory responses. *Nat Immunol* **2**:523–529.

Cuccurullo C, Iezzi A, Fazia ML, De Cesare D, Di Francesco A, Muraro R, Bei R, Ucchino S, Spigonardo F, Chiarelli F, et al. (2006) Suppression of RAGE as a basis of simvastatin-dependent plaque stabilization in type 2 diabetes. *Arterioscler Thromb Vasc Biol* **26**:2716–2723.

Dohlsten M, Sjogren HO, and Carlsson R (1987) Histamine acts directly on human T cells to inhibit interleukin-2 and interferon-gamma production. *Cell Immunol* **109**:65–70.