Mruthinti et al., 2002). Direct immunochemical evidence for the existence of four distinct AGE structures, AGE-2, AGE-3, AGE-4, and AGE-5, is provided from the analysis of AGEs within modified proteins and peptides (Takeuchi and Yamagishi, 2004). It has been reported that toxic AGE structures, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3), are the main structures of AGEs detectable in the serum of diabetic patients (Takeuchi and Yamagishi, 2004).

Loss of glycemic control induces the vascular complications of diabetes, leading to allograft loss (Miles et al., 1998). Immunosuppressive medications used after transplantation are risk factors for aggravating posttransplant diabetes mellitus (PTDM) among recipients who have insulin resistance before transplantation (Cosio et al., 2002). It is known that many patients develop DM early after transplantation, when exposure to tacrolimus and steroids is highest (Filler et al., 2000; van Hooff et al., 2004). Cyclosporine A (CsA) and tacrolimus are reported to prevent the proliferation but not the transendothelial migration of alloreactive lymphocytes into donor organs (Blaheta et al., 2000). The cause of impaired graft survival in patients with PTDM depends on the use of lower dosages of immunosuppressive agents. The accumulation of AGEs is elevated in recipients with chronic renal dysfunction and cardiovascular disease after renal transplantation (Hartog et al., 2006). Therefore, we suggested that the inhibition of AGE-induced actions should be a target for clinical use in patients with PTDM.

Monocyte-derived costimulatory signals are involved in eliciting maximal T cell growth, differentiation, T cell proliferation, and cytokine production, lowering the concentration of antigen required for stimulation, and promoting more sustained signaling from the T cell receptor. The interaction of ICAM-1, B7.1, B7.2, and CD40 on monocytes with their ligands on T cells produces important costimulatory signals (Dustin and Springer, 1989; Greenfield et al., 1998). Blockade of costimulatory signals has great therapeutic potential for controlling inflammatory and immune responses and prolongs allograft survival in a variety of animal models and human patients (Shimizu et al., 2000; Zhu et al., 2000), suggesting that the regulation of adhesion molecule expression on monocytes might decrease lymphocyte proliferation and cytokine production during MLR (Rizzo et al., 2000; Tamura et al., 2004; Takahashi et al., 2005). 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, production of IFN-γ and TNF-α, and lymphocyte proliferation during human MLR (Ohashi et al., 2010). Together with these results, we suggested that toxic AGE-dependent responses, including the enhancement of adhesion molecule expression on monocytes, might partially facilitate rejection in patients with PTDM.

PGE2, one of the major products of cyclooxygenase (COX)-initiated arachidonic acid metabolite released from monocytes, primes naive human T cells for the enhanced production of anti-inflammatory cytokines and inhibition of proinflammatory cytokines through COX-2 (Coleman et al., 1994; Hempel et al., 1994). There are four subtypes of PGE2 receptors: prostanoid EP1, EP2, EP3, and EP4 (Coleman et al., 1994). Activation of EP2 and EP4 receptors leads to an increase in cAMP levels (Coleman et al., 1994). In a previous study, we found that PGE2 inhibited AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation in PBMCs via EP2/EP4 receptors and the cAMP/PKA pathway (Takahashi et al., 2009a). However, little is known about the effect of PGE2 on the AGE-induced activation of monocytes during MLR. In the present study, we examined the effect of PGE2 on AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR.

Materials and Methods

Reagents. PGE2, AH6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid), AH23848 [(4*Z*)-7-[(rel-1*S*,2*S*,5*R*)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]- 4-heptenoic acid], H-89 [*N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride], and KT5720 [(9*S*,10*S*,12*R*)-2,3,9,10,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg: 3′,2′,1′-kl]pyrrolo[3,4-i][1,6]-benzo-diazocine-10-carboxylic acid hexyl ester] were purchased from Sigma-Aldrich (St. Louis, MO). Protein kinase inhibitor (PKI)(14–22) was purchased from Calbiochem (San Diego, CA). ONO-D1-004 (17*S*-2,5-ethano-6-oxo-17,20-dimethyl prostaglandin E1), ONO-AE1-259-01 (11,15-*O*-dimethyl prostaglandin E2), ONO-AE-248 (16*S*-9-deoxy-9β-chloro-15-deoxy-16-hyfroxy-17,17-trimethylene-19,20-didehydro prostaglandin F2), ONO-AE1-329 [16-(3-methoxymethyl)phenyl-omega-tetranor-3,7-dithia prostaglandin E1], and 11-deoxy-PGE1 were provided by Ono Pharmaceutical Co. Ltd. (Tokyo, Japan). Tacrolimus and CsA were purchased from Astellas Pharma (North Deerfield, IL). AGE-modified bovine serum albumin (BSA) (Sigma-Aldrich) was prepared as described previously (Takahashi et al., 2009b; Ohashi et al., 2010). 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, Inc. (Tokyo, 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).

Culture Conditions during MLR. Normal human PBMCs were obtained from 10 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 PBMCs were prepared (Takahashi et al., 2009b; Ohashi et al., 2010). PBMCs at 1×10^6 cells/ml from an individual volunteer were mixed with cells from an unrelated person (mixed cells), and the final concentration of cells was adjusted to 2.0×10^6 cells/ml. The mixed cells were subsequently suspended in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 20 μg/ml kanamycin, and 100 μg/ml streptomycin and penicillin (Sigma-Aldrich), and they were incubated under various conditions for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. All reagents were added to the media at the start of MLR.

Flow Cytometric Analysis for Adhesion Molecule Expression. Changes in the expression of human leukocyte antigens, ICAM-1, B7.1, B7.2, and CD40, were determined by fluorescein isothiocyanate-conjugated mouse IgG1 monoclonal antibodies against ICAM-1/CD54 (Dako Denmark A/S, Glostrup, Denmark), B7.1 (Immunotech, Marseille, France), B7.2 and CD40 (BD Biosciences Pharmingen, San Diego, CA), and IgG1 isotype-matched control (Sigma-Aldrich) with phycoerythrin-conjugated anti-CD14 antibody (monocyte) (Dako Denmark A/S). PBMCs and mixed cells at 2×10^6 cells/ml were incubated for 48 h. Cultured cells at 5×10^5 cells/ml were prepared for flow cytometric analysis as described previously (Takahashi et al., 2009b; Ohashi et al., 2010) and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA). Data were processed with the CellQuest software program.

ELISAs. PBMCs at 1×10^6 cells/ml were used for analyzing IFN-γ and TNF-α production. After being cultured for 24 h at 37°C in a 5% CO2/air mixture, the cell-free supernatant was assayed for IFN-γ and TNF-α protein by ELISA using the multiple antibodies sandwich principle (R&D Systems, Minneapolis, MN). The detection limit of ELISA for IFN-γ and TNF-α was 10 pg/ml.

Proliferation Assay. The mixed cells were treated under various conditions. Cultures were incubated for 48 h, during which they were pulsed with [^{3}H]thymidine (3.3 Ci/well) for the final 16 h. Cells were then divided into 96-well microplates (200 μl/well), resulting in 1μCi [^{3}H]thymidine per well, and harvested with the Micro-Mate 196 Cell Harvester (PerkinElmer Life and Analytical Sciences, Waltham, MA). Thymidine incorporation was measured with a β-counter (Matrix 9600; PerkinElmer Life and Analytical Sciences).

Statistical Analysis. Statistical significance was evaluated by 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

The Effects of PGE2 on AGE-2 and AGE-3-Induced Expression of ICAM-1, B7.1, B7.2, and CD40 on Monocytes, Production of IFN-γ and TNF-α, and Lymphocyte Proliferation during MLR. In a previous study, to evaluate the binding of AGE subtypes to RAGE, we established an in vitro assay using the immobilized AGE subspecies and the His-tagged soluble form of RAGE (sRAGE) protein (Takahashi et al., 2009b). AGE-2 and AGE-3 showed relatively high affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed moderate affinity for sRAGE. To determine the appropriate incubation time, we examined the kinetics at 0, 4, 16, 24, 48, and 72 h. AGE-2 and AGE-3 at 100 μg/ml significantly induced the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN-γ, and proliferation during MLR at 16 h and thereafter up to 24 and 48 h, whereas AGE-4, AGE-5, and BSA at 100 μg/ml had no effect (Ohashi et al., 2010). Moreover, to determine the appropriate concentration of AGEs, the effects of AGE-2 and AGE-3 at concentrations ranging from 100 ng/ml to 100 μg/ml for 48 h were examined. AGE-2 and AGE-3 at 1, 10, and 100 μg/ml significantly induced the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN-γ, and proliferation during MLR (Ohashi et al., 2010).

As shown in Fig. 1, we established the effect of PGE2 at concentrations ranging from 1 nM to 1 μM on the expression of ICAM-1, B7.1, B7.2, and CD40 and its impact on the production of IFN-γ and TNF-α and lymphocyte proliferation in the presence of AGE-2 and AGE-3 at 100 μg/ml during MLR. PGE2 concentration-dependently inhibited the effect of AGE-2 and AGE-3 on adhesion molecule expression, cytokine production, and lymphocyte proliferation. IC_{50} values for the inhibitory effect of PGE2 on the expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation in the presence of AGE-2 and AGE-3 are shown in Table 1. PGE2 concentration-dependently inhibited basal expression of adhesion molecule, cytokine production, and lymphocyte proliferation in the absence of AGE-2 and AGE-3. Moreover, we found that PGE2 had no effect on adhesion molecule expression, cytokine production, and lymphocyte proliferation in the presence of AGE-4 and AGE-5 (data not shown).

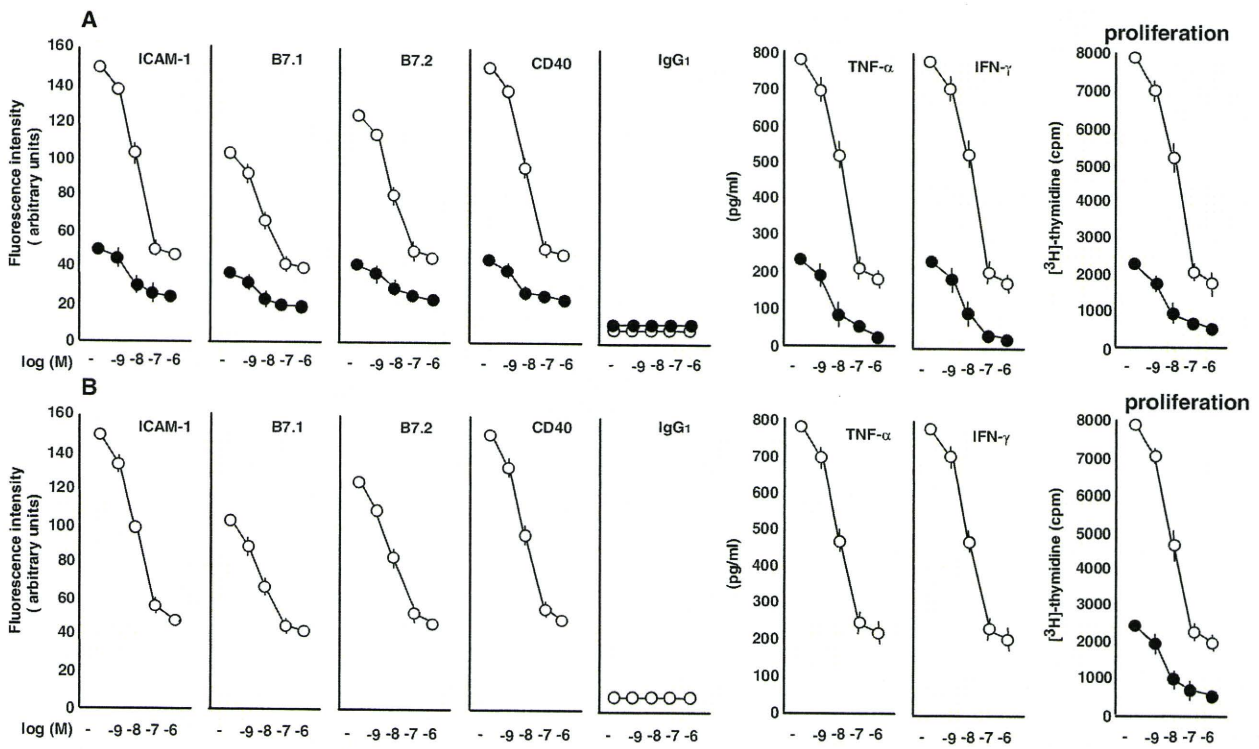

Fig. 1. The effects of PGE2 on AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR. Mixed cells at 2×10^6 cells/ml were incubated with PGE2 at increasing concentrations from 1 nM to 1 μM in the presence or absence of AGE-2 (A) and AGE-3 (B) at 100 μg/ml for 48 h. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. Isotype-matched control represents fluorescein isothiocyanate-conjugated IgG1. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [^{3}H]thymidine uptake as described under *Materials and Methods*. ●, the effect of PGE2 in the absence of AGEs. ○, the effect of PGE2 in the presence of AGEs. 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.

TABLE 1
The IC_{50} values for the inhibitory effect of PGE2 and EP 2/4 receptor agonists in the presence of AGE-2 and AGE-3
The results are expressed as the means ± S.E.M. of five donors with triplicate determinations.

	ICAM-1	B7.1	B7.2	CD40	TNF-α	IFN-γ	Proliferation
				nm			
AGE-2							
PGE2	15 ± 0.5	10 ± 0.5	10 ± 0.4	11 ± 0.5	11 ± 0.4	15 ± 0.6	12 ± 0.8
ONO-AE1-259-01	9 ± 0.3	8 ± 0.2	9 ± 0.3	8 ± 0.4	7 ± 0.2	7 ± 0.3	10 ± 0.4
ONO-AE1-329	10 ± 0.5	9 ± 0.2	10 ± 0.1	9 ± 0.2	7 ± 0.2	8 ± 0.5	7 ± 0.4
AGE-3							
PGE2	12 ± 0.2	9 ± 0.4	10 ± 0.5	10 ± 0.5	9 ± 0.4	10 ± 0.6	9 ± 0.7
ONO-AE1-259-01	8 ± 0.3	9 ± 0.2	8 ± 0.4	6 ± 0.4	7 ± 0.2	8 ± 0.3	8 ± 0.5
ONO-AE1-329	9 ± 0.5	9 ± 0.2	9 ± 0.5	8 ± 0.2	8 ± 0.2	9 ± 0.4	7 ± 0.6

The Involvement of Prostanoid EP2 and EP4 Receptors in the Actions of PGE2. To determine the involvement of PGE2 receptor subtypes in the effects of PGE2 on the expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation, the effects of an EP1 receptor agonist, ONO-D1-004, an EP2 receptor agonist, ONO-AE1-259-01, an EP3 receptor agonist, ONO-AE-248, and an EP4 receptor agonist, ONO-AE1-329 (Suzawa et al., 2000; Noguchi et al., 2001), at concentrations ranging from 1 nM to 1 μM on adhesion molecule expression, cytokine production, and lymphocyte proliferation in the presence of AGE-2 and AGE-3 at 100 μM during MLR were determined (Fig. 2). IC_{50} values for the inhibitory effect of ONO-AE1-259-01 and ONO-AE1-329 on the expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation in the presence of AGE-2 and AGE-3 are shown in Table 1. Apparently the EP2 and EP4 receptor agonists concentration-dependently inhib-

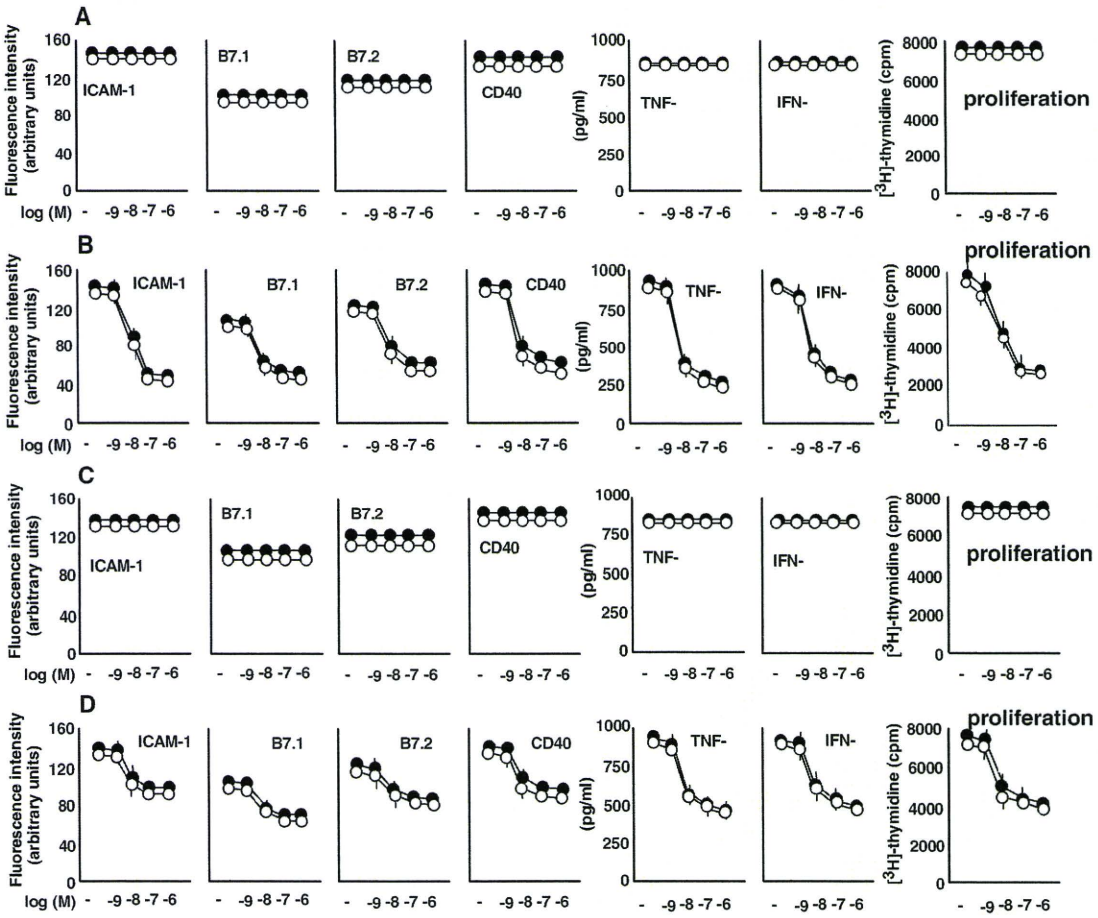

Fig. 2. The effect of prostanoid receptor agonists on AGE2- and AGE3-induced expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR. Mixed cells at 2×10^6 cells/ml were incubated with the EP1 receptor agonist, ONO-D-1-004 (A), the EP2 receptor agonist, ONO-AE1-259-01 (B), the EP3 receptor agonist, ONO-AE-248 (C), and the EP4 receptor agonist, ONO-AE1-329 (D), at increasing concentrations from 1 nM to 1 μM in the presence of AGE-2 (●) and AGE-3 (○) at 100 μg/ml for 48 h. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [^{3}H]thymidine uptake as described under *Materials and Methods*. 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. When an error bar was within a symbol, the bar was omitted.

ited AGE-2- and AGE-3-induced effects on adhesion molecule expression, cytokine production, and lymphocyte proliferation, but EP1 and EP3 receptor agonists had no effect. Moreover, we confirmed that a mixed EP2/EP4 receptor agonist, 11-deoxy-PGE1 (Suzawa et al., 2000; Noguchi et al., 2001), inhibited AGE-2- and AGE-3-induced adhesion molecule expression in a concentration-dependent manner (Fig. 3). In the absence of AGE-2 and AGE-3, EP2 and EP4 receptor agonists concentration-dependently inhibited the basal expression of adhesion molecule, cytokine production, and lymphocyte proliferation, but EP1 and EP3 receptor agonists had no effect (data not shown).

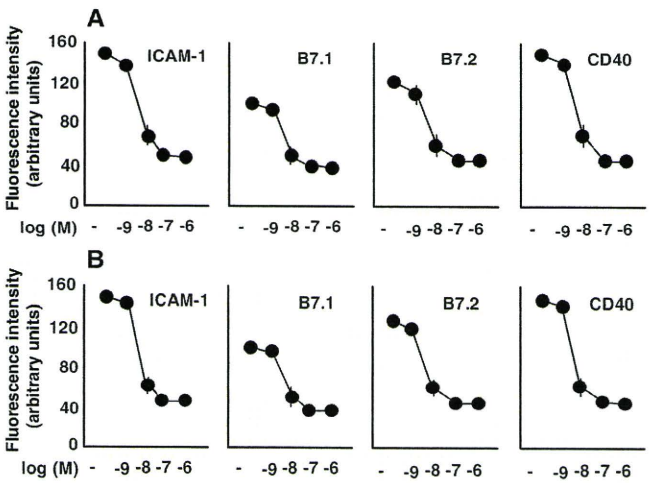

Fig. 3. The effects of 11-deoxy-PGE1 on AGE2- and AGE3-induced ICAM-1, B7.1, B7.2, and CD40 expression on human monocytes during MLR. Mixed cells at 2×10^6 cells/ml were incubated with increasing concentrations of the EP2/EP4 receptor agonist, 11-deoxy-PGE1, at increasing concentrations from 1 nM to 1 μM in the presence of AGE-2 (A) and AGE-3 (B) at 100 μg/ml for 48 h. 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 values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

The effect of an EP2 receptor antagonist, AH6809 (Kay et al., 2009), and an EP4 receptor antagonist, AH23848 (Kay et al., 2009), at concentrations ranging from 0.1 to 100 μM on adhesion molecule expression, cytokine production, and lymphocyte proliferation were examined in the presence of PGE2 at 1 μM (Fig. 4). AH6809 and AH23848 reversed the inhibitory effect of PGE2 on AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation in a concentration-dependent manner. On the other hand, AH6809 and AH23848 had no effect on the actions of AGE-2 and AGE-3 in the absence of PGE2. In the absence of AGE-2 and AGE-3, EP2 and EP4 receptor antagonists reversed the inhibitory effects of PGE2 on the basal expression of adhesion molecule, cytokine production, and lymphocyte proliferation (data not shown).

The Involvement of cAMP in the Actions of PGE2. To investigate the involvement of the cAMP/PKA pathway in the effects of PGE2 on the expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation, the effect of PKA inhibitors, H-89, PKI(14–22), and KT5720, at concentrations ranging from 0.1 to 100 μM on the actions of PGE2 in the presence of AGE-2 and AGE-3 at 100 μg/ml during MLR was determined (Fig. 5). H-89, PKI, and KT5720 reversed the inhibitory effect of PGE2 on AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation. On the other hand, the inhibitors had no effect on the actions of AGE-2 and AGE-3 in the absence of PGE2. In addition, H-89, PKI, and KT5720 reversed the

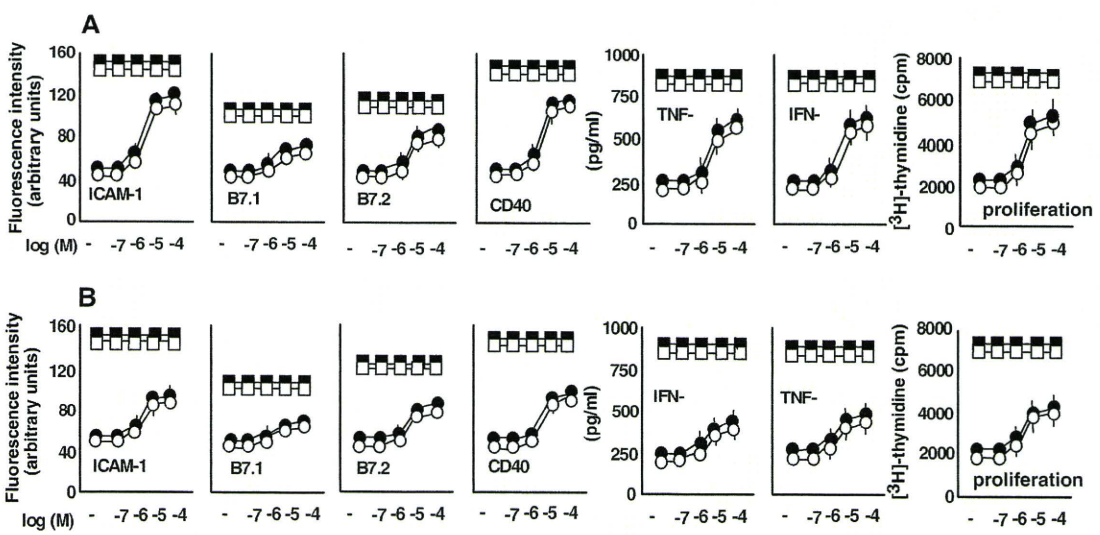

Fig. 4. The effects of prostanoid receptor antagonists on the inhibitory effect of PGE2 on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR. Mixed cells at 2×10^6 cells/ml treated with PGE2 at 1 μM were incubated with the EP2 receptor antagonist, AH6809 (A), and the EP4 receptor antagonist, AH23848 (B), at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 at 100 μg/ml for 48 h. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [^{3}H]thymidine uptake as described under *Materials and Methods*. ●, the effect of antagonists on PGE2-inhibited adhesion molecule expression in the presence of AGE-2; ○, the effect of antagonists on PGE2-inhibited adhesion molecule expression in the presence of AGE-3; ■, the effect of antagonists on the actions of AGE-2 in the absence of PGE2; □, the effect of antagonists on the actions of AGE-3 in the absence of PGE2. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $P < 0.01$ compared with the values for PGE2 in the presence of AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

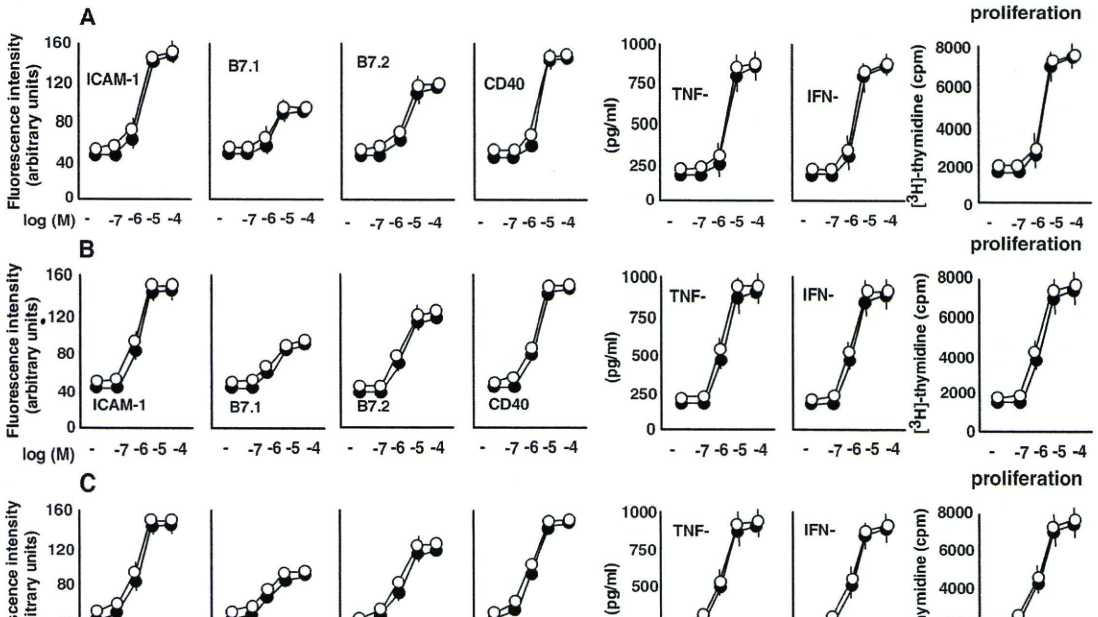

Fig. 5. The effects of PKA inhibitors on PGE2-inhibited ICAM-1, B7.1, B7.2, and CD40 monocyte expression, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR. The effect of PKA inhibitors, H-89 (A), KT5720 (B), and PKI(14–22) (C), at increasing concentrations from 0.1 to 100 μM, on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes treated with PGE2 at 10 nM in the presence of AGE-2 and AGE-3 at 100 μg/ml was determined by flow cytometry. The production of TNF-α and IFN-γ was determined by ELISA. Lymphocyte proliferation was determined by [^{3}H]thymidine uptake as described under *Materials and Methods*. ●, the effects of PKA inhibitors on the PGE2-induced inhibition of responses in the presence of AGE-2; ○, the effects of PKA inhibitors on the PGE2-induced inhibition of responses in the presence of AGE-3. The results are expressed as the means ± S.E.M. of triplicate findings from five donors. **, $P < 0.01$ compared with the value for PGE2 in the presence of AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

inhibitory effects of PGE2 on the basal expression of adhesion molecule, cytokine production, and lymphocyte proliferation in the absence of AGE-2 and AGE-3 (data not shown).

As shown in Fig. 6, 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 expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR in the presence of AGE-2 and AGE-3 at 100 μg/ml were examined. Both dbcAMP and forskolin inhibited AGE-2- and AGE-3-induced adhesion molecule expression, cytokine production, and lymphocyte proliferation

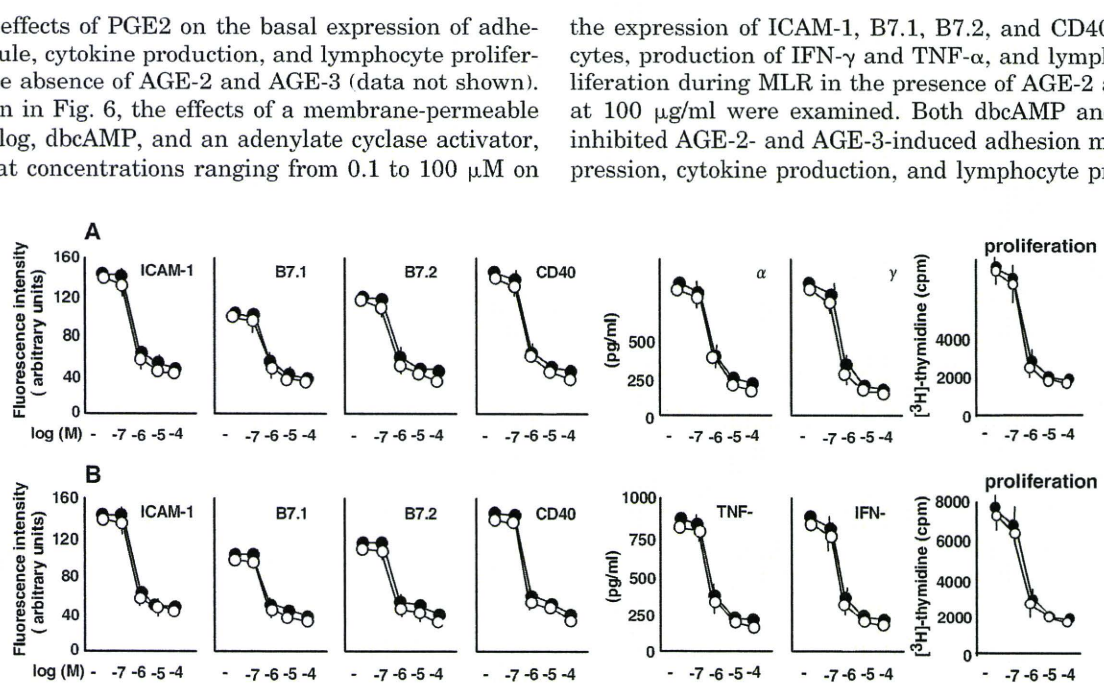

Fig. 6. The effects of forskolin and dbcAMP on AGE-induced ICAM-1, B7.1, B7.2, and CD40 expression on human monocytes, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR. Mixed cells at 2×10^6 cells/ml were incubated with an adenylate cyclase activator, forskolin (A), and a cAMP analog, dbcAMP (B), at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 (●) and AGE-3 (○) at 100 μg/ml for 48 h. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [^{3}H]thymidine uptake as described under *Materials and Methods*. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $P < 0.01$ compared with the values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

in a concentration-dependent manner. Moreover, dbcAMP and forskolin inhibited basal expression of adhesion molecule, cytokine production, and lymphocyte proliferation in the absence of AGE-2 and AGE-3 (data not shown).

The Effects of Tacrolimus and CsA on AGE-2- and AGE-3-Induced Expression of ICAM-1, B7.1, B7.2, and CD40, Production of IFN-γ and TNF-α, and Lymphocyte Proliferation during MLR. As shown in Fig. 7, we examined the effects of tacrolimus and CsA at increasing concentrations ranging from 1 nM to 1 μM on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN-γ and TNF-α, and lymphocyte proliferation in the presence or absence of AGE-2 and AGE-3 at 100 μg/ml during MLR. Tacrolimus and CsA had no effect on adhesion molecule expression in the presence or absence of AGE-2 and AGE-3; however, the calcineurin inhibitors inhibited cytokine production and lymphocyte proliferation.

Discussion

It has been reported that the level of AGE-2 is 17 μg/ml in the serum of a patient with diabetes (Enomoto et al., 2006; Nakamura et al., 2007). AGEs at concentrations ranging from 50 to 200 μg/ml markedly induce human monocyte adhesion to bovine retinal endothelial cells (Mamputu and Renier, 2004). AGEs at 200 μg/ml induce the expression of CD40, CD80, and CD86 and production of IFN-γ in dendritic cells (Ge et al., 2005). In a previous study, we found that AGE-2 and AGE-3 at 10 and 100 μg/ml significantly upregulated the expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR (Ohashi et al., 2010). Therefore, the concentration (100 μg/ml) used in the present study may not be far above the pathological concentration of AGEs in the serum of patients with diabetes reported in other studies (Enomoto et al., 2006; Nakamura et al., 2007).

In the present study, we found, for the first time, that PGE2 inhibited the expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR in the presence or absence of AGE-2 and AGE-3 (Fig. 1). Nataraj et al. (2001) reported that the antiproliferative action of PGE2 on mouse MLR was caused by the stimulation of EP2 and EP4 receptors using EP1- and EP4-deficient splenocytes from knockout mice. It has been reported that the selective EP1, EP2, EP3, and EP4 receptor agonists used in the present study were highly selective for their respective receptors (Suzawa et al., 2000). For example, the EP2 receptor agonist, ONO-AE1-259-01, and the EP4 receptor agonist, ONO-AE1-329, were demonstrated to be highly selective for mouse EP2 and EP4 receptors, respectively, using a receptor binding assay for Chinese hamster ovary cells transfected with each EP's cDNA (Suzawa et al., 2000). The EP2 receptor agonist, ONO-AE1-259, had at least

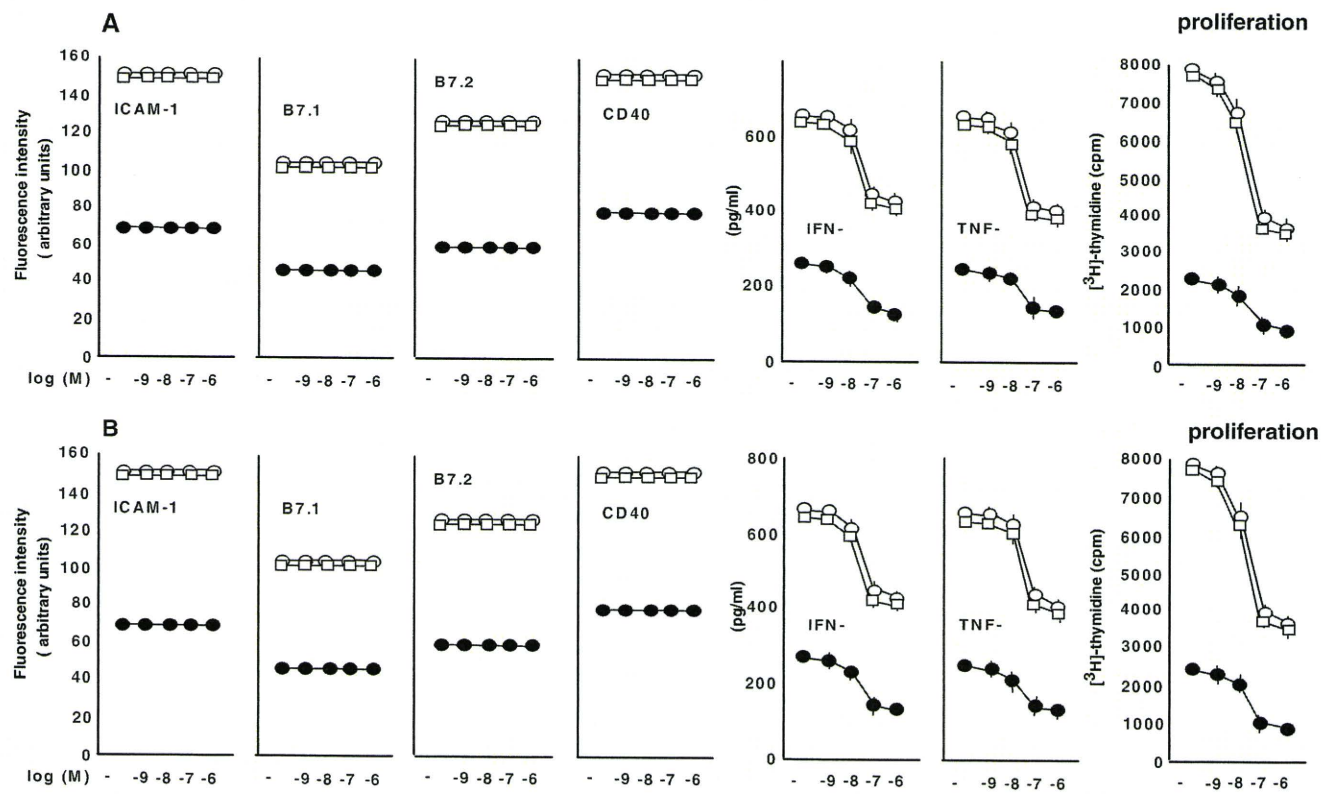

Fig. 7. The effect of tacrolimus and CsA on the expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR. Mixed cells at 2×10^6 cells/ml were incubated with tacrolimus (A) and CsA (B) in the presence or absence of AGE-2 and AGE-3 at 100 μg/ml for 48 h, and the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [^{3}H]thymidine uptake as described under *Materials and Methods*. ○, the effect of tacrolimus and CsA on the actions of AGE-2; □, the effect of tacrolimus and CsA on the actions of AGE-3 during MLR; ●, the effect of tacrolimus and CsA in the absence of AGEs. The results are expressed as the means ± S.E.M. of triplicate findings from five distinct responder/stimulator pairs. **, $P < 0.01$ compared with the value for medium. #, $P < 0.05$ and ##, $P < 0.01$ compared with the value for AGE-2 and AGE-3, respectively. When an error bar was within a symbol, the bar was omitted.

700-fold higher affinity for EP2 receptors compared with other receptor agonists (Suzawa et al., 2000). As shown in Fig. 2, ONO-AE1-259 and ONO-AE-1-329 mimicked the effects of PGE2 on adhesion molecule expression, cytokine production, and lymphocyte proliferation. In the present study, IC_{50} values for the inhibitory effects of ONO-AE1-259 and ONO-AE-1-329 on the expression of ICAM-1 on monocytes induced by AGE-2 and AGE-3, respectively, were similar (Table 1). It is unlikely that either receptor agonist stimulated the other receptors at the concentration range used judging from the selectivity of each agonist. As shown in Fig. 3, the observation that the mixed EP2/EP4 receptor agonist, 11-deoxy-PGE1 (Noguchi et al., 2001), mimicked the inhibitory effects of PGE2 was consistent with the above conclusion. The IC_{50} values of PGE2 to prevent the up-regulation of adhesion molecule expression, cytokine production, and lymphocyte proliferation were consistent with the affinity of those agonists to typical EP2 and EP4 receptors (Morichika et al., 2003; Takahashi et al., 2009a; Table 1). Moreover, the EP2 receptor antagonist, AH6809, and the EP4 receptor antagonist, AH23848, inhibited the actions of PGE2 (Fig. 4). Therefore, it was suggested that the inhibitory effect of PGE2 was mediated by stimulation with EP2 and EP4 receptors but not EP1 and EP3 receptors.

It is known that stimulation with EP2 and EP4 receptors induces the production of cAMP (Coleman et al., 1994). In a previous study, we found that PGE2, EP2, and EP4 receptor agonists induced the production of cAMP in monocytes irrespective of the presence of AGE-2 and AGE-3 (Takahashi et al., 2009a). The PKA inhibitors, H-89, PKI(14–21), and KT5720, inhibited the action of PGE2 (Fig. 5), and the cAMP analog, dbcAMP, and the adenylate cyclase activator, forskolin, mimicked the effect of PGE2 (Fig. 6). These results suggested the involvement of the cAMP/PKA pathway in the actions of PGE2. We observed a similar pattern of inhibitory effects of PGE2 on IL-18-induced activation of monocytes in humans via EP2 and EP4 receptors during MLR (Morichika et al., 2003). Thus, there may be a common pathway triggered by IL-18 and AGEs that was regulated by the EP2/EP4 receptor cAMP/PKA system. Additional work is necessary on this issue.

In a previous study using an in vitro binding assay, we found that AGE-2 and AGE-3 had higher affinity for RAGE than AGE-4 and AGE-5 (Takahashi et al., 2009b). 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 in PBMCs and MLR (Takahashi et al., 2009b; Ohashi et al., 2010). PGE2 had no effect on the expression of RAGE in the presence and absence of AGE-2 and AGE-3 (data not shown), suggesting that there might be distinct signal transduction pathways of RAGE activation, leading to enhanced expression of adhesion molecule and RAGE, which were differentially regulated by the cAMP–PKA system.

In a previous study, we confirmed that AGE-2, AGE-3, AGE-4, and AGE-5 at 100 μg/ml had no effect on the expression of COX-2 mRNA and protein in human monocytes (Takahashi et al., 2009a). In the present study, we examined the effect of a nonselective COX-2 inhibitor, indomethacin, and a selective COX-2 inhibitor, NS398, on the actions of PGE2 during MLR in the presence or absence of AGE-2 and AGE-3. COX-2 inhibitors had no effect on the expression of adhesion molecule, cytokine production, and lymphocyte proliferation (data not shown). In addition, AGE-2, AGE-3, AGE-4, and AGE-5 had no effect on PGE2 production (data not shown). Therefore, it is likely that the endogenous production of PGE2 in monocytes did not occur under the present conditions.

Atherosclerotic disease is documented with higher frequency among patients with PTDM than nondiabetic patients (Sezer et al., 2006), and atherosclerosis plays a role in the development of chronic allograft nephropathy and graft loss (Carvalho and Soares, 2001). It has been reported that PGE2 induced by monocytes inhibits procollagen secretion by human vascular smooth muscle cells, leading to extracellular matrix remodeling and resistance to rupture during atherosclerosis (Fitzsimmons et al., 1999). Elevation of cAMP in endothelial cells inhibits proliferation, leading to the inhibition of atherosclerosis in patients with diabetes (Lorenowicz et al., 2007). Together with previous studies (Fitzsimmons et al., 1999; Lorenowicz et al., 2007; Takahashi et al., 2009a), it is suggested that PGE2 induces intracellular cAMP production upon binding to their cognate G protein-coupled receptors and might regulate the activation of monocytes, vascular smooth muscle cells, and endothelial cells. Therefore, through the inhibition of toxic AGE-dependent responses in monocytes, the stimulation of EP2 and EP4 receptors might partially contribute to regulation of the development of atherosclerotic plaques in patients with PTDM.

It has been reported that PGE2 markedly inhibits the rejection of organ and tissue transplants in a rat cardiac or small intestinal transplantation model (Kamei et al., 1991; Koh et al., 1992). CsA suppresses COX-2 in cultured vascular smooth muscle cells, whereas systemic prostacyclin is not suppressed by CsA and tacrolimus in vivo (Jespersen et al., 2009). However, CsA and tacrolimus are known to inhibit gene transcription directed by cAMP (Siemann et al., 1999). In the present study, we found that CsA and tacrolimus had no effect on the actions of PGE2 during MLR in the presence or absence of AGE-2 and AGE-3 (Fig. 7). In conclusion, PGE2 inhibited AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR via EP2/EP4 receptors and the cAMP/PKA pathway. The present study might lead to an exploration of the therapeutic potential of PGE2 on the rejection response evoked by PTDM.

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RESEARCH PAPER

Histamine inhibits adhesion molecule expression in human monocytes, induced by advanced glycation end products, during the mixed lymphocyte reaction

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Background and purpose: Post-transplant diabetes mellitus is a frequent complication among transplant recipients. Ligation of advanced glycation end products (AGEs) with their receptor on monocytes/macrophages plays important roles in the genesis of diabetic complications. The enhancement of adhesion molecule expression on monocytes/macrophages activates T-cells, reducing allograft survival. Out of four distinct AGE subtypes (AGE-2, AGE-3, AGE-4 and AGE-5), only AGE-2 and AGE-3 induced expression of intercellular adhesion molecules (ICAMs), output of cytokines and proliferation of lymphocytes, during the mixed lymphocyte reaction (MLR). Here we have assessed the role of histamine in the actions of AGEs during the MLR.
Experimental approach: Human peripheral blood cells were used in these experiments. Flow cytometry was used to examine the expression of the ICAM-1, B7.1, B7.2 and CD40. Production of the cytokine interferon-γ, and levels of cAMP were determined by ELISA. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake.
Key results: Histamine concentration dependently inhibited the action of AGE-2 and AGE-3. The actions of histamine were antagonized by an H_2-receptor antagonist, famotidine, and mimicked by H_2/H_4-receptor agonists, dimaprit and 4-methylhistamine. The effects of histamine were reversed by a protein kinase A (PKA) inhibitor, H89, and mimicked by dibutyryl cAMP and an adenylate cyclase activator, forskolin.
Conclusions and implications: Histamine down-regulated AGE-2- and AGE-3-induced expression of adhesion molecules, cytokine production and lymphocyte proliferation via histamine H_2 receptors and the cAMP/PKA pathway.
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Keywords: histamine; advanced glycation end products; adhesion molecule; human mixed lymphocyte reaction; transplantation; diabetes; monocytes; cyclic adenosine monophosphate; H_2 receptor

Abbreviations: AGEs, advanced glycation end products; ICAM, intercellular adhesion molecule; MLR, mixed lymphocyte reaction; PTDM, post-transplant diabetes mellitus

Introduction

Diabetes mellitus is characterized by hyperglycemia, which facilitates the formation of advanced glycation end products (AGEs), both in blood and intracellularly (Vlassara and Palace, 2002; Schiekofer *et al.*, 2003). AGEs are formed by a non-enzymatic reaction between a carbonyl group of reducing sugars and free amino groups from macromolecules such as proteins, lipoproteins and nucleic acids. AGEs accumulate in the plasma and tissues of patients with diabetes, leading to the pathogenesis of many of the complications of diabetes (Brownlee *et al.*, 1988; Cooper, 2004). Tissue deposition of AGEs induces macrophage-mediated injury in diabetic complications that correlate with the severity and duration of hyperglycemia (Tanji *et al.*, 2000; Swamy-Mruthinti *et al.*, 2002). Direct immunochemical evidence for the existence of

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four distinct AGE structures, AGE-2, AGE-3, AGE-4 and AGE-5, is provided from the analysis of AGEs within modified proteins and peptides (Takeuchi and Yamagishi, 2004). Recently, two AGE structures, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3), have been shown to exert diverse biological activities on vascular endothelial cells, vascular smooth muscle cells, mesangial cells, Schwann cells, malignant melanoma cells and cortical neurons (Okamoto *et al.*, 2002; Yamagishi *et al.*, 2002). AGE-2 and AGE-3 are the main forms of AGEs detectable in the serum of diabetic patients (Takeuchi and Yamagishi, 2004). Loss of glycemic control induces vascular complications of diabetes, leading to allograft loss (Sumrani *et al.*, 1991; Miles *et al.*, 1998). Many risk factors for atherosclerosis, including hypertension, hyperlipidemia and hyperglycemia, play important roles in the development of chronic allograft nephropathy and graft loss (Arnalich *et al.*, 2000; Thomas *et al.*, 2001). However, the mechanism of impaired graft survival in patients with post-transplant diabetes mellitus (PTDM) is uncertain.

Monocyte-derived co-stimulatory signals are important in eliciting maximal T-cell growth, differentiation, T-cell proliferation and cytokine production, lowering the concentration of antigen required for stimulation and promoting more sustained signalling from T-cell receptors. The interaction of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes with their ligands on T-cells produces important co-stimulatory signals (Dustin and Springer, 1989; Greenfield *et al.*, 1998). Blockade of these co-stimulatory signals has great therapeutic potential for controlling inflammatory and immune responses, and prolongs allograft survival in a variety of animal models and human patients (Shimizu *et al.*, 2000; Zhu *et al.*, 2000). These results suggested that the down-regulation of adhesion molecule expression on monocytes might decrease lymphocyte proliferation and cytokine production during a mixed lymphocyte reaction (MLR) (Rizzo *et al.*, 2000; Tamura *et al.*, 2004; Takahashi *et al.*, 2005). In an earlier study, we found that AGE-2 and AGE-3, but not AGE-4 and AGE-5, induced the expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of interferon (IFN)-γ and lymphocyte proliferation during human MLR (Ohashi *et al.*, 2010).

Histamine is known to modulate cytotoxic T-cell activity (Khan *et al.*, 1989), NK-cell activity (Hellstrand *et al.*, 1994) and cytokine production in human peripheral mononuclear cells (PBMCs) (Elenkov *et al.*, 1998; van der Pouw Kraan *et al.*, 1998), through the stimulation of H_1, H_2, H_3 and H_4 receptors (Elenkov *et al.*, 1998; van der Pouw Kraan *et al.*, 1998; receptor nomenclature follows Alexander *et al.*, 2009). Immunoregulatory effects of histamine are reported to depend on the stimulation of H_2 receptors (Elenkov *et al.*, 1998; van der Pouw Kraan *et al.*, 1998; Hough, 2001), and such stimulation is coupled with the activation of adenylate cyclase and the cAMP/protein kinase A (PKA) pathway in monocytes (Shayo *et al.*, 1997). We have found histamine to inhibit AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40 and the production of IFN-γ in PBMCs via H_2 receptors and the cAMP/PKA pathway (Wake *et al.*, 2009). However, little is known about the effect of histamine on the AGE-induced activation of monocytes during the MLR, which is frequently used to assess immune reactions in transplantation. In the present study, we examined the effect of histamine on AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and lymphocyte proliferation during MLR, using human cells.

Methods

Culture conditions during the MLR

Normal human PBMCs were obtained from 10 healthy volunteers with consent and institutional review board (IRB) approval (Okayama Univ. IRB No.106). Samples of 20–50 mL peripheral blood were withdrawn from the forearm vein, after which PBMCs were prepared (Ohashi *et al.*, 2010; Takahashi *et al.*, 2009). PBMCs at 1×10^6 cells·mL^{-1} from an individual volunteer were mixed with cells from an unrelated person (mixed cells), and the final concentration was adjusted to 2.0×10^6 cells·mL^{-1}. PBMCs (not mixed cells) and mixed cells were subsequently suspended in RPMI 1640 medium (Nissui, Co. Ltd, Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 20 μg·mL^{-1} kanamycin and 100 μg·mL^{-1} streptomycin and penicillin (Sigma Aldrich, St Louis, MO, USA). Cells were incubated under various conditions for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. All reagents were added to the media at the start of the MLR.

Flow cytometric analysis for adhesion molecule expression

For flow cytometric analysis, fluoresceinisothiocyanate isomer-1 (FITC)-conjugated mouse IgG1 monoclonal antibody (mAb) against ICAM-1/CD54 and phycoerythrin (PE)-conjugated anti-CD3, CD14 and CD19 mAb were purchased from DAKO (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 were from Pharmingen (San Diego, CA, USA), and FITC-conjugated IgG1 isotype-matched control was obtained from Sigma Chemical. Changes in the expression of human leucocyte antigens, ICAM-1, B7.1, B7.2 and CD40, were determined by anti-ICAM-1, anti-B7.1, anti-B7.2 or anti-CD40 Ab with anti-CD14 Ab. In addition, changes in the expression of the receptor for AGEs (RAGE) on human monocytes were examined by multicoloured flow cytometry using a combination of PE-conjugated anti-CD14 (monocyte) mouse IgG (DAKO) and anti-human RAGE rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by FITC-conjugated anti-rabbit IgG goat IgG (DAKO) respectively. Mixed cells at 2×10^6 cells·mL^{-1} were incubated for 48 h. Cultured cells at 5×10^5 cells·mL^{-1} were prepared for flow cytometric analysis as previously described (Ohashi *et al.*, 2010; Takahashi *et al.*, 2009) and analyzed with FACS Calibur (BD Biosciences, San Jose, CA, USA). Data were processed using the CELL QUEST program.

ELISA assays

Mixed cells at 2×10^6 cells·mL^{-1} were used for analyzing IFN-γ production. After culturing for 48 h at 37°C in a 5% CO_2/air mixture, the cell-free supernatant was assayed for IFN-γ protein by ELISA employing the multiple Abs sandwich

principle (R&D Systems, Minneapolis, MN, USA). The detection limit of ELISA for IFN-γ was 10 pg·mL^{-1}.

Cell proliferation assay

Mixed cells were treated under various conditions. Cultures were incubated for 48 h, during which they were pulsed with [^{3}H]-thymidine (3·3 Ci per well) for the final 16 h. Cells were then divided into 96-well microplates, 200 μL per well, resulting in 1 μCi [^{3}H]-thymidine per well, and harvested by a Micro-Mate 196 Cell Harvester (Perkin Elmer Life Science, Inc., Boston, MA, USA). Thymidine incorporation was measured by a beta-counter (Matrix 9600, Perkin Elmer Life Science, Inc.).

Statistical analysis

Statistical significance was evaluated using ANOVA followed by Dunnett's test. A probability value of less than 0.05 was considered significant. The results are expressed as the means ± SEM of triplicate findings from five donors

Materials

Histamine dihydrochloride was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Dimaprit dihydrochloride and 4-methylhistamine dihydrochloride were gifts from Drs WAM Duncan and DJ Durant (The Research Institute, Smith Kline and French Laboratories, Welwyn Garden City, Herts, 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) was prepared as previously described (Ohashi *et al.*, 2010; Takahashi *et al.*, 2009). Briefly, 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 contamination in the AGEs prepared as

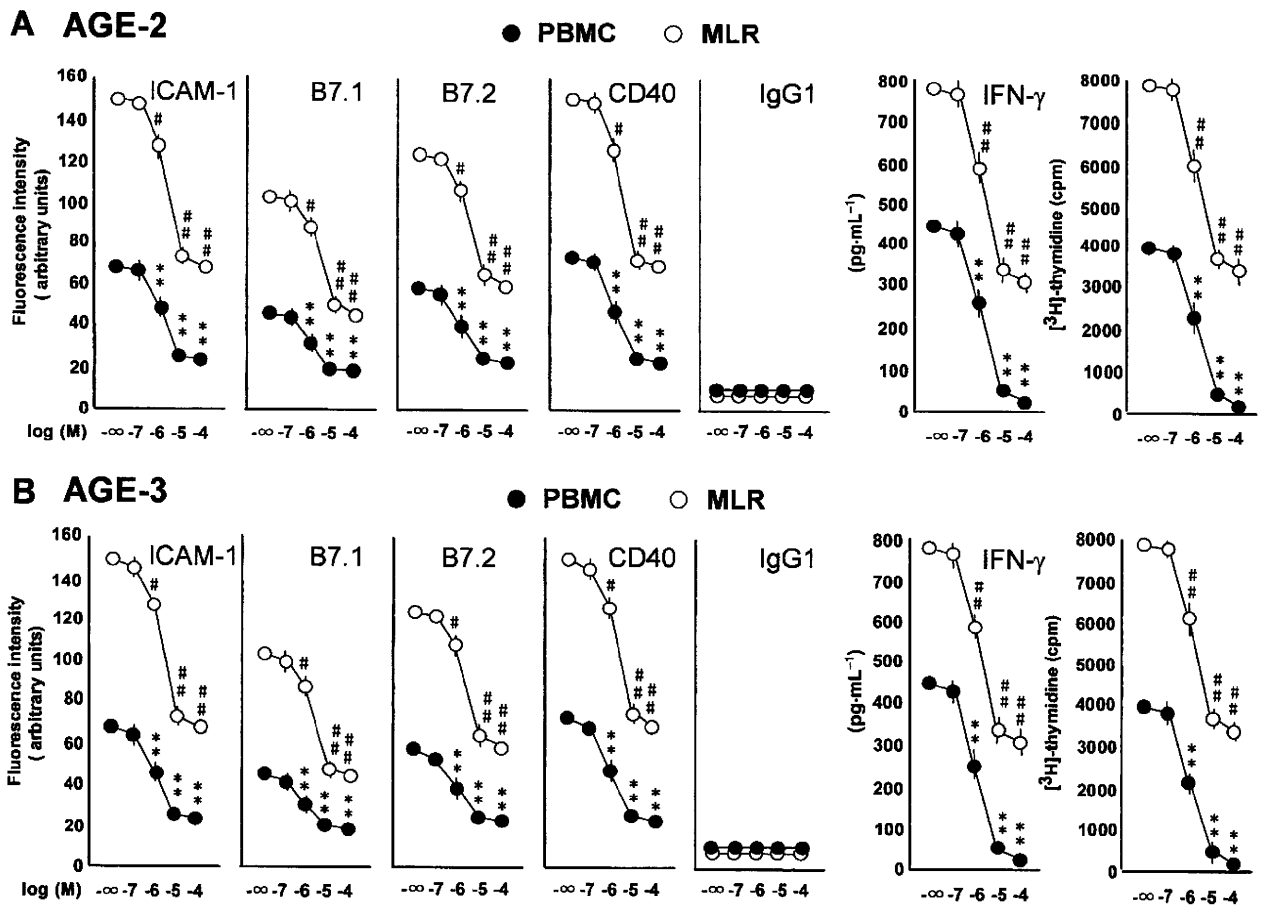

Figure 1 The effects of histamine on advanced glycation end product (AGE)-2- and AGE-3-induced expression of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes, the production of interferon (IFN)-γ and lymphocyte proliferation during the mixed lymphocyte reaction (MLR). Peripheral mononuclear cells (PBMCs) (not mixed cells) and mixed cells at 2×10^6 cells·mL^{-1} were incubated with AGE-2 (A) and AGE-3 (B) (100 μg·mL^{-1}) and histamine (0.1–100 μM) for 48 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. FITC-conjugated IgG_1 was used as an isotype-matched control Ab. IFN-γ concentration in conditioned media was determined by ELISA. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake. The results are expressed as the means ± SEM of five donors with triplicate determinations. $^{**}P < 0.01$ compared with the value for AGE-2 and AGE-3 in PBMCs. $\#P < 0.5$; $\#\#P < 0.01$ compared with the value for AGE-2 and AGE-3 during MLR. When an error bar is within a symbol, the bar is omitted.

described above was 1.2 pg per 100 μg AGE in 1 mL (determined by SRL, Inc., Okayama, Japan). AGE-specific fluorescence was measured at 450 nm after excitation at 390 nm with a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Dibutyryl cAMP (dbcAMP) and forskolin were purchased from Wako Co., Ltd. (Tokyo, Japan). H89 was purchased from Sigma Chemical.

Results

The effects of histamine on AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and lymphocyte proliferation during MLR

In a previous study, to evaluate the binding of AGE subtypes to RAGE, we established an *in vitro* assay using immobilized

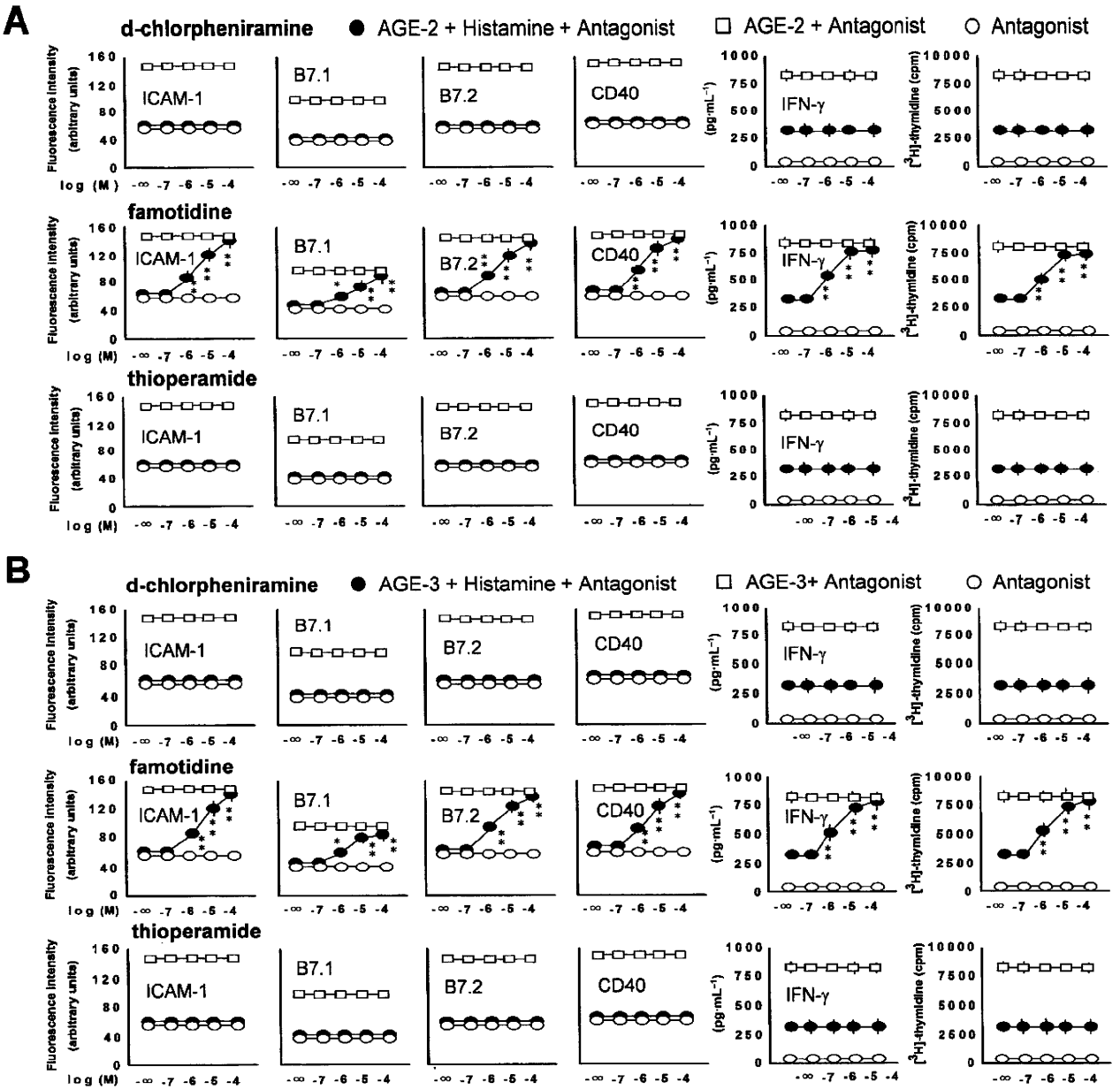

Figure 2 The effects of histamine receptor antagonists on the actions of histamine. Mixed cells at 2×10^6 cells·mL^{-1} were incubated with different classes of histamine receptor antagonists, including *d*-chlorpheniramine (H_1 antagonist), famotidine (H_2 antagonist) and thioperamide (H_3/H_4 antagonist), at increasing concentrations from 0.1 to 100 μM. The antagonists were used alone (antagonist), with advanced glycation end product (AGE)-2 or AGE-3 (100 μg·mL^{-1}; AGE + antagonist) or with AGEs and histamine (100 μM; AGE + Hist + Ant). The expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. Interferon (IFN)-γ concentration in conditioned media was determined by ELISA. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake The results are expressed as the means ± SEM of five donors with triplicate determinations. $^*P < 0.05$; $^{**}P < 0.01$ compared with the value for histamine. When an error bar is within a symbol, the bar is omitted.

AGE subspecies and His-tagged soluble RAGE (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. To determine the appropriate incubation time, we examined the kinetics at 0, 4, 16, 24, 48 and 72 h. AGE-2 and AGE-3 (100 μg·mL^{-1}) induced the expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and the proliferation during MLR at 16 h and thereafter up to 24 and 48 h, whereas AGE-4, AGE-5 and BSA at the same concentration had no effect (Ohashi *et al.*, 2010). Moreover, to determine the appropriate concentration of AGEs, the effects of AGE-2 and AGE-3 at concentrations ranging from 100 ng·mL^{-1} to 100 μg·mL^{-1} for 48 h were examined. AGE-2 and AGE-3 at 1, 10 and 100 μg·mL^{-1} significantly induced the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and proliferation during MLR (Ohashi *et al.*, 2010).

As shown in Figure 1, we observed the effects of histamine (0.1–100 μM) on AGE-enhanced expression of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and lymphocyte proliferation, using 100 μg·mL^{-1} AGE-2 or AGE-3 for 48 h in PBMC and MLR. The expression levels of ICAM-1, B7.1, B7.2 and CD40, production of IFN-γ and lymphocyte proliferation were higher during the MLR than in PBMCs cultured alone. Histamine concentration dependently inhibited the effect of the AGEs on adhesion molecule expression, cytokine production and lymphocyte proliferation in both cell systems. IC_{50} values for the inhibitory effect of histamine in the presence of AGE-2 during MLR were 2, 2, 2, 1.5, 1.5 and 1.5 μM, and those in the presence of AGE-3 were 2, 2, 2, 1.5, 1.5 and 1.5 μM respectively. Incubation with histamine alone had no effect on the variables we measured in PBMC or in the MLR (data not shown).

The involvement of H_2 receptors in the actions of histamine

To determine the histamine receptor subtypes involved in the effects of histamine on these cellular responses to AGE-2 and AGE-3, the effects of an H_1-receptor antagonist, we used a range of histamine receptor antagonists, *d*-chlorpheniramine, an H_2-receptor antagonist, famotidine, and an H_3/H_4-receptor antagonist, thioperamide, at concentrations ranging from 0.1–100 μM. In these experiments, we used a fixed concentration of histamine (100 μM). As shown in Figure 2, among these histamine receptor antagonists, only famotidine inhibited the effects of histamine. Another H_2-receptor antagonist, ranitidine, exerted a substantially similar effect to famotidine (data not shown). As shown in Figure 3, the effects of the H_2/H_4-receptor agonists, dimaprit and 4-methylhistamine (Parsons *et al.*, 1977), at concentrations ranging from 0.1 to

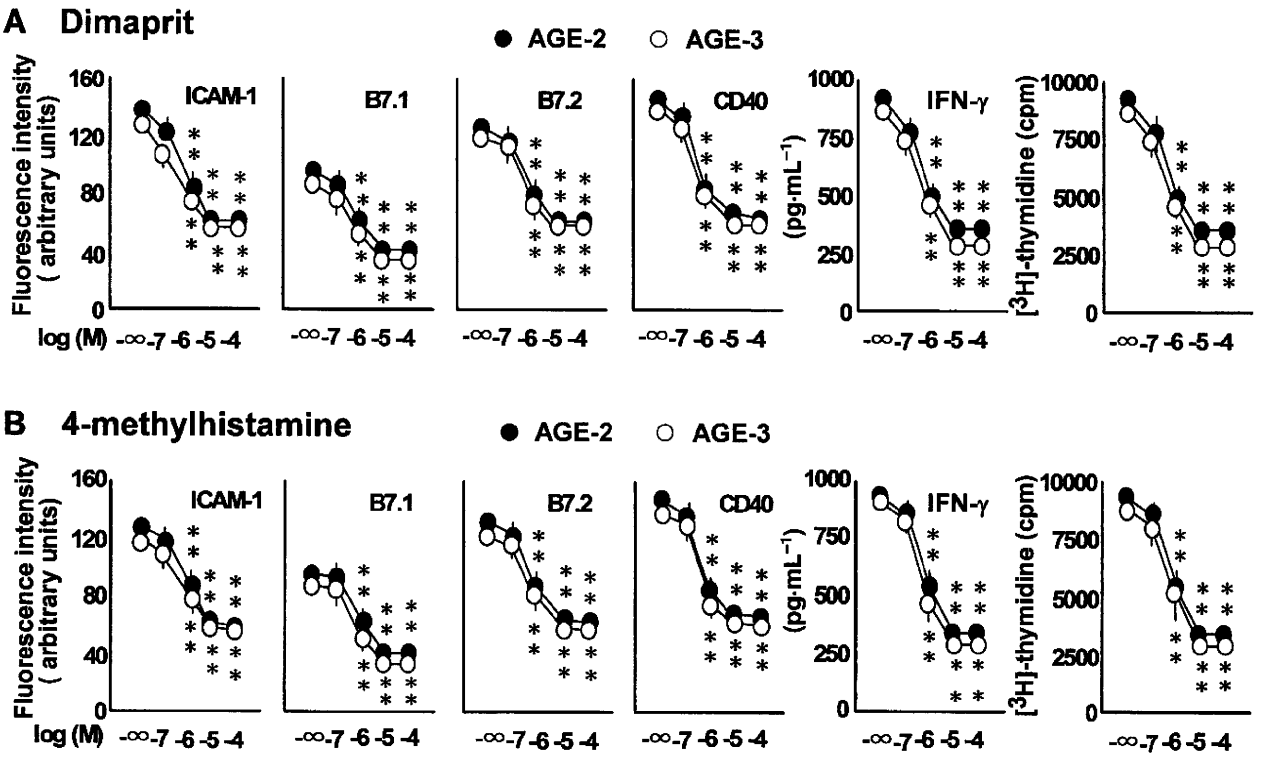

Figure 3 The effects of histamine receptor agonists on the advanced glycation end product (AGE)-2- and AGE-3-induced expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40, the production of interferon (IFN)-γ and lymphocyte proliferation. Mixed cells at 2×10^6 cells·mL^{-1} were incubated with histamine H_2/H_4-receptor agonists, dimaprit (A) and 4-methylhistamine (B) at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 (100 μg·mL^{-1}; 48 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. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake The results are expressed as the means ± SEM of five donors with triplicate determinations. $^{**}P < 0.01$ compared with the value for AGE-2 or AGE-3 alone. When an error bar is within a symbol, the bar is omitted.

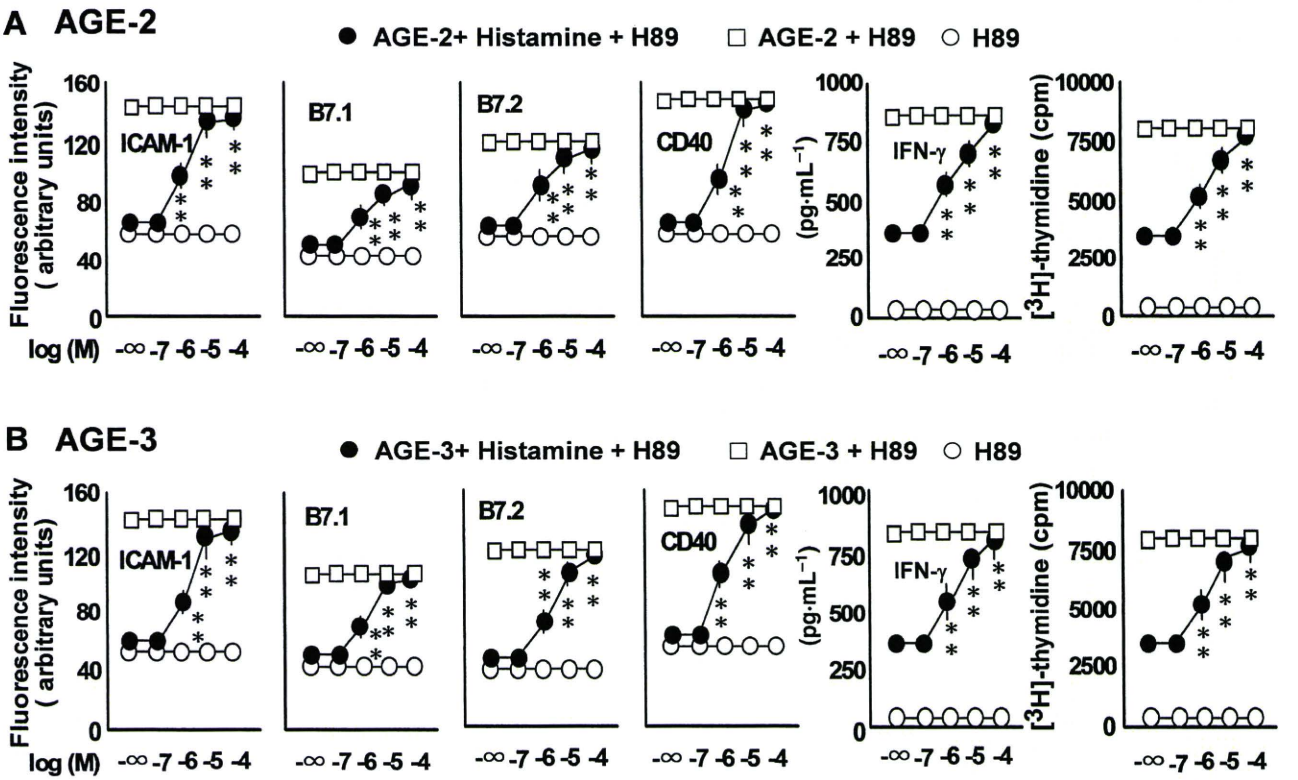

Figure 4 The effects of the protein kinase A (PKA) inhibitor, H89, on the histamine-induced down-regulation of expression of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40, the production of interferon (IFN)-γ and lymphocyte proliferation. Mixed cells at 2×10^6 cells·mL^{-1} were incubated with a PKA inhibitor, H89, (0.1–100 μM) in the presence of advanced glycation end product (AGE)-2 (A) and AGE-3 (B) (100 μg·mL^{-1}) and histamine (100 μM) for 48 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. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake. The results are expressed as the means ± SEM of triplicate findings from five donors. $^{**}P < 0.01$ compared with the value in the presence of histamine and AGEs. When an error bar is within a symbol, the bar is omitted.

100 μM were determined in the presence of AGE-2 or AGE-3 (100 μg·mL^{-1}). Both agonists inhibited the expression of adhesion molecules, the production of IFN-γ and lymphocyte proliferation in a concentration-dependent manner. The potency and efficacy of the two agonists were quite similar to those of histamine in each response. Moreover, we found that an H_1 agonist, 2-(2-pyridyl)ethylamine dihydrochloride (Durant *et al.*, 1975) and an H_3 agonist (*R*)-α-methylhistamine dihydrochloride (Arrang *et al.*, 1987) had no effect on the responses to AGE-2 and AGE-3 in our system (data not shown).

The 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 (0.1–100 μM), on the action of histamine (100 μM) were determined (Figure 4). In the absence of histamine, the PKA inhibitor had no effect on adhesion molecule expression, cytokine production and lymphocyte proliferation, but it did reverse the inhibitory effect of histamine on these responses to AGE-2 or AGE-3. We also assessed the effects of a membrane-permeable cAMP analogue, dbcAMP, and an adenylate cyclase activator, forskolin (0.1–100 μM), on these responses. As shown in Figure 5, both dbcAMP and forskolin inhibited AGE-2- and AGE-3-induced adhesion molecule expression, cytokine production and lymphocyte proliferation in a concentration-dependent manner.

Discussion

The levels of AGE-2 are about 17 μg·mL^{-1} in the serum of diabetic patients (Enomoto *et al.*, 2006; Nakamura *et al.*, 2007). AGEs at concentrations ranging from 50 to 200 μg·mL^{-1} markedly induced human monocyte adhesion to bovine retinal endothelial cells (Mamputu and Renier, 2004). AGEs at 200 μg·mL^{-1} induced the expressions of CD40, CD80 and CD86 and the production of IFN-γ in dendritic cells (Ge *et al.*, 2005). In the previous study, we found that AGE-2 and AGE-3 over a range of concentrations (1–100 μg·mL^{-1}) significantly up-regulated the expression of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and lymphocyte proliferation during MLR (Ohashi *et al.*, 2010). Therefore, the concentration (100 μg·mL^{-1}) used in the present study may not be far above the pathological concentration of AGEs in the serum of diabetic patients previously reported (Enomoto *et al.*, 2006; Nakamura *et al.*, 2007).

In the present study, we clearly demonstrated for the first time that histamine inhibited AGE-2- and AGE-3-induced

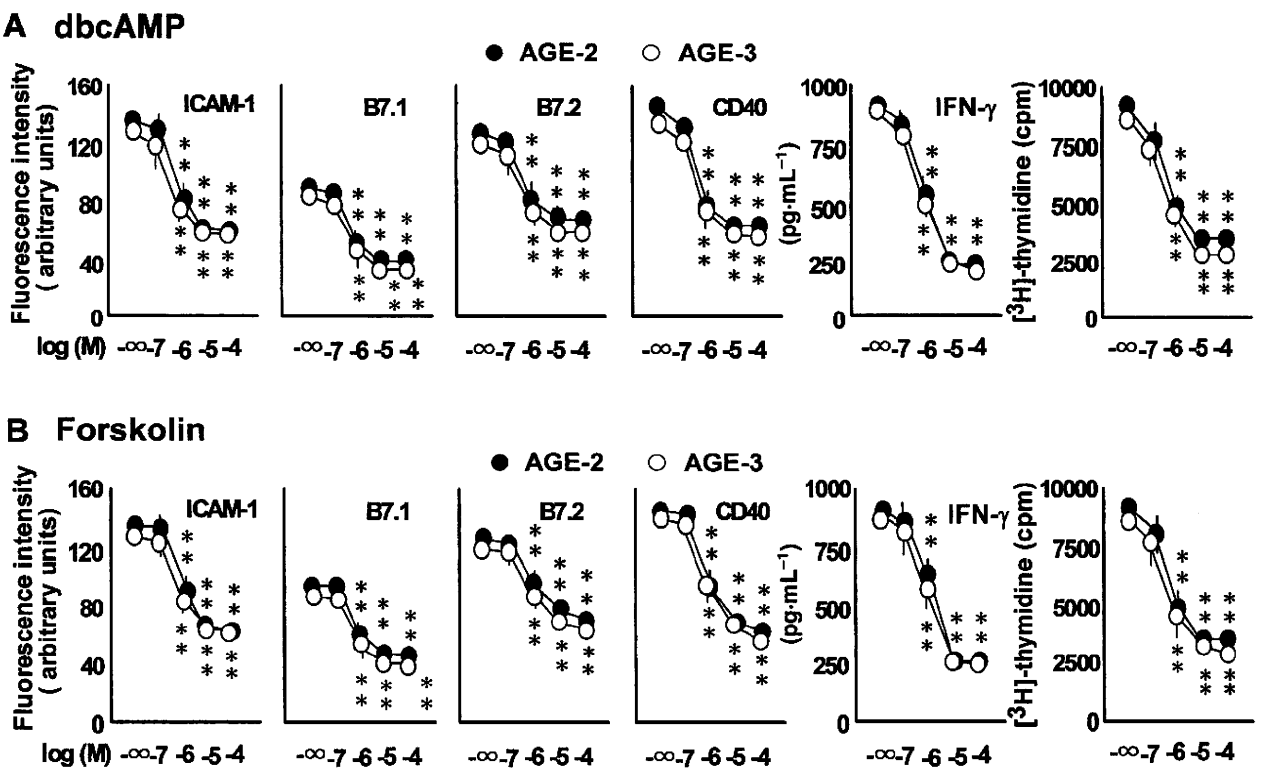

Figure 5 The effects of forskolin and dibutyryl cAMP (dbcAMP) on advanced glycation end product (AGE)-induced expression of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40, the production of interferon (IFN)-γ and lymphocyte proliferation. Mixed cells at 2×10^6 cells·mL^{-1} were incubated with a cAMP analogue, dbcAMP (A), or an adenylate cyclase activator, forskolin (B), at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 (100 μg·mL^{-1}; 48 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. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake The results are expressed as the means ± SEM of five donors with triplicate determinations. $^{**}P < 0.01$ compared with the value for AGE-2 or AGE-3 alone. When an error bar is within a symbol, the bar is omitted.

expressions of ICAM-1, B7.1, B7.2 and CD40 on human monocytes, the production of IFN-γ and lymphocyte proliferation during MLR (Figure 1). The action of histamine was inhibited by an H_2-receptor antagonist, famotidine, but not an H_1-receptor antagonist, *d*-chlorpheniramine, or an H_3/H_4 antagonist, thioperamide (Figure 2). The histamine H_2/H_4-receptor agonists dimaprit and 4-methylhistamine mimicked the action of histamine (Figure 3). As the IC_{50} values of histamine and the H_2/H_4-receptor agonists to prevent the up-regulation of adhesion molecule expression and cytokine production were consistent with the affinity of those agonists to typical histamine H_2 receptors (Elenkov *et al.*, 1998; Itoh *et al.*, 2002; Wake *et al.*, 2009), it was concluded that the inhibitory effect of histamine was mediated by the stimulation of H_2 receptors rather than the H_1, H_3 and H_4 receptors for histamine.

In the previous study, we found that histamine induced the production of cAMP in monocytes via H_2 receptors (Wake *et al.*, 2009). Here we found that the PKA inhibitor, H89, inhibited the action of histamine (Figure 4) and that the cAMP analogue, dbcAMP, and the adenylate cyclase activator, forskolin, mimicked the effect of histamine (Figure 5), suggesting the involvement of the cAMP/PKA pathway in these actions of histamine. We observed a similar pattern of inhibitory effects of histamine on IL-18-induced activation of monocytes during MLR via H_2 receptors (Itoh *et al.*, 2002). Thus, a common pathway triggered by IL-18 and AGEs may be regulated by the H_2-receptor cAMP/PKA system. Further work is necessary on this issue.

Although histamine alone had no effect on basal levels of the adhesion molecules, the production of IFN-γ and lymphocyte proliferation during MLR, that is, in the absence of AGE-2 and AGE-3, dbcAMP and forskolin inhibited these basal responses (data not shown). Moreover, none of the histamine receptor antagonists affected this lack of response to histamine alone, and no histamine receptor agonists altered the basal responses (data not shown). These results indicated that histamine used alone inhibited cAMP-initiated regulation during the MLR, in a histamine receptor-independent manner.

It is known that PTDM is a common side effect of cyclosporin A and tacrolimus (Marchetti, 2005). Many patients develop diabetes mellitus early after transplantation when they are exposed to tacrolimus and steroids (Filler *et al.*, 2000; van Hooff *et al.*, 2004). The cause of impaired graft survival in PTDM patients depends on the use of lower dosages of immunosuppressive agents in diabetic patients. The accumulation of AGEs was elevated in recipients with chronic renal dysfunction and cardiovascular disease after renal transplantation (Hartog *et al.*, 2006). Immunological

rejection and ischaemia was reported as mechanisms of the induction of mast cell infiltration in renal allograft (Shoskes *et al.*, 1990). The relation between mast cells and rejection has been described in the heart (Ly *et al.*, 1992), lung (Yousem, 1997), intestine (Walgenbach *et al.*, 1996), graft-versus-host disease (Nagler *et al.*, 1995) and acute cellular rejection of the human kidney (Lajoie *et al.*, 1996). However, little is known about the effect of histamine and H_2-receptor stimulation on AGE-induced monocyte activation during MLR.

In conclusion, histamine inhibited the AGE-2- and AGE-3-induced expression of the adhesion molecules ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and lymphocyte proliferation, via histamine H_2 receptors and the cAMP/PKA pathway. Through the inhibition of toxic AGE-induced responses in monocytes, the stimulation of histamine H_2 receptors may partially contribute to down-regulating the immune response in patients with PTDM.

Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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RESEARCH PAPER

Ciprofloxacin inhibits advanced glycation end products-induced adhesion molecule expression on human monocytes

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BACKGROUND AND PURPOSE

Advanced glycation end products (AGEs) subtypes, proteins or lipids that become glycated after exposure to sugars, can induce complications in diabetes. Among the various AGE subtypes, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3) are involved in inflammation in diabetic patients; monocytes are activated by these AGEs. Ciprofloxacin (CIP), a fluorinated 4-quinolone, is often used clinically to treat infections associated with diabetis due to its antibacterial properties. It also modulates immune responses in human peripheral blood mononuclear cells (PBMC) therefore we investigated the involvement of AGEs in these effects.

EXPERIMENTAL APPROACH

Expression of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 was examined by flow cytometry. The production of tumour necrosis factor (TNF)-α, interferon (IFN)-γ, prostaglandin E_2 (PGE_2) and cAMP were determined by enzyme-linked immunosorbent assay. Cyclooxygenase (COX)-2 expression was determined by Western blot analysis. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake.

KEY RESULTS

CIP induced PGE_2 production in monocytes, irrespective of the presence of AGE-2 and AGE-3, by enhancing COX-2 expression; this led to an elevation of intracellular cAMP in monocytes. Non-selective and selective COX-2 inhibitors, indomethacin and NS398, inhibited CIP-induced PGE_2 and cAMP production. In addition, CIP inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40 in monocytes, the production of TNF-α and IFN-γ and lymphocyte proliferation in PBMC. Indomethacin, NS398 and a protein kinase A inhibitor, H89, inhibited the actions of CIP.

CONCLUSIONS AND IMPLICATIONS

CIP exerts immunomodulatory activity via PGE_2, implying therapeutic potential of CIP for the treatment of AGE-2- and AGE-3-induced inflammatory responses.

Abbreviations

AGEs, advanced glycation end products; AH6809, 6-isopropoxy-9-oxaxanthene-2-carboxylic acid; AH23848, (4Z)-7-[(rel-1S,2S,5R)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid; BSA, bovine serum albumin; CIP, ciprofloxacin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; H-89, N-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulphonamide dihydrochloride; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; m, monoclonal; PBMC, peripheral blood mononuclear cells; PGE_2, prostaglandins E_2; PKA, protein kinase A; RAGE, receptor for AGEs; TNF, tumour necrosis factor

Introduction

It is known that advanced glycation end products (AGEs) are products of the non-enzymatic glycation 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). The formation and accumulation of AGEs occur at an accelerated rate in diabetic patients and may participate in the pathogenesis of diabetic microvascular and macrovascular complications (Bierhaus *et al.*, 1998; Fukami *et al.*, 2004). Direct immunochemical evidence for the existence of some AGE structures was provided within AGE-modified proteins and peptides (Takeuchi and Yamagishi, 2004). Among the various subtypes of AGE, it has been shown that toxic AGE structures, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3), are the main AGE structures detectable in the serum of diabetic patients. AGE-2 plays a role in the development of atherosclerosis (Takeuchi *et al.*, 2000). The interaction between AGEs and the receptor for AGEs (RAGE) perturbs a variety of vascular homeostatic functions, and thus may contribute to diabetic vasculopathy (Schmidt *et al.*, 1994; Park *et al.*, 1998). AGEs and RAGE are detected in atherosclerotic plaque of diabetic patients (Cuccurullo *et al.*, 2006). A recent study reported that RAGE expression is associated with the apoptotis of smooth muscle cells and macrophages, suggesting that RAGE may promote plaque destabilization (Burke *et al.*, 2004). AGEs up-regulate RAGE expression in various tissues, facilitating the AGE–RAGE response by forming a positive feedback loop (Yamagishi and Imaizumi, 2005). In a previous study, we found that AGE-2 and AGE-3 induced the expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes, the production of tumour necrosis factor (TNF)-α and interferon (IFN)-γ, and lymphocyte proliferation in human peripheral blood mononuclear cells (PBMC), but AGE-4 and AGE-5 had no effect (Takahashi *et al.*, 2009a). The effect of AGE-2 and AGE-3 on the production of IFN-γ and TNF-α was mediated by RAGE and dependent on cell-to-cell interaction via the engagement between ICAM-1, B7.1, B7.2 and CD40 on monocytes and their ligands on T-cells (Wake *et al.*, 2009; Takahashi *et al.*, 2009a).

A major product of cyclooxygenase (COX)-initiated arachidonic acid metabolism, prostaglandin E_2 (PGE_2), which is released from antigen-presenting cells, primes naive human T-cells and enhances production of anti-inflammatory cytokines while inhibiting synthesis of pro-inflammatory cytokines (Coleman *et al.*, 1994). Among the four PGE_2 receptor subtypes E-prostanoid $(EP)_1$, EP_2, EP_3 and EP_4, activation of the EP_2 and EP_4 receptors leads to an increase in cAMP levels and protein kinase A (PKA) activity (Bastien *et al.*, 1994). It has been reported that the enhanced expression of adhesion molecules, including ICAM-1, B7.1, B7.2 and CD40, on monocytes results in the enhanced 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 on T-cells were involved in T-cell activation, inducing the production of IFN-γ and TNF-α in PBMC (Takahashi *et al.*, 2003). In a previous study, we found that PGE_2 inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation via EP_2 and EP_4 receptors (Takahashi *et al.*, 2009b).

Fluoroquinolone antibacterial agents are known to exert their bactericidal activity by inhibiting bacterial type II topoisomerases, a major component of mitotic chromosomes. It has been well documented that this group of agents have the immunomodulatory effects (Riesbeck, 2002). The synthesis of interleukin (IL)-1β and TNF-α by lipopolysaccharide-stimulated human monocytes is significantly inhibited by ciprofloxacin (CIP) (Riesbeck and Forsgren, 1990). Recently, we found that CIP induced the production of PGE_2 in monocytes in a concentration-dependent manner, regardless of the presence of IL-18, by enhancing the expression of COX-2 protein and the elevation of intracellular cAMP in monocytes (Takahashi *et al.*, 2005).

In the present study, we examined the effects of CIP on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation in human PBMC to better understand the immunomodulatory effects of CIP.

Methods

Isolation of PBMC and monocytes

Normal human PBMC were obtained from 10 healthy volunteers after acquiring Institutional Review Board (IRB) approval (Okayama Univ. IRB No.106). Samples of 20–50 mL peripheral blood were withdrawn from a forearm vein; after which, PBMC were prepared and monocytes isolated from PBMC were separated by counterflow centrifugal elution as previously described (Takahashi *et al.*, 2003). PBMC and monocytes were then suspended at a final concentration of

1×10^6 cells·mL^{-1} in the medium as previously described (Takahashi *et al.*, 2003).

Flow cytometric analysis for adhesion molecule expression

Changes in the expressions of human leukocyte antigens, ICAM-1, B7.1, B7.2, CD40 and CD40L, on monocytes were examined by multicolour flow cytometry using a combination of anti-CD14 Ab with anti-ICAM-1, anti-B7.1, anti-B7.2, anti-CD40 or anti-CD40L Ab. PBMC at 1×10^6 cells·mL^{-1} were incubated for 24 h. Cultured cells at 5×10^5 cells·mL^{-1} were prepared for flow cytometric analysis as previously described (Takahashi *et al.*, 2003) and analysed with a FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were processed using the CELL QUEST program.

Enzyme-linked immunosorbent assay

PBMC at 1×10^6 cells·mL^{-1} were used to analyse IFN-γ and TNF-α production, and monocytes at 1×10^6 cells mL^{-1} were used to analyse PGE_2 production. After being cultured for 24 h at 37°C in a 5%CO_2/air mixture, the cell-free supernatant was assayed for IFN-γ, TNF-α (R&D Systems, Minneapolis, MN, USA) and PGE_2 protein (Cayman Chemical, Ann Arbor, MI, USA) by enzyme-linked immunosorbent assay (ELISA) employing the multiple Abs sandwich principle. The detection limits of ELISA for IFN-γ, TNF-α and PGE_2 were 10 pg·mL^{-1}.

Proliferation assay

PBMC were treated under various conditions. Cultures were incubated for 48 h, during which they were pulsed with [^{3}H]-thymidine (3 Ci per well) for the final 16 h. Cells were then divided into 96-well microplates, 200 μL per well, resulting in 1 μCi [^{3}H]-thymidine per well, and harvested by the Micro-Mate 196 Cell Harvester (Perkin Elmer Life Science Inc., Boston, MA, USA). Thymidine incorporation was measured by a beta-counter (Matrix 9600; Perkin Elmer Life Science Inc.).

Western immunoblotting

Monocytes at 1×10^6 cells·mL^{-1} were incubated with CIP in the presence or absence of AGE-2 and AGE-3 at 37°C in a 5% CO_2–air mixture for 30 min. After the incubation, the cells were washed twice in phosphate-buffered saline before the addition of 60 mL ice-cold lysis buffer (HEPES-buffered Hank's balanced salt solution, pH 7.4, 0.5% Triton X-100, 10 mg·mL^{-1} leupeptin, 10 mg·mL^{-1} aprotinin) and 60 μL sample buffer (0.125 M Trizma base, pH 6.8, 20% glycerol, 4% sodium dodecyl sulphate, 10% 2-mercaptoethanol). The samples were then heated at 95°C for 7 min before being stored at 20°C. Sample proteins (50 μL per lane) were separated on 9% acrylamide gel and transferred onto Trans-Blot membranes at 4°C for 16 h at 300 mA; after which, the membranes were blocked for 1 h at 25°C in Tris-buffered saline (25 mM Tris–HCl, 0.2 M NaCl, 0.15% Tween 20, pH 7.6) containing 5% dried milk (wt. vol^{-1}). Next, the membranes were treated with horseradish peroxidase-conjugated rabbit polyclonal Ab against human COX-2 (Cayman Chemical) and β-actin (Sigma Chemical).

Measurement of cAMP production in monocytes

Monocytes at 1×10^6 cells·mL^{-1} were incubated at 37°C in a 5%CO_2/air mixture. After 1 h, cells at 2×10^5 cells 200 μL^{-1} per 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) according to the manufacturer's instructions, for which no acetylation procedures were performed.

Statistical analysis

Statistical significance was evaluated using ANOVA 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 ± SEM of triplicate findings from five donors.

Reagents and drugs

AGE-modified bovine serum albumin (BSA) (Sigma Aldrich, St Louis, MO, USA) was prepared as previously described (Takeuchi *et al.*, 2000; Takahashi *et al.*, 2009a). Briefly, 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). CIP (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid hydrochloride hydraye) was