

back loop (Yamagishi and Imaizumi, 2005). In a previous study, we found that AGE-2 and AGE-3 induced the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation in human PBMCs, 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- $\gamma$  and TNF- $\alpha$  depended 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, and the stimulation of RAGEs on monocytes was involved in the actions of AGE-2 and AGE-3 (Takahashi et al., 2009a; Wake et al., 2009).

Acetylcholine effectively deactivates peripheral macrophages and inhibits the release of proinflammatory mediators. Nicotine activates nAChR belonging to a family of ionotropic receptors consisting of five transmembrane subunits constituting ion channels. Non-neuronal cells such as monocytes and macrophages express nAChR (Wang et al., 2003, 2004). ACh-dependent macrophage deactivation is mediated by  $\alpha 7$ -nAChR, which is expressed in peripheral macrophages and has been described as being essential for the cholinergic anti-inflammatory pathway (Wang et al., 2003, 2004). On the other hand, a major product of COX-initiated arachidonic acid metabolism, PGE<sub>2</sub>, which is released from antigen-presenting cells, primes naive human T cells and enhances their production of anti-inflammatory cytokines while inhibiting their synthesis of pro-inflammatory cytokines (Coleman et al., 1994; Hempel et al., 1994). Among the four PGE<sub>2</sub> receptor subtypes EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub>, and EP<sub>4</sub>, activation of EP<sub>2</sub> and EP<sub>4</sub> receptors leads to an increase in cAMP levels and PKA activity (Bastien et al., 1994; Nataraj et al., 2001). In a previous study, we found that PGE<sub>2</sub> inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.2, and CD40, the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation via the EP<sub>2</sub> and EP<sub>4</sub> receptors (Takahashi et al., 2009b). The cAMP/PKA pathway was involved in the actions of PGE<sub>2</sub>. Nicotine is reported to induce the expressions of COX-2 and PGE<sub>2</sub> in whole blood and microglia through  $\alpha 7$ -nAChR stimulation (Saareks et al., 1998; De Simone et al., 2005).

In the present study, we examined the effects of nicotine on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.2, and CD40, the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation in human PBMCs; we also investigated the involvement of PGE<sub>2</sub> production in mediating these effects.

## Materials and Methods

**Reagents and Drugs.** 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  $\mu$ g/ml described above was measured at SRL Co. (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). Nicotine (1-methyl-2-(3-pyridyl)pyrrolidine),  $\alpha$ -bungarotoxin, mecamlamine, H-89, AH6809, and AH23848 (Kay et al., 2006) were purchased from Sigma-Aldrich. NS398 and indomethacin were from Cayman Chemical (Ann Arbor, MI). For flow cytometric analysis, FITC-conjugated mouse

IgG1 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 from BD Pharmingen (San Diego, CA), and FITC-conjugated IgG1, an isotype-matched control, was obtained from Sigma-Aldrich.

**Isolation of PBMCs and Monocytes.** Normal human PBMCs were obtained from 10 healthy volunteers after acquiring institutional review board approval (Okayama University IRB 106). Samples of 20 to 50 ml of peripheral blood were withdrawn from a forearm vein, after which PBMCs were prepared, and monocytes isolated from PBMCs were separated by counterflow centrifugal elutriation as described previously (Takahashi et al., 2003). The PBMCs and monocytes were then suspended at a final concentration of  $1 \times 10^6$  cells/ml in the medium as described previously (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 multicolor flow cytometry by use of a combination of anti-CD14 Ab with anti-ICAM-1, anti-B7.1, anti-B7.2, anti-CD40, or anti-CD40L Ab. PBMCs at  $1 \times 10^6$  cells/ml were incubated for 24 h. Cultured cells at  $5 \times 10^5$  cells/ml were prepared for flow cytometric analysis as described previously (Takahashi et al., 2003) and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA). The data were processed by use of the CellQuest program.

**ELISA Assays.** PBMCs at  $1 \times 10^6$  cells/ml were used to analyze IFN- $\gamma$  and TNF- $\alpha$  production, and monocytes at  $1 \times 10^6$  cells/ml were used to analyze PGE<sub>2</sub> production. After culturing for 24 h at 37°C in a 5% CO<sub>2</sub>/air mixture, cell-free supernatants were assayed for IFN- $\gamma$ , TNF- $\alpha$  (R&D Systems, Minneapolis, MN) and PGE<sub>2</sub> protein (Cayman Chemical) by ELISA using the multiple Abs sandwich principle. The detection limits of ELISA for IFN- $\gamma$ , TNF- $\alpha$ , and PGE<sub>2</sub> were 10 pg/ml.

**Proliferation Assay.** PBMCs were divided into 96-well microplates, 200  $\mu$ l/well, and were incubated with various conditions for 48 h, during which they were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine per well for the final 16 h. Then, the cells were harvested by the Micro-Mate 196 Cell Harvester (PerkinElmer Life and Analytical Sciences, Waltham, MA). Thymidine incorporation was measured by a  $\beta$ -counter (Matrix 9600; PerkinElmer Life and Analytical Sciences).

**Western Immunoblotting.** Monocytes at  $1 \times 10^6$  cells/ml were incubated with nicotine in the presence or absence of AGE-2 and AGE-3 at 37°C in a 5% CO<sub>2</sub>-air mixture for 30 min. After incubation, the cells were washed twice in phosphate-buffered saline before the addition of 60  $\mu$ l of ice-cold lysis buffer (HEPES-buffered Hanks' balanced salt solution, pH 7.4, 0.5% Triton X-100, 10 mg/ml leupeptin, 10 mg/ml aprotinin) and 60  $\mu$ l of sample buffer (0.125 M Trizma base, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol). The samples were then heated at 95°C for 7 min before being stored at 20°C. Sample proteins (50 ml/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). Next, the membranes were treated with horseradish peroxidase-conjugated rabbit polyclonal Ab against human COX-2 (Cayman Chemical) and  $\beta$ -actin (Sigma-Aldrich).

**Measurement of cAMP Production in Monocytes.** Monocytes at  $1 \times 10^6$  cells/ml were incubated at 37°C in a 5% CO<sub>2</sub>/air mixture. After 30 min, cells at  $2 \times 10^5$  cells/200  $\mu$ l/well were supplemented with trichloroacetic acid to a final concentration of 5% and 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase at 100  $\mu$ M and frozen at -80°C. Frozen samples were subsequently sonicated and assayed for cAMP by use of a cAMP enzyme immunoassay kit (Cayman Chemical) according to the manufacturer's instructions, for which no acetylation procedures were performed.

**Statistical Analysis.** Statistical significances were 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  $\pm$  S.E.M. of triplicate findings from five donors.

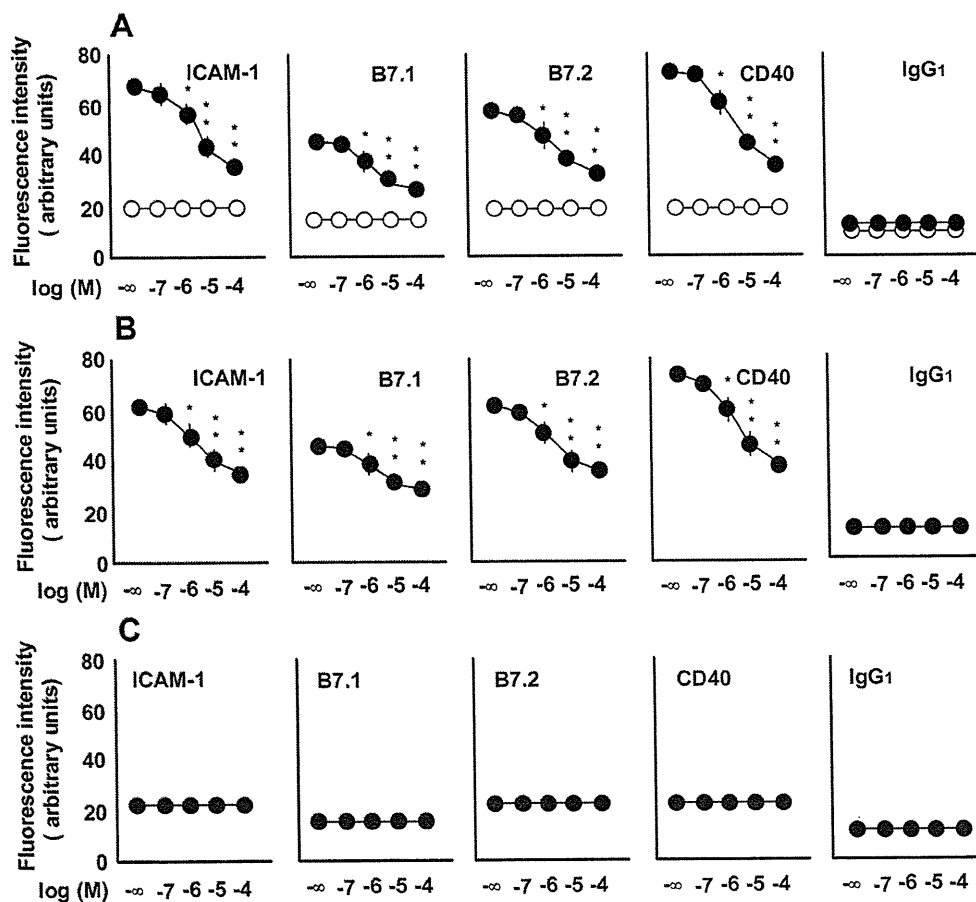
## Results

**Effects of Nicotine on AGE-2- and AGE-3-Induced Expression of ICAM-1, B7.1, B7.2, and CD40 on Monocytes, the Production of IFN- $\gamma$  and TNF- $\alpha$  in PBMCs, and the Proliferation in PBMCs.** In the previous study, to evaluate the binding of AGE subtypes to RAGE, we established an in vitro assay using immobilized AGE subspecies and the His-tagged soluble form of RAGE (sRAGE) protein (Takahashi et al., 2009a). AGE-2 and AGE-3 showed relatively high-affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed moderate affinity for sRAGE. The appropriate incubation time and concentration of AGEs were determined according to the studies reported (Takahashi et al., 2009a; Wake et al., 2009). To determine appropriate incubation time, we examined the kinetics at 0, 4, 16, 24, and 48 h. In the absence of AGEs, the expression of ICAM-1, B7.1, B7.2, and CD40 moderately increased at 16 h and thereafter up to 24 and 48 h. AGE-2 and AGE-3 at 100  $\mu$ g/ml significantly induced the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN- $\gamma$  and TNF- $\alpha$  in PBMCs, and the proliferation in PBMCs at 16 h and thereafter up to 24 and 48 h, whereas AGE-4, AGE-5, and BSA at 100  $\mu$ g/ml had no effect at all (Takahashi et al., 2009; Wake et al., 2009). Moreover, to determine the appropriate concentration of AGEs, the effects of AGE-2 and AGE-3 at concentrations

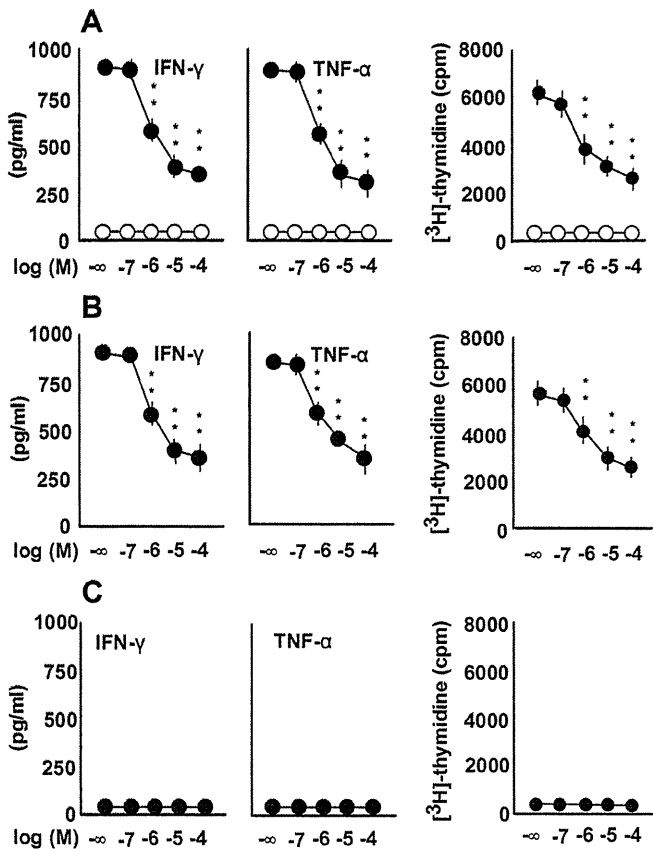
ranging from 100 ng/ml to 100  $\mu$ g/ml for 24 h were examined. AGE-2 and AGE-3 at 10 and 100  $\mu$ g/ml significantly induced the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN- $\gamma$  and TNF- $\alpha$  in PBMCs, and the proliferation in PBMCs (Takahashi et al., 2009a; Wake et al., 2009).

As shown in Figs. 1 and 2, we established the effect of nicotine at concentrations ranging from 0.1 to 100  $\mu$ M on 100  $\mu$ g/ml AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN- $\gamma$  and TNF- $\alpha$  and the lymphocyte proliferation in PBMCs. Nicotine concentration-dependently inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 (Fig. 1, A and B), the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation (Fig. 2, A and B). IC<sub>50</sub> values for the inhibitory effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40, the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation in the presence of AGE-2 were 2, 1, 1, 2, 0.9, 1, and 0.9  $\mu$ M, and those in the presence of AGE-3 were 1, 1, 2, 1, 0.9, 1, and 1  $\mu$ M, respectively. In the presence of BSA at 100  $\mu$ g/ml, nicotine had no effect on the expressions of adhesion molecule (Fig. 1C), cytokine production, and lymphocyte proliferation (Fig. 2C).

**Effects of  $\alpha$ 7-nAChR Antagonists on the Actions of Nicotine.** To determine the involvement of  $\alpha$ 7-nAChR in nicotine activity, we examined the effect of a nonselective  $\alpha$ 7-nAChR antagonist, mecamylamine, and a selective  $\alpha$ 7-nAChR antagonist,  $\alpha$ -bungarotoxin, ranging from 0.1 to 100  $\mu$ M on 100  $\mu$ M nicotine-induced inhibition of ICAM-1, B7.1,



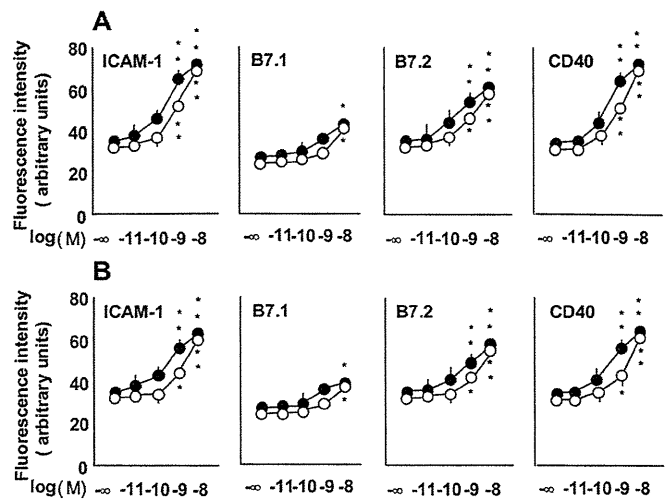
**Fig. 1.** The effects of nicotine on AGE-2-, AGE-3-, and BSA-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes. PBMCs at  $1 \times 10^6$  cells/ml were incubated with AGE-2 (A), AGE-3 (B), and BSA (C) at 100  $\mu$ g/ml and nicotine at increasing concentrations from 0.1 to 100  $\mu$ M 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. Filled circles represent the effect of nicotine on the adhesion molecule expression and cytokine production in the presence of AGE-2 and AGE-3. Open circles represent the effect of nicotine in the absence of AGE-2 and AGE-3. The results are expressed as the means  $\pm$  S.E.M. of five donors with triplicate determinations. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with the value for AGE-2 and AGE-3. When an error bar is within a symbol, the bar was omitted.



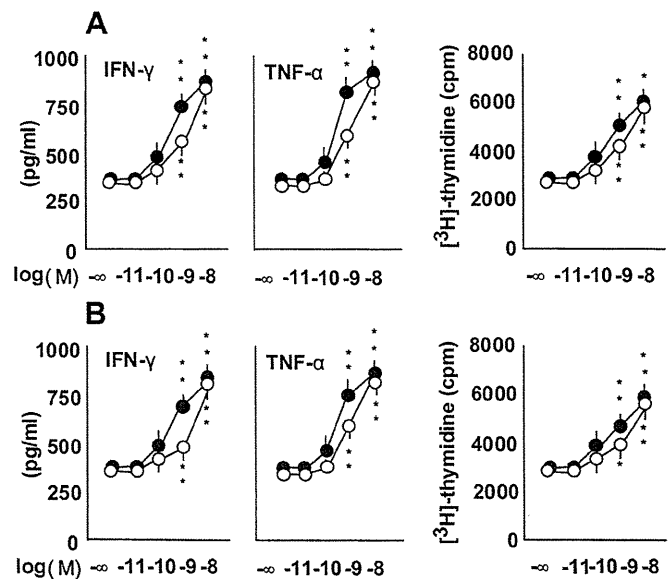
**Fig. 2.** The effects of nicotine on AGE-2-, AGE-3-, and BSA-induced production of IFN- $\gamma$  and TNF- $\alpha$  and the lymphocyte proliferation in PBMCs. PBMCs at  $1 \times 10^6$  cells/ml were incubated with AGE-2 (A), AGE-3 (B), and BSA (C) at 100  $\mu$ g/ml and nicotine at increasing concentrations from 0.1 to 100  $\mu$ M for 24 h. IFN- $\gamma$  and TNF- $\alpha$  concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [ $^3$ H]thymidine uptake as described under *Materials and Methods*. A–C, filled circles represent the effect of nicotine on adhesion molecule expression and cytokine production in the presence of AGE-2 and AGE-3. A, open circles represent the effect of nicotine in the absence of AGE-2 and AGE-3. The results are expressed as the means  $\pm$  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 is within a symbol, the bar was omitted.

B7.2, and CD40 expressions on monocytes (Fig. 3), the production of TNF- $\alpha$  and IFN- $\gamma$ , and the lymphocyte proliferation in PBMCs (Fig. 4) in the presence or absence of AGE-2 and AGE-3 at 100  $\mu$ g/ml. The  $\alpha 7$ -nAChR antagonists reversed the inhibitory effects of nicotine. Mecamylamine and  $\alpha$ -bungarotoxin had no effect in the absence of nicotine (data not shown).

**Effects of Nicotine on Expression of COX-2 and Production of PGE<sub>2</sub> in Monocytes.** As shown in Fig. 5A, nicotine at 100  $\mu$ M induced the expression of COX-2 in monocytes in the presence or absence of AGE-2 and AGE-3 at 100  $\mu$ g/ml, but AGE-2 or AGE-3 alone had no effect. As shown in Fig. 5, B and C, nicotine induced PGE<sub>2</sub> production in monocytes in the presence or absence of AGE-2 and AGE-3, but AGE-2 or AGE-3 alone had no effect. Mecamylamine (Fig. 5D) and  $\alpha$ -bungarotoxin (Fig. 5E) prevented nicotine-stimulated PGE<sub>2</sub> production in the presence or absence of AGE-2 and AGE-3. Without nicotine,  $\alpha 7$ -nAChR antagonist inhibitors had no effect on PGE<sub>2</sub> production (data not shown). A nonselective or a selective COX-2 inhibitor, indomethacin

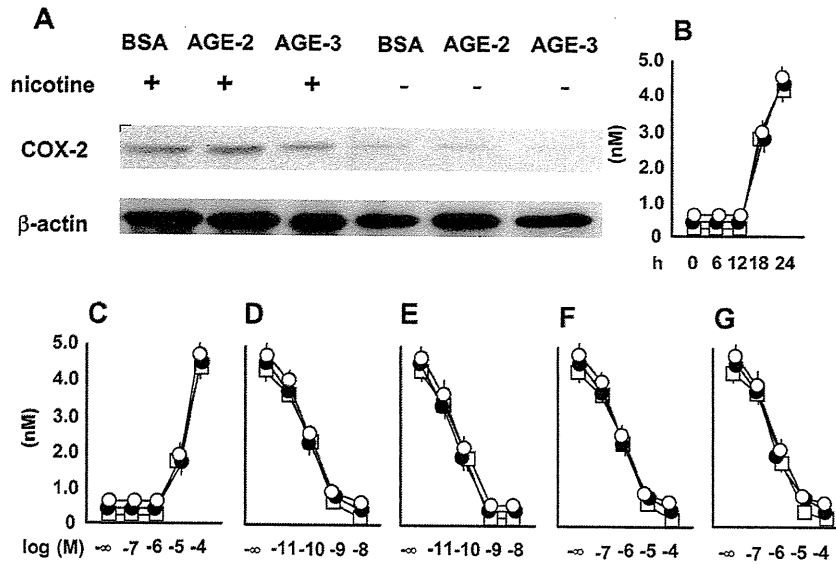


**Fig. 3.** The effect of  $\alpha 7$ -nAChR antagonists on the effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40. The effect of a nonselective  $\alpha 7$ -nAChR antagonist, mecamylamine ( $\bullet$ ), or a selective  $\alpha 7$ -nAChR antagonist,  $\alpha$ -bungarotoxin ( $\circ$ ), at increasing concentrations ranging from 0.01 to 10 nM on the actions of nicotine at 100  $\mu$ M in the presence of AGE-2 (A) and AGE-3 (B) at 100  $\mu$ g/ml. The expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes were determined by flow cytometry. The results are expressed as the means  $\pm$  S.E.M. of triplicate findings from five donors. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with the value for AGE-2 and AGE-3. When an error bar is within a symbol, the bar was omitted.



**Fig. 4.** The effect of  $\alpha 7$ -nAChR antagonists on the effect of nicotine on the production of IFN- $\gamma$  and TNF- $\alpha$  and the lymphocyte proliferation. The effect of a nonselective  $\alpha 7$ -nAChR antagonist, mecamylamine ( $\bullet$ ), or a selective  $\alpha 7$ -nAChR antagonist,  $\alpha$ -bungarotoxin ( $\circ$ ), at increasing concentrations ranging from 0.01 to 10 nM on the actions of nicotine at 100  $\mu$ M in the presence of AGE-2 (A) and AGE-3 (B) at 100  $\mu$ g/ml. IFN- $\gamma$  and TNF- $\alpha$  concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [ $^3$ H]thymidine uptake as described in *Materials and Methods*. The results are expressed as the means  $\pm$  S.E.M. of triplicate findings from five donors. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with the value for AGE-2 and AGE-3. When an error bar is within a symbol, the bar was omitted.

(Fig. 5F) or NS398 (Fig. 5G), inhibited nicotine-induced PGE<sub>2</sub> production in the presence or absence of AGE-2 and AGE-3, but a PKA inhibitor, H89, had no effect (data not shown). Without nicotine, these inhibitors had no effect on PGE<sub>2</sub> production (data not shown).

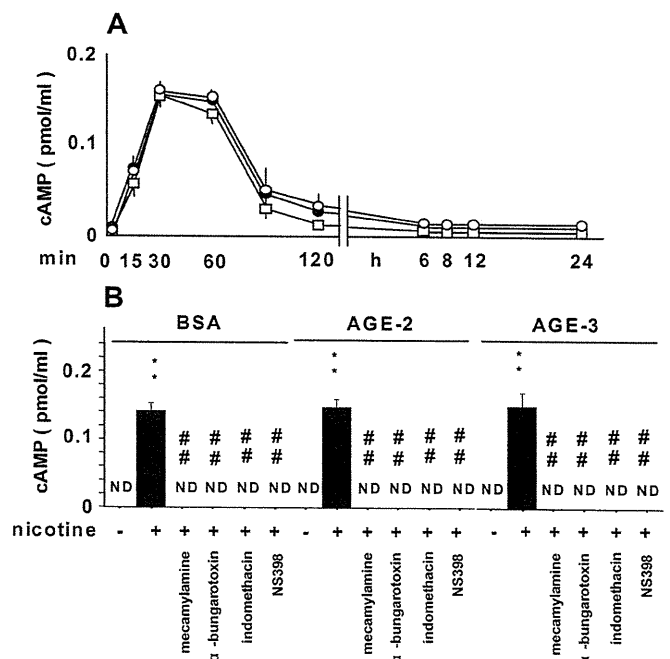


**Fig. 5.** The effect of nicotine on the expression of COX-2 and the production of PGE<sub>2</sub> in monocytes. **A**, monocytes at  $1 \times 10^6$  cells/ml were incubated with nicotine at  $100 \mu\text{M}$  in the presence or absence of AGE-2, AGE-3, and BSA at  $100 \mu\text{g/ml}$  for 30 min. The expression of COX-2 protein was determined by Western immunoblotting as described under *Materials and Methods*.  $\beta$ -Actin was used as control for loading. The concentration of PGE<sub>2</sub> was determined by ELISA. **B**, the time course effect of nicotine on PGE<sub>2</sub> production was determined. Monocytes at  $1 \times 10^6$  cells/ml were incubated with nicotine at  $100 \mu\text{M}$  in the presence of AGE-2, AGE-3, and BSA at  $100 \mu\text{g/ml}$  for the indicated periods. **C**, the effect of nicotine at increasing concentrations from 0.1 to  $100 \mu\text{M}$  in the presence of AGE-2, AGE-3, and BSA at  $100 \mu\text{g/ml}$  for 24 h was determined. **D–G**, the effect of a nonselective  $\alpha 7$ -nAChR antagonist, mecamylamine (**D**); a selective  $\alpha 7$ -nAChR antagonist,  $\alpha$ -bungarotoxin (**E**); a nonselective COX-2 inhibitor, indomethacin (**F**); and a selective COX-2 inhibitor, NS398 (**G**), on the actions of nicotine at  $100 \mu\text{M}$  was determined. Filled circles represent the effect of nicotine on PGE<sub>2</sub> production in the presence of AGE-2, open circles represent that effect in the presence of AGE-3, and open squares represent that effect in the presence of BSA. The results are expressed as the means  $\pm$  S.E.M. of five donors with triplicate determinations. When an error bar is within a symbol, the bar is omitted.

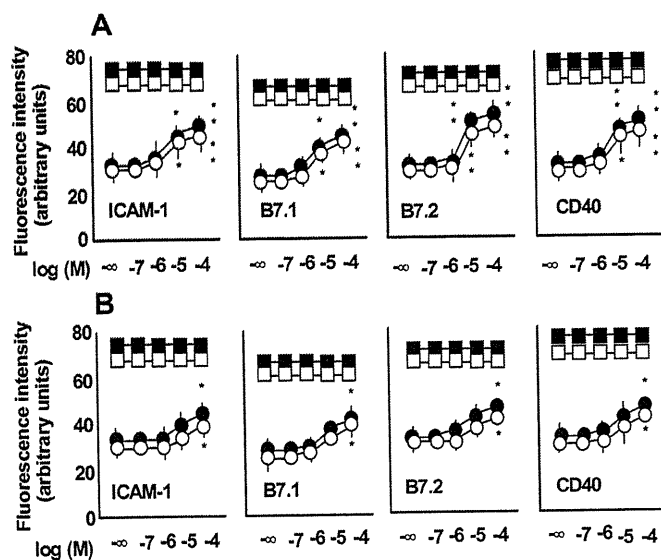
**Effects of Nicotine on the Activation of cAMP in Monocytes.** In the presence or absence of AGE-2 and AGE-3 at  $100 \mu\text{g/ml}$ , nicotine at  $100 \mu\text{M}$  significantly activated intracellular cAMP in monocytes at 15 min and thereafter up to 30 and 60 min (Fig. 6A). As shown in Fig. 6B,  $\alpha 7$ -nAChR antagonists, mecamylamine and  $\alpha$ -bungarotoxin, prevented nicotine-induced activation of cAMP in the presence or absence of AGE-2 and AGE-3. COX-2 inhibitors, indomethacin and NS398, also reversed the nicotine-enhanced activation of cAMP in the presence or absence of AGE-2 and AGE-3. In the absence of nicotine,  $\alpha 7$ -nAChR antagonists and COX-2 inhibitors had no effect on the activation of cAMP (data not shown).

**Involvement of Prostanoid EP<sub>2</sub> and EP<sub>4</sub> Receptors in the Actions of Nicotine.** To determine the involvement of PGE<sub>2</sub> receptor subtypes in the effects of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40, the production of IFN- $\gamma$  and TNF- $\alpha$  and the lymphocyte proliferation, the effect of an EP<sub>2</sub>-receptor antagonist, AH6809 (Kay et al., 2006; Takahashi et al., 2009b) and an EP<sub>4</sub>-receptor antagonist, AH23848 (Kay et al., 2006; Takahashi et al., 2009b) at concentrations ranging from 0.1 to  $100 \mu\text{M}$  in the presence of nicotine at  $100 \mu\text{M}$  were examined (Figs. 7 and 8). AH6809 and AH23848 reversed the inhibitory effect of nicotine on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 (Fig. 7), the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation (Fig. 8) in a concentration-dependent manner. The effect of AH6809 on the actions of nicotine was stronger than that of AH23848. On the other hand, AH6809 and AH23848 had no effect on the actions of AGE-2 and AGE-3 in the absence of nicotine.

**Involvement of COX-2 and PKA in the Action of Nicotine.** To investigate the involvement of COX-2 and PKA in



**Fig. 6.** The effect of nicotine on the activation of cAMP in monocytes. **A**, the time course effect of nicotine at  $100 \mu\text{M}$  on production of cAMP was determined by ELISA. Monocytes at  $1 \times 10^6$  cells/ml were incubated with AGE-2 ( $\bullet$ ), AGE-3 ( $\circ$ ), and BSA ( $\square$ ) at  $100 \mu\text{g/ml}$  and nicotine at  $100 \mu\text{M}$  for 24 h. **B**, the effect of  $\alpha 7$ -nAChR antagonists, mecamylamine or  $\alpha$ -bungarotoxin, at  $10 \text{ nM}$  and the COX-2 inhibitors, indomethacin and NS398, at  $100 \mu\text{M}$  on  $100 \mu\text{M}$  nicotine-induced production of cAMP in the presence of AGE-2, AGE-3, and BSA at  $100 \mu\text{g/ml}$  was determined. \*\*,  $P < 0.01$  compared with the value for BSA alone; ##,  $P < 0.01$  compared with the value for nicotine. The results are the means  $\pm$  S.E.M. of triplicate findings from five donors. ND, not detected.

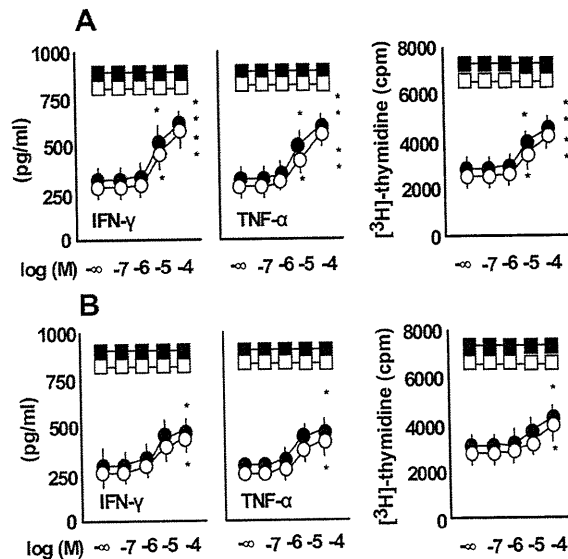


**Fig. 7.** The effects of prostanoid receptor antagonists on the inhibitory effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40. PBMCs at  $1 \times 10^6$  cells/ml treated with nicotine at  $100 \mu\text{M}$  were incubated with the  $\text{EP}_2$ -receptor antagonist AH6809 (A) and the  $\text{EP}_4$ -receptor antagonist AH23848 (B) at increasing concentrations from 0.1 to  $100 \mu\text{M}$  in the presence of AGE-2 and AGE-3 at  $100 \mu\text{g/ml}$ . The expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes were determined by flow cytometry. Filled circles represent the effect of antagonists on nicotine-inhibited adhesion molecule expression in the presence of AGE-2. Open circles represent the effect of antagonists on nicotine-inhibited adhesion molecule expression in the presence of AGE-3. Filled squares represent the effect of antagonists on the actions of AGE-2 in the absence of nicotine. Open squares represent the effect of antagonists on the actions of AGE-3 in the absence of nicotine. The results are expressed as the means  $\pm$  S.E.M. of five donors with triplicate determinations. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with the value for nicotine in the presence of AGE-2 and AGE-3. When an error bar is within a symbol, the bar was omitted.

the inhibitory effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40, the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation, we examined the effect of COX-2 and PKA inhibitors (Figs. 9 and 10). COX-2 inhibitors, indomethacin and NS398, and the PKA inhibitor, H89, reversed the inhibitory effect of nicotine on the expressions of adhesion molecule (Fig. 9), the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation (Fig. 10) in the presence of AGE-2 and AGE-3. COX-2 and PKA inhibitors had no effect on AGE-2- and AGE-3-induced adhesion molecule expression and cytokine production in the absence of nicotine (data not shown).

## Discussion

The level of AGE-2 is reported to be  $17 \mu\text{g/ml}$  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  $200 \mu\text{g/ml}$  remarkably induce human monocyte adhesion to bovine retinal endothelial cells (Mamputu and Renier, 2004). AGEs at  $200 \mu\text{g/ml}$  induce the expression of CD40, CD80 and CD86 and the production of IFN- $\gamma$  in dendritic cells (Ge et al., 2005). In the previous study, we found that AGE-2 and AGE-3 at 10 and  $100 \mu\text{g/ml}$  significantly up-regulated the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN- $\gamma$  and TNF- $\alpha$  and the lymphocyte proliferation (Takahashi et al., 2009a; Wake et

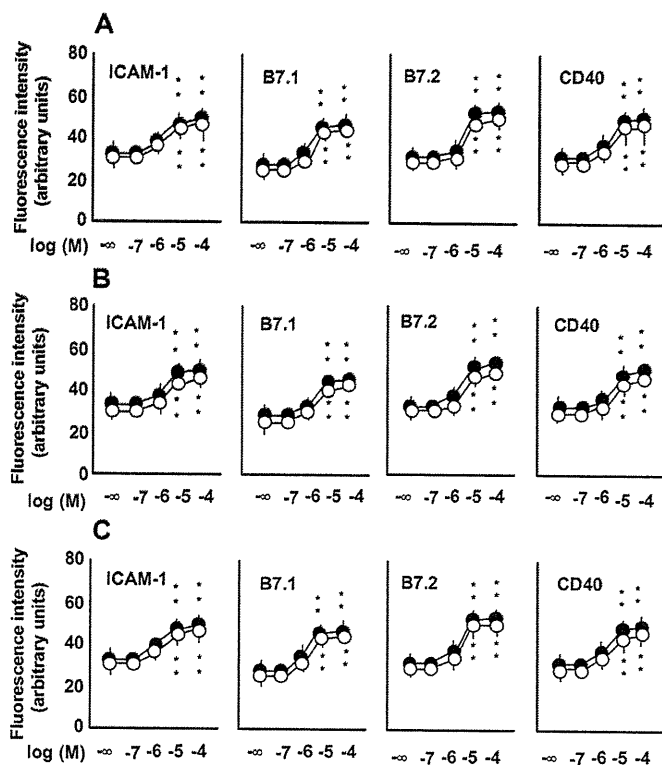


**Fig. 8.** The effects of prostanoid receptor antagonists on the inhibitory effect of nicotine on the production of IFN- $\gamma$  and TNF- $\alpha$  and the lymphocyte proliferation. PBMCs at  $1 \times 10^6$  cells/ml treated with nicotine at  $100 \mu\text{M}$  were incubated with the  $\text{EP}_2$ -receptor antagonist AH6809 (A) and the  $\text{EP}_4$ -receptor antagonist AH23848 (B) at increasing concentrations from 0.1 to  $100 \mu\text{M}$  in the presence of AGE-2 and AGE-3 at  $100 \mu\text{g/ml}$ . IFN- $\gamma$  and TNF- $\alpha$  concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [ $^3\text{H}$ ]thymidine uptake as described under *Materials and Methods*. Filled circles represent the effect of antagonists on nicotine-inhibited adhesion molecule expression in the presence of AGE-2. Open circles represent the effect of antagonists on nicotine-inhibited adhesion molecule expression in the presence of AGE-3. Filled squares represent the effect of antagonists on the actions of AGE-2 in the absence of nicotine. Open squares represent the effect of antagonists on the actions of AGE-3 in the absence of nicotine. The results are expressed as the means  $\pm$  S.E.M. of five donors with triplicate determinations. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with the value for nicotine in the presence of AGE-2 and AGE-3. When an error bar is within a symbol, the bar was omitted.

al., 2009). Therefore, the concentration ( $100 \mu\text{g/ml}$ ) used in the present study may be not far above the pathological concentration of AGEs in the serum of patient with diabetes reported in the studies (Enomoto et al., 2006; Nakamura et al., 2007).

We found that AGE-2 and AGE-3 induced the production of IFN- $\gamma$  and TNF- $\alpha$  in monocytes isolated from PBMCs, exhibiting 20% of the amount obtained in PBMCs (Takahashi et al., 2009a). AGE-2 and AGE-3 had no effect on the production of IFN- $\gamma$  and TNF- $\alpha$  in T cells isolated from PBMCs. Anti-ICAM-1, anti-B7.1, anti-B7.2, and anti-CD40 Abs inhibited the AGE-2- and AGE-3-induced production of IFN- $\gamma$  and TNF- $\alpha$  in PBMCs. We suggested that AGE-2- and AGE-3-induced cytokine production required the enhancement of cell-to-cell interaction between monocytes and T cells through the induction of plural adhesion molecule expression on monocytes. Together with these results, we examined the effect of nicotine on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN- $\gamma$  and TNF- $\alpha$  in PBMCs, and the lymphocyte proliferation in PBMCs.

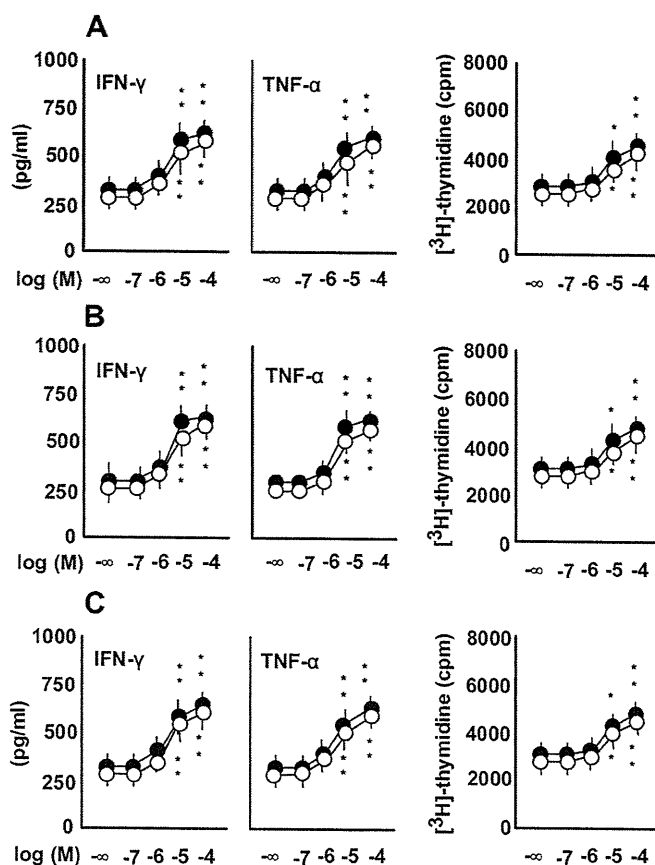
As shown in Figs. 1 and 2, we found that nicotine prevented  $100 \mu\text{g/ml}$  AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation in PBMCs. In the presence of AGE-2 and AGE-3 at  $10 \mu\text{g/ml}$ , nicotine also inhibited expressions of ICAM-1, B7.1, B7.2, and CD40, the produc-



**Fig. 9.** The involvement of COX-2 and PKA in the effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40. The effect of COX-2 inhibitors, indomethacin (A) and NS398 (B), and a PKA inhibitor, H89 (C), at increasing concentrations ranging from 0.1 to 100  $\mu$ M on the actions of nicotine at 100  $\mu$ M in the presence of AGE-2 (●) and AGE-3 (○) at 100  $\mu$ g/ml. The expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes were determined by flow cytometry. The results are expressed as the means  $\pm$  S.E.M. of triplicate findings from five donors. \*\*,  $P < 0.01$  compared with the value for nicotine. When an error bar is within a symbol, the bar was omitted.

tion of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation in a concentration-dependent manner (data not shown). The  $IC_{50}$  values of nicotine for the inhibition of adhesion molecule and cytokine production were within the range of the concentration reported to be effective in recent animal studies (Wang et al., 2003, 2004). It is suggested that vagus nerve stimulation modulates the immune response and controls inflammation through a nicotinic anti-inflammatory pathway dependent on  $\alpha 7$ -nAChR (de Jonge et al., 2005; Saeed et al., 2005).  $\alpha 7$ -nAChR is required for acetylcholine inhibition of lipopolysaccharide-induced TNF- $\alpha$  production in human macrophages (Wang et al., 2003). In the present study, we found that  $\alpha 7$ -nAChR antagonists reversed the inhibitory effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40, the production of IFN- $\gamma$  and TNF- $\alpha$  and the lymphocyte proliferation in the presence of AGE-2 and AGE-3 (Figs. 3 and 4). This suggests that inhibitory effects of nicotine depend on the stimulation of  $\alpha 7$ -nAChR.

We further investigated the action mechanism of nicotine. PGE<sub>2</sub>, which is released from antigen-presenting cells, acts on naive human T cells to enhance their production of anti-inflammatory cytokines (Hempel et al., 1994; Coleman et al., 1994). PGE<sub>2</sub>, a product of COX-2-initiated metabolism, is known to activate the cAMP/PKA pathway (Bastien et al., 1994; Nataraj et al., 2001). As shown in Fig. 5, A and B, we found that nicotine induced the expression of COX-2 and the production of PGE<sub>2</sub> in the presence or absence of AGE-2 and



**Fig. 10.** The involvement of COX-2 and PKA in the effect of nicotine on the production of IFN- $\gamma$  and TNF- $\alpha$  and the lymphocyte proliferation. The effect of COX-2 inhibitors, indomethacin (A) and NS398 (B), and a PKA inhibitor, H89 (C), at increasing concentrations ranging from 0.1 to 100  $\mu$ M on the actions of nicotine at 100  $\mu$ M in the presence of AGE-2 (●) and AGE-3 (○) at 100  $\mu$ g/ml. IFN- $\gamma$  and TNF- $\alpha$  concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [<sup>3</sup>H]thymidine uptake as described under *Materials and Methods*. The results are expressed as the means  $\pm$  S.E.M. of triplicate findings from five donors. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with the value for nicotine. When an error bar is within a symbol, the bar was omitted.

AGE-3. The  $\alpha 7$ -nAChR and COX-2 inhibitors prevented nicotine-initiated PGE<sub>2</sub> production in the presence or absence of AGE-2 and AGE-3 (Fig. 5, C–F). These results suggest that the effect of nicotine on the expression of COX-2 and the production of PGE<sub>2</sub> depends on the stimulation of  $\alpha 7$ -nAChR. We also determined the levels of other COX-2 metabolites, including PGE<sub>1</sub>, PGD<sub>2</sub>, PGF<sub>2</sub>, PGI<sub>2</sub>, PGJ<sub>2</sub>, and thromboxane, measured in monocyte medium treated with nicotine in the presence or absence of AGE-2 and AGE-3, but all were under the level of detection (data not shown). As shown in Fig. 6A, nicotine activated intracellular cAMP in monocytes in the presence or absence of AGE-2 and AGE-3.  $\alpha 7$ -nAChR antagonists and COX-2 inhibitors prevented this nicotine-induced activation of cAMP in the presence or absence of AGE-2 and AGE-3 (Fig. 6B), suggesting the involvement of  $\alpha 7$ -nAChR stimulation and COX-2 production in nicotine-induced elevation of cAMP.

In the previous study, we reported that PGE<sub>2</sub> inhibited AGE-2- and AGE-3-enhanced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation in PBMCs (Taka-



hashi et al., 2009b). Among the four subtypes of receptors: prostanoid EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub>, and EP<sub>4</sub> receptors, the EP<sub>2</sub> and EP<sub>4</sub> receptors were involved in the actions of PGE<sub>2</sub>. In the previous study, we found that the EP<sub>2</sub>-receptor antagonist, AH6809 and the EP<sub>4</sub>-receptor antagonist, AH23848, inhibited the actions of PGE<sub>2</sub> (Takahashi et al., 2009b). An EP<sub>2</sub>-receptor agonist, ONO-AE1-259-01 and an EP<sub>4</sub>-receptor agonist, ONO-AE1-329 mimicked the actions of PGE<sub>2</sub>. In the presence of AGE-2 and AGE-3, PGE<sub>2</sub> induced the elevation of cAMP via EP<sub>2</sub> and EP<sub>4</sub> receptor. Moreover, the PKA inhibitor, H89, inhibited the action of PGE<sub>2</sub>, and a membrane-permeable cAMP analog, dibutyryl cAMP, and an adenylate cyclase activator, forskolin, mimicked the effect of PGE<sub>2</sub>. These results suggested the involvement of the EP<sub>2</sub>/EP<sub>4</sub>-receptors-cAMP/PKA pathway in the actions of PGE<sub>2</sub>.

In the present study, we found that the EP<sub>2</sub>-receptor antagonist, AH6809 and the EP<sub>4</sub>-receptor antagonist, AH23848, inhibited the actions of nicotine (Figs. 7 and 8). As shown in Figs. 9 and 10, COX-2 or PKA inhibitors partially reversed the inhibitory effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN- $\gamma$  and TNF- $\alpha$ , and the lymphocyte proliferation in PBMCs in the presence of AGE-2 and AGE-3. These results imply that the additional increase in PGE<sub>2</sub> by the combination of nicotine and AGEs might, at least partially account for the suppressive effect of nicotine on AGE-2- and AGE-3-induced enhancement of adhesion molecule expressions and cytokine production. We observed a similar pattern of inhibitory effects of nicotine on lipopolysaccharide (LPS)- and interleukin (IL)-18-induced activation of monocytes in human PBMCs via  $\alpha$ 7-nAChR (Hamano et al., 2006; Takahashi et al., 2006). Nicotine induced PGE<sub>2</sub> production in monocytes treated with LPS and IL-18 via  $\alpha$ 7-nAChR. COX-2 and PKA inhibitors prevented the effects of nicotine on adhesion molecule expression and cytokine production. Thus, there may be a partially common pathway triggered by LPS, IL-18, and AGEs that is regulated by the  $\alpha$ 7-nAChR-PGE<sub>2</sub>-cAMP/PKA system. Further work is necessary on this issue.

Nicotine by its own did not inhibit basal production of IFN- $\gamma$  and TNF- $\alpha$  in the absence of AGE-2 and AGE-3 (Fig. 2). As shown in Fig. 5, nicotine at 100  $\mu$ M induced production of PGE<sub>2</sub> at 4.7 nM in the absence of AGE-2 and AGE-3. Exogenous PGE<sub>2</sub> at 5 nM had no effect on the production of IFN- $\gamma$  and TNF- $\alpha$  in the absence of AGE-2 and AGE-3 (data not shown). Therefore, 100  $\mu$ M nicotine-induced PGE<sub>2</sub> might not effect on IFN- $\gamma$  and TNF- $\alpha$  production.

In the previous study, using of an in vitro binding assay, we found that AGE-2 and AGE-3 had higher affinity for RAGEs than AGE-4 and AGE-5 (Takahashi et al., 2009a). 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. Nicotine had no effect on the expression of RAGEs 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 expressions of adhesion molecule and RAGEs, which were differentially regulated by the cAMP/PKA system.

It is reported that AGEs and RAGEs are detected in atherosclerotic plaque of diabetic patients (Cuccurullo et al., 2006). RAGEs are reported to be expressed by smooth muscle cells (SMCs) as well as endothelial cells, monocytes, macrophages,

mesangial cells, and nerve cells (Brett et al., 1993). AGEs directly stimulate proliferation not in endothelial cells, but in SMCs (Satoh et al., 1997). It is reported that PGE<sub>2</sub> induced by monocytes inhibits procollagen secretion by human vascular SMCs, 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). Nicotine exerts its atherogenic effects in part through the increase of SMC proliferation and migration (Carty et al., 1997; Di Luozzo et al., 2005).  $\alpha$ 7-nAChR is detected in the rat arterial system and in SMCs derived from brain basilar arteries (Brüggmann et al., 2003; Li et al., 2004), indicating that stimulation of  $\alpha$ 7-nAChR induces the proliferation of SMCs. Together with these results and our data, other extracellular stimuli, which induce intracellular cAMP production upon binding to their cognate G protein-coupled receptors, may regulate the activation of vascular smooth muscle cells and endothelial cells. However,  $\alpha$ 7-nAChR stimulation may inhibit the activation of monocytes. Further study of the role of  $\alpha$ 7-nAChR in the stimulation of SMCs and monocytes should be continued.

In conclusion, we found that nicotine inhibited AGE-2- and AGE-3-enhanced adhesion molecule expressions, the cytokine production, and the lymphocyte proliferation via  $\alpha$ 7-nAChR. The COX-2-PGE<sub>2</sub>-cAMP/PKA system may be involved, in part, in the actions of nicotine. Through the inhibition of toxic AGE-dependent responses in monocytes, the stimulation of  $\alpha$ 7-nAChR may partially contribute to regulation of the development of atherosclerotic plaques in diabetes.

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#### References

- Bastien L, Sawyer N, Grygorczyk R, Metters KM, and Adam M (1994) Cloning, functional expression, and characterization of the human prostaglandin E<sub>2</sub> receptor EP<sub>2</sub> subtype. *J Biol Chem* 269:11873–11877.
- Brett J, Schmidt AM, Yan SD, Zou YS, Weidman E, Pinsky D, Nowygro R, Neepor M, Przysiecki C, and Shaw A (1993) Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. *Am J Pathol* 143:1699–1712.
- Brownlee M (1995) Advanced protein glycosylation in diabetes and aging. *Annu Rev Med* 46:223–234.
- Brüggmann D, Lips KS, Pfeil U, Haberberger RV, and Kummer W (2003) Rat arteries contain multiple nicotinic acetylcholine receptor  $\alpha$ -subunits. *Life Sci* 72: 2095–2099.
- Carty CS, Huribal M, Marsan BU, Ricotta JJ, and Dryjski M (1997) Nicotine and its metabolite cotinine are mitogenic for human vascular smooth muscle cells. *J Vasc Surg* 25:682–688.
- Coleman RA, Smith WL, and Narumiya S (1994) International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptor and their subtypes. *Pharmacol Rev* 46:205–229.
- Cuccurullo C, Iezzi A, Fazio ML, De Cesare D, Di Francesco A, Muraro R, Bei R, Uechino 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.
- de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bennink RJ, Berthoud HR, Uematsu S, Akira S, van den Wijngaard RM, et al. (2005) Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat Immunol* 6:844–851.
- De Simone R, Ajmone-Cat MA, Carnevale D, and Minghetti L (2005) Activation of  $\alpha$ 7 nicotinic acetylcholine receptor by nicotine selectively up-regulates cyclooxygenase-2 and prostaglandin E<sub>2</sub> in rat microglial cultures. *J Neuroinflammation* 2:4.
- Di Luozzo G, Pradhan S, Dhadwal AK, Chen A, Ueno H, and Sumpio BE (2005) Nicotine induces mitogen-activated protein kinase dependent vascular smooth muscle cell migration. *Atherosclerosis* 178:271–277.
- 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.

- Fitzsimmons C, Proudfoot D, and Bowyer DE (1999) Monocyte prostaglandins inhibit procollagen secretion by human vascular smooth muscle cells: implications for plaque stability. *Atherosclerosis* 142:287-293.
- 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.
- Hamano R, Takahashi HK, Iwagaki H, Yoshino T, Nishibori M, and Tanaka N (2006) Stimulation of alpha7 nicotinic acetylcholine receptor inhibits CD14 and the toll-like receptor 4 expression in human monocytes. *Shock* 26:358-364.
- Hempel SL, Monick MM, and Hunninghake GW (1994) Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J Clin Invest* 93:391-396.
- Kay LJ, Yeo WW, and Peachell PT. (2006) Prostaglandin E<sub>2</sub> activates EP<sub>2</sub> receptors to inhibit human lung mast cell degranulation. *Br J Pharmacol* 147:707-713.
- Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, and Schmidt AM (1997) Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *J Biol Chem* 272:17810-17814.
- Li S, Zhao T, Xin H, Ye LH, Zhang X, Tanaka H, Nakamura A, and Kohama K (2004) Nicotinic acetylcholine receptor alpha7 subunit mediates migration of vascular smooth muscle cells toward nicotine. *J Pharmacol Sci* 94:334-338.
- Lorenovicz MJ, Fernandez-Borja M, and Hordijk PL (2007) cAMP signaling in leukocyte transendothelial migration. *Arterioscler Thromb Vasc Biol* 27:1014-1022.
- Mamputu JC and Renier G (2004) Advanced glycation end-products increase monocyte adhesion to retinal endothelial cells through vascular endothelial growth factor-induced ICAM-1 expression: inhibitory effect of antioxidants. *J Leukoc Biol* 75:1062-1069.
- 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.
- Nataraj C, Thomas DW, Tilley SL, Nguyen MT, Mannon R, Koller BH, and Coffman TM (2001) Receptors for prostaglandin E<sub>2</sub> that regulate cellular immune responses in the mouse. *J Clin Invest* 108:1229-1235.
- 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.
- Saareks V, Mucha I, Sievi E, Vapaatalo H, and Riutta A (1998) Nicotine stereoisomers and cotinine stimulate prostaglandin E<sub>2</sub> but inhibit thromboxane B<sub>2</sub> and leukotriene E<sub>4</sub> synthesis in whole blood. *Eur J Pharmacol* 353:87-92.
- Saeed RW, Varma S, Peng-Nemeroff T, Sherry B, Balakhaneh D, Huston J, Tracey KJ, Al-Abed Y, and Metz CN (2005) Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. *J Exp Med* 201:1113-1123.
- Satoh H, Togo M, Hara M, Miyata T, Han K, Maekawa H, Ohno M, Hashimoto Y, Kurokawa K, and Watanabe T (1997) Advanced glycation endproducts stimulate mitogenactivated protein kinase and rabbit vascular smooth muscle cells. *Biochem Biophys Res Commun* 239:111-115.
- 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.
- Takahashi HK, Iwagaki H, Hamano R, Yoshino T, Tanaka N, and Nishibori M (2006) Effect of nicotine on IL-18-initiated immune response in human monocytes. *J Leukoc Biol* 80:1388-1394.
- 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.
- Takahashi HK, Liu K, Wake H, Mori S, Zhang J, Liu R, Yoshino T, and Nishibori M (2009b) Prostaglandins E<sub>2</sub> inhibits advanced glycation end products-induced adhesion molecule expression, cytokine production and lymphocyte proliferation in human peripheral blood mononuclear cells. *J Pharmacol Exp Ther* 331:656-670.
- Takahashi HK, Mori S, Wake H, Liu K, Yoshino T, Ohashi K, Tanaka N, Shikata K, Makino H, and Nishibori M (2009a) Advanced glycation end products subselectively induce adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells. *J Pharmacol Exp Ther* 330:89-98.
- 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 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.
- 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.
- Wake H, Takahashi HK, Mori S, Liu K, Yoshino T, and Nishibori M (2009) Histamine inhibits advanced glycation end products-induced adhesion molecule expression on human monocytes. *J Pharmacol Exp Ther* 330:826-833.
- Wang H, Liao H, Ochani M, Justiniani M, Lin X, Yang L, Al-Abed Y, Wang H, Metz C, Miller EJ, et al. (2004) Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. *Nat Med* 10:1216-1221.
- Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, et al. (2003) Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature* 421:384-388.

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# Prostaglandin E2 Inhibits Advanced Glycation End Product-Induced Adhesion Molecule Expression on Monocytes, Cytokine Production, and Lymphocyte Proliferation during Human Mixed Lymphocyte Reaction

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## ABSTRACT

Posttransplant diabetes mellitus is a frequent complication among transplant recipients. Ligation of advanced glycation end products (AGEs) with their receptor on monocytes/macrophages plays a role in diabetes complications. The enhancement of adhesion molecule expression on monocytes/macrophages activates T cells, reducing allograft survival. In previous work, we found that toxic AGEs, AGE-2 and AGE-3, induced the expression of intracellular adhesion molecule-1, B7.1, B7.2, and CD40 on monocytes, production of interferon- $\gamma$  and tumor necrosis factor  $\alpha$ , and lymphocyte proliferation during human mixed lymphocyte reaction. AGE-induced up-regulation of adhesion molecule expression was involved in cytokine production and lymphocyte proliferation. Prostaglandin E2 (PGE2) concentration-dependently inhibited the actions of AGE-2 and

AGE-3. The effects of PGE2 were mimicked by an EP2 receptor agonist, ONO-AE1-259-01 (11,15-O-dimethyl PGE2), and an EP4 receptor agonist, ONO-AE1-329 [16-(3-methoxymethyl)phenyl-omega-tetranor-3,7dithia PGE1]. An EP2 receptor antagonist, AH6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid), and an EP4 receptor antagonist, AH23848 [(4Z)-7-[(rel-1S,2S,5R)-5-((1,1'-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid], inhibited the actions of PGE2. The stimulation of EP2 and EP4 receptors is reported to increase cAMP levels. The effects of PGE2 were reversed by protein kinase A (PKA) inhibitors and mimicked by dibutyl cAMP and an adenylate cyclase activator, forskolin. These results as a whole indicate that PGE2 inhibited the actions of AGE-2 and AGE-3 via EP2/EP4 receptors and the cAMP/PKA pathway.

## Introduction

It is known that diabetes mellitus (DM) facilitates the formation of AGEs, which are formed by a nonenzymatic

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reaction between a carbonyl group of reducing sugars and free amino groups from macromolecules such as proteins, lipoproteins, and nucleic acids, both in blood and intracellularly (Vlassara and Palace, 2002; Schiekofer et al., 2003). Accumulation of AGEs is shown in the plasma and tissues of patients with diabetes, leading to the pathogenesis of diabetes complications (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 (Swamy-

**ABBREVIATIONS:** DM, diabetes mellitus; PTDM, posttransplant diabetes mellitus; AGE, advanced glycation end product; BSA, bovine serum albumin; COX, cyclooxygenase; CsA, cyclosporine A; dbcAMP, dibutyl cAMP; ELISA, enzyme-linked immunosorbent assay; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cell; PGE2, prostaglandin E2; PKA, protein kinase A; PKI, protein kinase inhibitor; RAGE, receptor for advanced glycation end products; sRAGE, soluble RAGE; TNF, tumor necrosis factor; ONO-DI-004, 17S-2,5-ethano-6-oxo-17,20-dimethyl prostaglandin E1; ONO-AE1-259-01, 11,15-O-dimethyl prostaglandin E2; ONO-AE-248, 16S-9-deoxy-9 $\beta$ -chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-didehydro prostaglandin F2; ONO-AE1-329, 16-(3-methoxymethyl)phenyl-omega-tetranor-3,7dithia prostaglandin E1; 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; H-89, N-[2-(p-bromocinnamyl-amino)ethyl]-5-isoquinoline-sulfonamide dihydrochloride; PKI, protein kinase inhibitor; KT5720, (9S,10S,12R)-2,3,9,10,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]-benzo-diazocine-10-carboxylic acid hexyl ester.

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- $\gamma$  and TNF- $\alpha$ , 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- $\gamma$  and TNF- $\alpha$ , 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- $\gamma$  and TNF- $\alpha$ , and lymphocyte proliferation during MLR.

## Materials and Methods

**Reagents.** PGE2, 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], H-89 [N-[2-(p-bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride], and KT5720 [(9S,10S,12R)-2,3,9,10,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-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 (17S-2,5-ethano-6-oxo-17,20-dimethyl prostaglandin E1), ONO-AE1-259-01 (11,15-O-dimethyl prostaglandin E2), ONO-AE-248 (16S-9-deoxy-9 $\beta$ -chloro-15-deoxy-16-hydroxy-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  $\mu$ g/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  $1 \times 10^6$  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  $2.0 \times 10^6$  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  $\mu$ g/ml kanamycin, and 100  $\mu$ g/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<sub>2</sub> 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  $2 \times 10^6$  cells/ml were incubated for 48 h. Cultured cells at  $5 \times 10^5$  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  $1 \times 10^6$  cells/ml were used for analyzing IFN- $\gamma$  and TNF- $\alpha$  production. After being cultured for 24 h at 37°C in a 5% CO<sub>2</sub>/air mixture, the cell-free supernatant was assayed for IFN- $\gamma$  and TNF- $\alpha$  protein by ELISA using the multiple antibodies sandwich principle (R&D Systems, Minneapolis, MN). The detection limit of ELISA for IFN- $\gamma$  and TNF- $\alpha$  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 [<sup>3</sup>H]thymidine (3.3 Ci/well) for the final 16 h. Cells were then divided into 96-well microplates (200 μl/well), resulting in 1 μCi [<sup>3</sup>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).

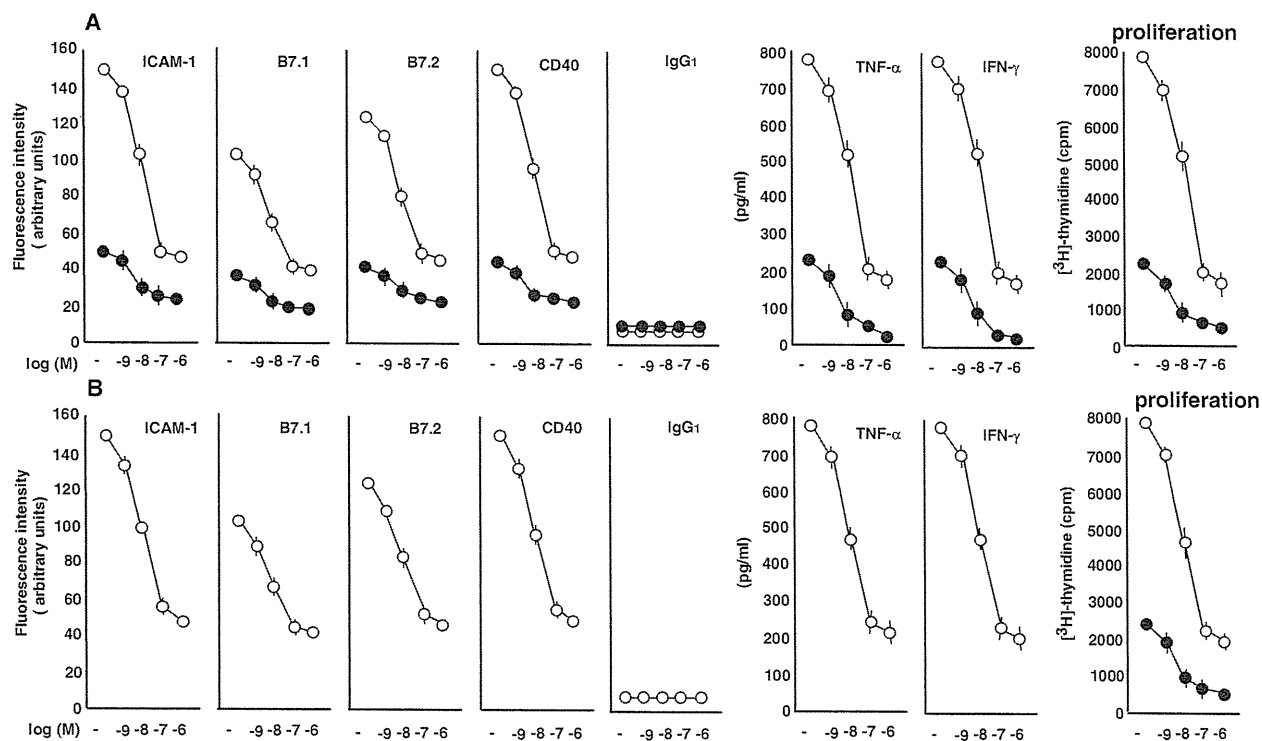
**Statistical Analysis.** Statistical significance was evaluated by analysis of variance followed by Dunnett's test. A probability value of less than 0.05 was considered to indicate statistical significance. The results are expressed as the means ± S.E.M. of triplicate findings from five donors.

## 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, pro-

duction 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<sub>50</sub> 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  $2 \times 10^6$  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 [<sup>3</sup>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.

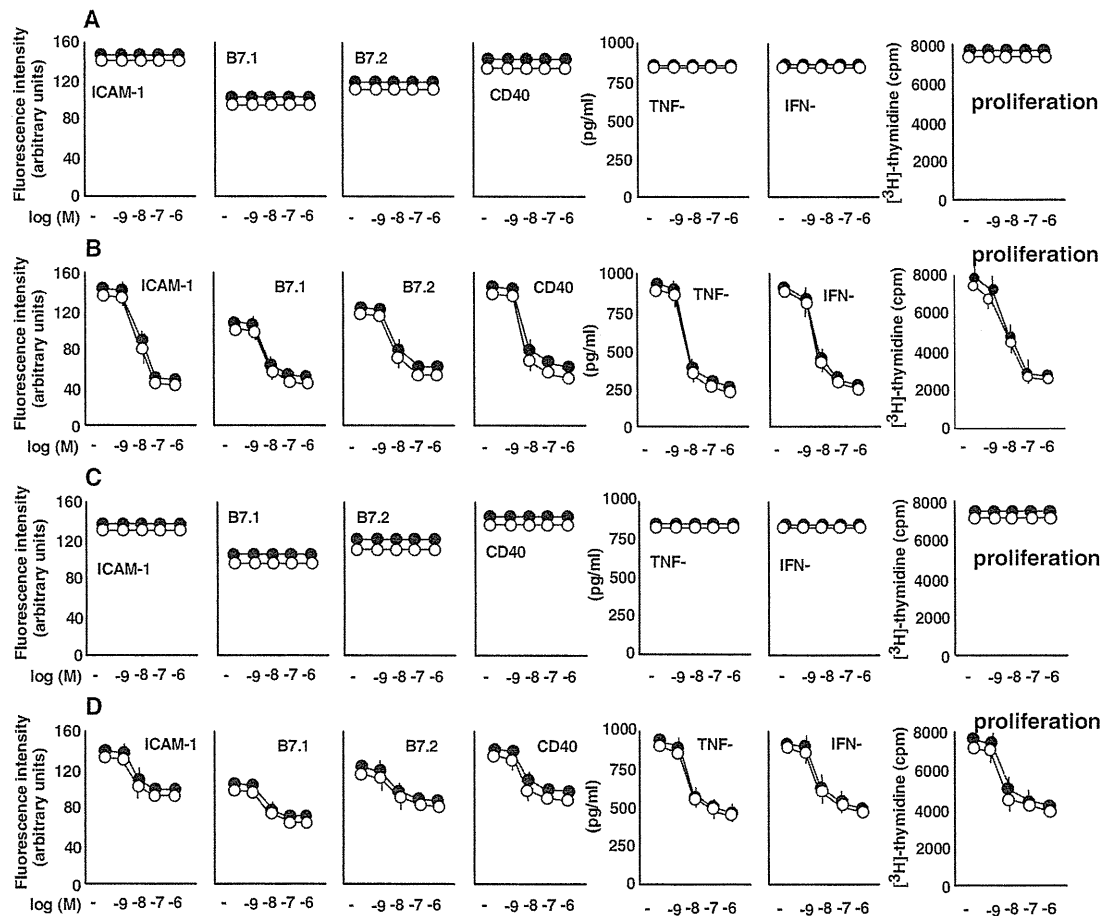
TABLE 1

The IC<sub>50</sub> 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
<i>nm</i>							
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<sub>50</sub> 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-



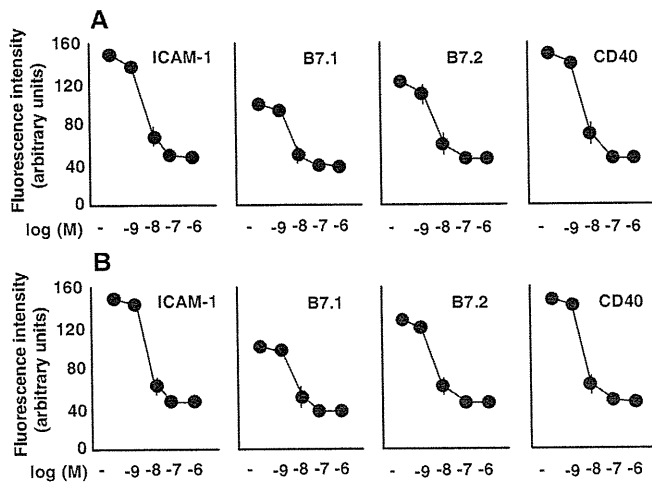
**Fig. 2.** The effect of prostanoid receptor agonists on AGE2- and AGE3-induced expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN-γ and TNF-α, and lymphocyte proliferation during MLR. Mixed cells at  $2 \times 10^6$  cells/ml were incubated with the EP1 receptor agonist, ONO-D-1-004 (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 [<sup>3</sup>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

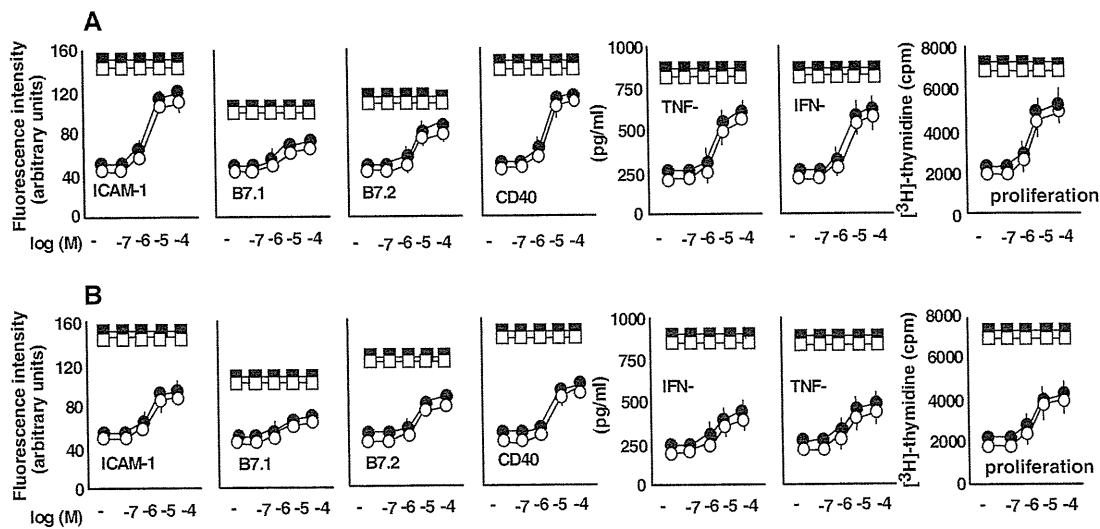
agonists concentration-dependently inhibited the basal expression of adhesion molecule, cytokine production, and lymphocyte proliferation, but EP1 and EP3 receptor agonists had no effect (data not shown).

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  $\mu$ M on adhesion molecule expression, cytokine production, and lymphocyte proliferation were examined in the presence of PGE2 at 1  $\mu$ M (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- $\gamma$  and TNF- $\alpha$ , 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).

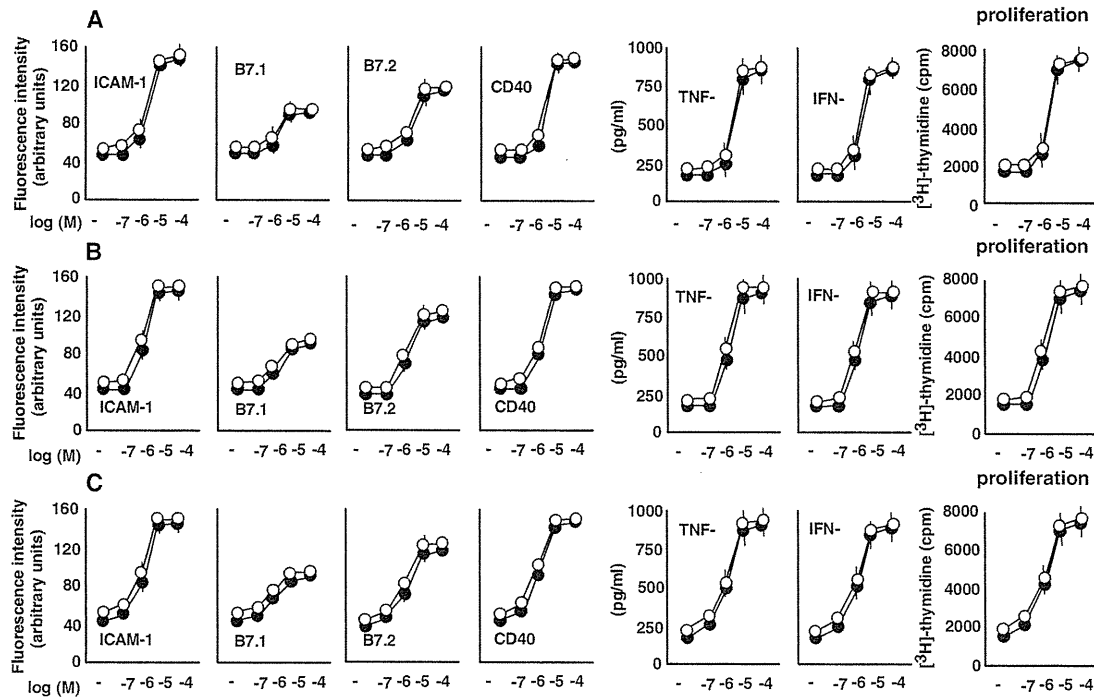
**The Involvement of cAMP in the Actions of PGE2.** To investigate the involvement of the cAMP/PKA pathway in the effects of PGE2 on the expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN- $\gamma$  and TNF- $\alpha$ , and lymphocyte proliferation, the effect of PKA inhibitors, H-89, PKI(14-22), and KT5720, at concentrations ranging from 0.1 to 100  $\mu$ M on the actions of PGE2 in the presence of AGE-2 and AGE-3 at 100  $\mu$ g/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- $\gamma$  and TNF- $\alpha$ , 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. 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  $2 \times 10^6$  cells/ml were incubated with increasing concentrations of the EP2/EP4 receptor agonist, 11-deoxy-PGE1, at increasing concentrations from 1 nM to 1  $\mu$ M in the presence of AGE-2 (A) and AGE-3 (B) at 100  $\mu$ g/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  $\pm$  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.



**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- $\gamma$  and TNF- $\alpha$ , and lymphocyte proliferation during MLR. Mixed cells at  $2 \times 10^6$  cells/ml treated with PGE2 at 1  $\mu$ M were incubated with the EP2 receptor antagonist, AH6809 (A), and the EP4 receptor antagonist, AH23848 (B), at increasing concentrations from 0.1 to 100  $\mu$ M in the presence of AGE-2 and AGE-3 at 100  $\mu$ g/ml for 48 h. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. IFN- $\gamma$  and TNF- $\alpha$  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  $\pm$  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.

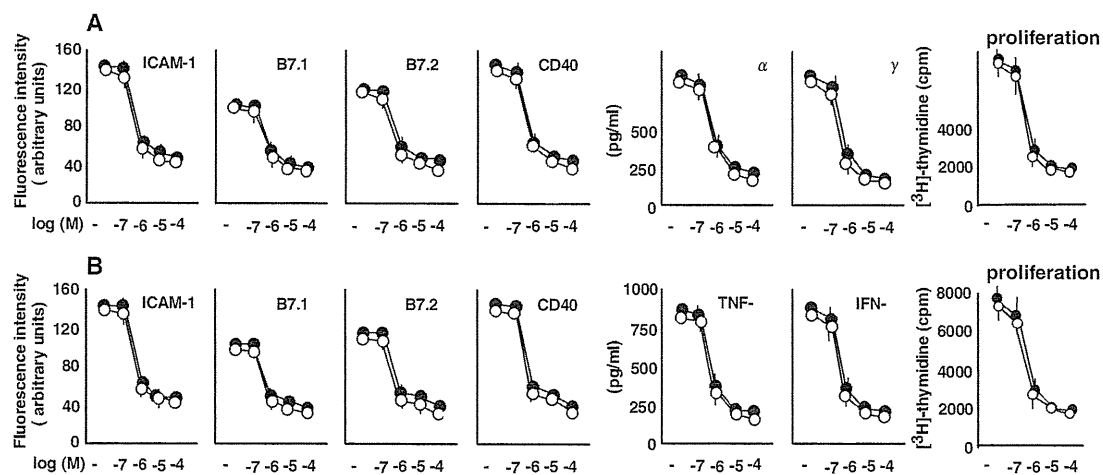


**Fig. 5.** The effects of PKA inhibitors on PGE2-inhibited ICAM-1, B7.1, B7.2, and CD40 monocytic expression, production of IFN- $\gamma$  and TNF- $\alpha$ , and lymphocyte proliferation during MLR. The effect of PKA inhibitors, H-89 (A), KT5720 (B), and PKI(14-22) (C), at increasing concentrations from 0.1 to 100  $\mu$ M, 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  $\mu$ g/ml was determined by flow cytometry. The production of TNF- $\alpha$  and IFN- $\gamma$  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  $\pm$  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  $\mu$ M on

the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN- $\gamma$  and TNF- $\alpha$ , and lymphocyte proliferation during MLR in the presence of AGE-2 and AGE-3 at 100  $\mu$ g/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- $\gamma$  and TNF- $\alpha$ , and lymphocyte proliferation during MLR. Mixed cells at  $2 \times 10^6$  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  $\mu$ M in the presence of AGE-2 (●) and AGE-3 (○) at 100  $\mu$ g/ml for 48 h. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. IFN- $\gamma$  and TNF- $\alpha$  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  $\pm$  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).

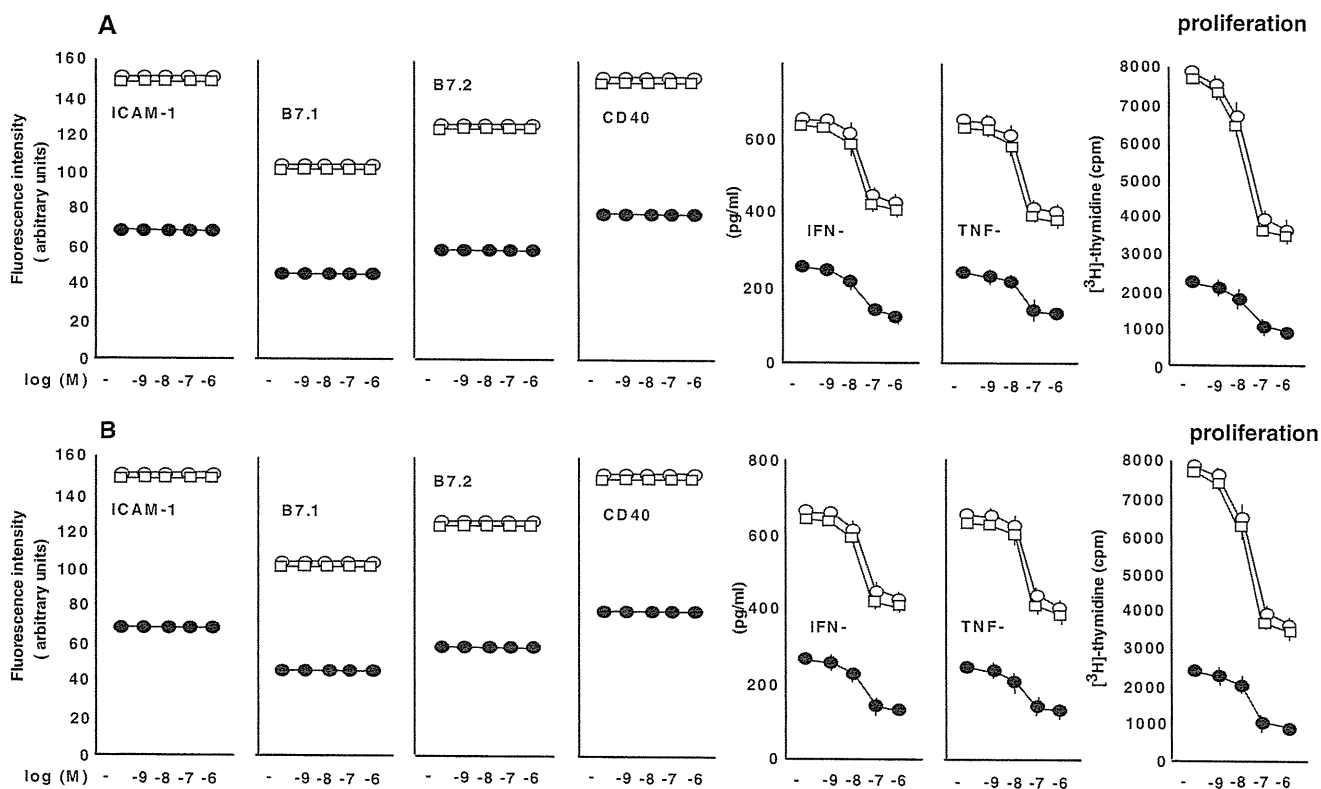
**The Effects of Tacrolimus and CsA on AGE-2- and AGE-3-Induced Expression of ICAM-1, B7.1, B7.2, and CD40, Production of IFN- $\gamma$  and TNF- $\alpha$ , and Lymphocyte Proliferation during MLR.** As shown in Fig. 7, we examined the effects of tacrolimus and CsA at increasing concentrations ranging from 1 nM to 1  $\mu$ M on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, production of IFN- $\gamma$  and TNF- $\alpha$ , and lymphocyte proliferation in the presence or absence of AGE-2 and AGE-3 at 100  $\mu$ g/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  $\mu$ g/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  $\mu$ g/ml markedly induce human monocyte adhesion to bovine retinal endothelial cells (Mamputu and Renier, 2004). AGEs at 200  $\mu$ g/ml induce the expression of CD40, CD80, and CD86 and production of IFN- $\gamma$  in dendritic

cells (Ge et al., 2005). In a previous study, we found that AGE-2 and AGE-3 at 10 and 100  $\mu$ g/ml significantly up-regulated the expression of ICAM-1, B7.1, B7.2, and CD40, production of IFN- $\gamma$  and TNF- $\alpha$ , and lymphocyte proliferation during MLR (Ohashi et al., 2010). Therefore, the concentration (100  $\mu$ g/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- $\gamma$  and TNF- $\alpha$ , 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- $\gamma$  and TNF- $\alpha$ , and lymphocyte proliferation during MLR. Mixed cells at  $2 \times 10^6$  cells/ml were incubated with tacrolimus (A) and CsA (B) in the presence or absence of AGE-2 and AGE-3 at 100  $\mu$ g/ml for 48 h, and the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. IFN- $\gamma$  and TNF- $\alpha$  concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [<sup>3</sup>H]thymidine uptake as described under *Materials and Methods*.  $\circ$ , the effect of tacrolimus and CsA on the actions of AGE-2;  $\square$ , the effect of tacrolimus and CsA on the actions of AGE-3 during MLR;  $\bullet$ , the effect of tacrolimus and CsA in the absence of AGEs. The results are expressed as the means  $\pm$  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-AE1-329 mimicked the effects of PGE2 on adhesion molecule expression, cytokine production, and lymphocyte proliferation. In the present study, IC<sub>50</sub> values for the inhibitory effects of ONO-AE1-259 and ONO-AE1-329 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<sub>50</sub> 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 produc-

tion, 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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#### References

- Blaheta RA, Hailer NP, Brude N, Wittig B, Leckel K, Oppermann E, Bachmann M, Harder S, Cinatl J, Scholz M, et al. (2000) In vitro analysis of verapamil-induced immunosuppression: potent inhibition of T cell motility and lymphocytic transmigration through allogeneic endothelial cells. *Transplantation* 69:588–597.
- Brownlee M, Cerami A, and Vlassara H (1988) Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 318:1315–1321.
- Carvalho MF and Soares V (2001) Hyperlipidemia as a risk factor of renal allograft function impairment. *Clin Transplant* 15:48–52.
- Coleman RA, Smith WL, and Narumiya S (1994) VIII. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptor and their subtypes. *Pharmacol Rev* 46:205–229.
- Cooper ME (2004) Importance of advanced glycation end products in diabetes-associated cardiovascular and renal disease. *Am J Hypertens* 17:31S–38S.
- Cosio FG, Pesavento TE, Kim S, Osei K, Henry M, and Ferguson RM (2002) Patient

- survival after renal transplantation: IV. Impact of post-transplant diabetes. *Kidney Int* 62:1440-1446.
- Dustin ML and Springer TA (1989) T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* 341:619-624.
- 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.
- Filler G, Neuschulz I, Vollmer I, Amendt P, and Hocher B (2000) Tacrolimus reversibly reduces insulin secretion in paediatric renal transplant recipients. *Nephrol Dial Transplant* 15:867-871.
- Fitzsimmons C, Proudfoot D, and Bowyer DE (1999) Monocyte prostaglandins inhibit procollagen secretion by human vascular smooth muscle cells: implications for plaque stability. *Atherosclerosis* 142:287-293.
- 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.
- Greenfield EA, Nguyen KA, and Kuchroo VK (1998) CD28/B7 costimulation: a review. *Crit Rev Immunol* 18:389-418.
- Hartog JW, de Vries AP, Bakker SJ, Graaff R, van Son WJ, van der Heide JJ, Gans RO, Wolfenbutter BH, de Jong PE, and Smit AJ (2006) Risk factors for chronic transplant dysfunction and cardiovascular disease are related to accumulation of advanced glycation end-products in renal transplant recipients. *Nephrol Dial Transplant* 21:2263-2269.
- Hempel SL, Monick MM, and Hunninghake GW (1994) Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J Clin Invest* 93:391-396.
- Jespersen B, Thiesson HC, Henriksen C, Therland K, Falk C, Poulsen T, Fogh B, Madsen K, Walther S, and Jensen BL (2009) Differential effects of immunosuppressive drugs on COX-2 activity in vitro and in kidney transplant patients in vivo. *Nephrol Dial Transplant* 24:1644-1655.
- Kamei T, Callery MP, and Flye MW (1991) Intra-graft delivery of 16,16-dimethyl PGE2 induces donor-specific tolerance in rat cardiac allograft recipients. *Transplantation* 51:242-246.
- Kay LJ, Yeo WW, and Peachell PT (2006) Prostaglandin E2 activates EP2 receptors to inhibit human lung mast cell degranulation. *Br J Pharmacol* 147:707-713.
- Koh IH, Kim PC, Chung SW, Waddell T, Wong PY, Gorczynski R, Levy GA, and Cohen Z (1992) The effects of 16,16-dimethyl prostaglandin E2 therapy alone and in combination with low-dose cyclosporine on rat small intestinal transplantation. *Transplantation* 54:592-598.
- Lorenowicz MJ, Fernandez-Borja M, and Hordijk PL (2007) cAMP signaling in leukocyte transendothelial migration. *Arterioscler Thromb Vasc Biol* 27:1014-1022.
- Mamputu JC and Renier G (2004) Advanced glycation end-products increase monocyte adhesion to retinal endothelial cells through vascular endothelial growth factor-induced ICAM-1 expression: inhibitory effect of antioxidants. *J Leukoc Biol* 75:1062-1069.
- Miles AM, Sumrani N, Horowitz R, Homel P, Maursky V, Markell MS, Distant DA, Hong JH, Sommer BG, and Friedman EA (1998) Diabetes mellitus after renal transplantation: as deleterious as non-transplant-associated diabetes? *Transplantation* 65:380-384.
- Morichika T, Takahashi HK, Iwagaki H, Yagi T, Saito S, Kubo S, Yoshino T, Akagi T, Mori S, Nishibori M, et al. (2003) Effect of prostaglandin E2 on intercellular adhesion molecule-1 and B7 expression in mixed lymphocyte reaction. *Transplantation* 75:2100-2105.
- 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.
- Nataraj C, Thomas DW, Tilley SL, Nguyen MT, Mannon R, Koller BH, and Coffman TM (2001) Receptors for prostaglandin E(2) that regulate cellular immune responses in the mouse. *J Clin Invest* 108:1229-1235.
- Noguchi K, Iwasaki K, Shitashige M, Umeda M, Izumi Y, Murota S, and Ishikawa I (2001) Down-regulation of lipopolysaccharide-induced intercellular adhesion molecule-1 expression via EP2/EP4 receptors by prostaglandin E2 in human fibroblasts. *Inflammation* 25:75-81.
- Ohashi K, Takahashi HK, Mori S, Liu K, Wake H, Sadamori H, Matsuda H, Yagi T, Yoshino T, Nishibori M, et al. (2010) Advanced glycation end products enhance monocyte activation during human mixed lymphocyte reaction. *Clin Immunol* 134:345-353.
- Rizzo M, SivaSai KS, Smith MA, Trulock EP, Lynch JP, Patterson GA, and Mohanakumar T (2000) Increased expression of inflammatory cytokines and adhesion molecules by alveolar macrophages of human lung allograft recipients with acute rejection: decline with resolution of rejection. *J Heart Lung Transplant* 19:858-865.
- Schiekofer S, Andrassy M, Chen J, Rudofsky G, Schneider J, Wendt T, Stefan N, Humpert P, Fritsche A, Stumvoll M, et al. (2003) Acute hyperglycemia causes intracellular formation of CML and activation of ras, p42/44 MAPK, and nuclear factor kappaB in PBMCs. *Diabetes* 52:621-633.
- Sezer S, Akgul A, Altinoglu A, Arat Z, Ozdemir FN, and Haberal M (2006) Post-transplant diabetes mellitus: Impact of good blood glucose regulation on renal transplant recipient outcome. *Transplant Proc* 38:533-536.
- Shimizu K, Schönbeck U, Mach F, Libby P, and Mitchell RN (2000) Host CD40 ligand deficiency induces long-term allograft survival and donor-specific tolerance in mouse cardiac transplantation but does not prevent graft arteriosclerosis. *J Immunol* 165:3506-3518.
- Siemann G, Blume R, Grapentin D, Oetjen E, Schwaninger M, and Knepel W (1999) Inhibition of cyclic AMP response element-binding protein/cyclic AMP response element-mediated transcription by the immunosuppressive drugs cyclosporin A and FK506 depends on the promoter context. *Mol Pharmacol* 55:1094-1100.
- Suzawa T, Miyaura C, Inada M, Maruyama T, Sugimoto Y, Ushikubi F, Ichikawa A, Narumiya S, and Suda T (2000) The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology* 141:1554-1559.
- Swamy-Mruthinti S, Miriam KC, Kumar SK, Biswas J, Ramakrishnan S, Nagaraj RH, and Sulochana KN (2002) Immunolocalization and quantification of advanced glycation end products in retinal neovascular membranes and serum: a possible role in ocular neovascularization. *Curr Eye Res* 25:139-145.
- Takahashi HK, Iwagaki H, Tamura R, Yagi T, Yoshino T, Mori S, Tanaka N, and Nishibori M (2005) Effect of antibodies against intercellular adhesion molecule-1, B7, and CD40 on interleukin-18-treated human mixed lymphocyte reaction. *J Pharmacol Sci* 97:447-450.
- Takahashi HK, Liu K, Wake H, Mori S, Zhang J, Liu R, Yoshino T, and Nishibori M (2009a) Prostaglandin E2 inhibits advanced glycation end product-induced adhesion molecule expression, cytokine production, and lymphocyte proliferation in human peripheral blood mononuclear cells. *J Pharmacol Exp Ther* 331:656-670.
- Takahashi HK, Mori S, Wake H, Liu K, Yoshino T, Ohashi K, Tanaka N, Shikata K, Makino H, and Nishibori M (2009b) Advanced glycation end products subselectively induce adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells. *J Pharmacol Exp Ther* 330:89-98.
- Takeuchi M and Yamagishi S (2004) TAGE (toxic AGEs) hypothesis in various chronic diseases. *Med Hypotheses* 63:449-452.
- Tamura R, Takahashi HK, Iwagaki H, Yagi T, Mori S, Yoshino T, Nishibori M, and Tanaka N (2004) Effect of  $\beta$ 2-adrenergic receptor agonists on intercellular adhesion molecule (ICAM)-1, B7, and CD40 expression in mixed lymphocyte reaction. *Transplantation* 77:293-301.
- van Hooff JP, Christiaans MH, and van Duijnhoven EM (2004) Evaluating mechanisms of post-transplant diabetes mellitus. *Nephrol Dial Transplant* 19:vi8-vi12.
- Vlassara H and Palace MR (2002) Diabetes and advanced glycation endproducts. *J Intern Med* 251:87-101.
- Zhu SN, Yamada J, Streilein JW, and Dana MR (2000) ICAM-1 deficiency suppresses host allosensitization and rejection of MHC-disparate corneal transplants. *Transplantation* 69:1008-1013.

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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- $\gamma$ , and levels of cAMP were determined by ELISA. Lymphocyte proliferation was determined by [<sup>3</sup>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<sub>2</sub>-receptor antagonist, famotidine, and mimicked by H<sub>2</sub>/H<sub>4</sub>-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<sub>2</sub> 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<sub>2</sub> 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)- $\gamma$  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<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub> and H<sub>4</sub> 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<sub>2</sub> 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- $\gamma$  in PBMCs via H<sub>2</sub> 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- $\gamma$  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  $1 \times 10^6$  cells·mL<sup>-1</sup> from an individual volunteer were mixed with cells from an unrelated person (mixed cells), and the final concentration was adjusted to  $2.0 \times 10^6$  cells·mL<sup>-1</sup>. 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  $\mu$ g·mL<sup>-1</sup> kanamycin and 100  $\mu$ g·mL<sup>-1</sup> 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<sub>2</sub> 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, fluorescein isothiocyanate 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  $2 \times 10^6$  cells·mL<sup>-1</sup> were incubated for 48 h. Cultured cells at  $5 \times 10^5$  cells·mL<sup>-1</sup> 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  $2 \times 10^6$  cells·mL<sup>-1</sup> were used for analyzing IFN- $\gamma$  production. After culturing for 48 h at 37°C in a 5% CO<sub>2</sub>/air mixture, the cell-free supernatant was assayed for IFN- $\gamma$  protein by ELISA employing the multiple Abs sandwich

principle (R&D Systems, Minneapolis, MN, USA). The detection limit of ELISA for IFN- $\gamma$  was 10 pg·mL<sup>-1</sup>.

#### Cell proliferation assay

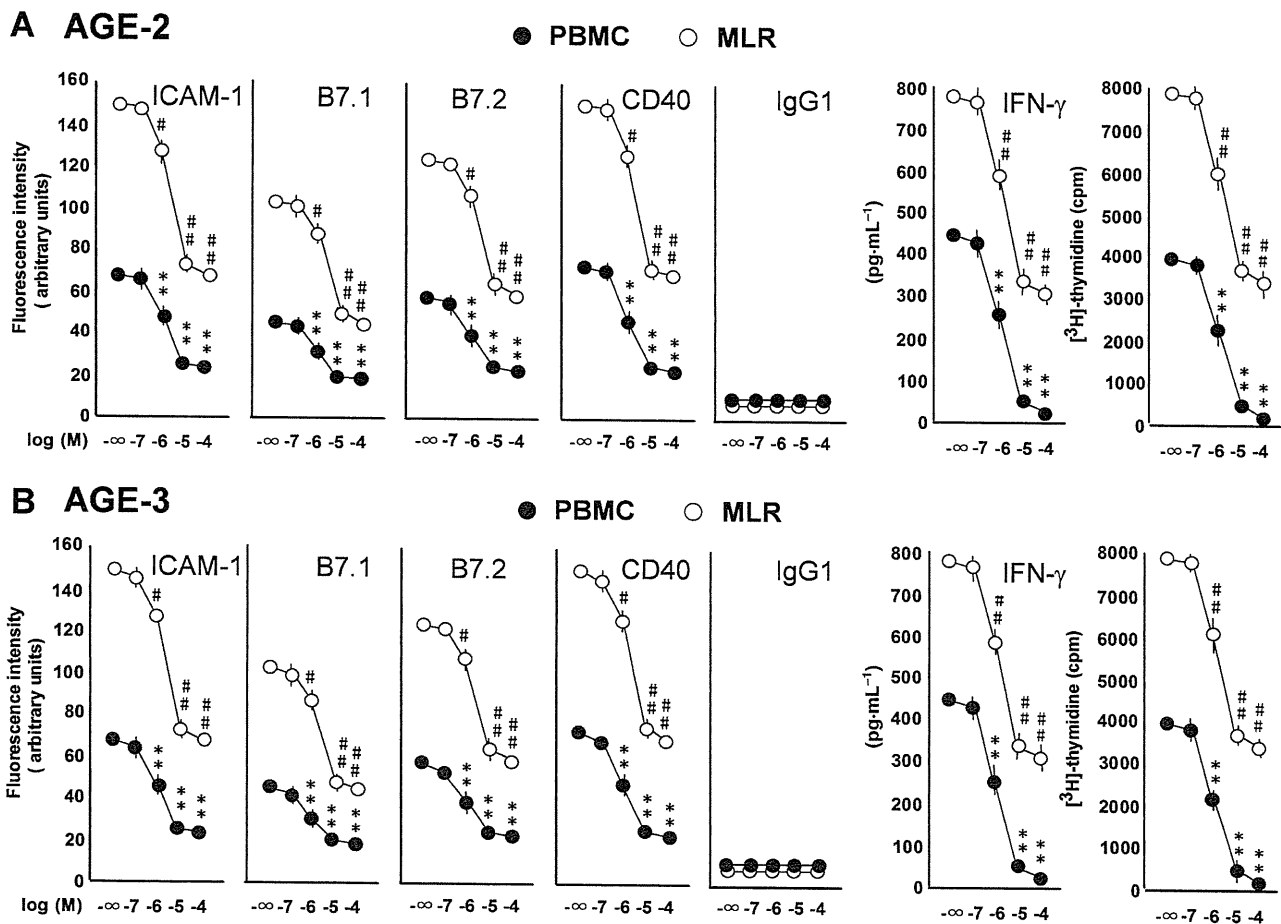
Mixed cells were treated under various conditions. Cultures were incubated for 48 h, during which they were pulsed with [<sup>3</sup>H]-thymidine (3.3 Ci per well) for the final 16 h. Cells were then divided into 96-well microplates, 200  $\mu$ L per well, resulting in 1  $\mu$ Ci [<sup>3</sup>H]-thymidine 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  $\pm$  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)- $\gamma$  and lymphocyte proliferation during the mixed lymphocyte reaction (MLR). Peripheral mononuclear cells (PBMCs) (not mixed cells) and mixed cells at  $2 \times 10^6$  cells·mL<sup>-1</sup> were incubated with AGE-2 (A) and AGE-3 (B) ( $100 \mu\text{g}\cdot\text{mL}^{-1}$ ) and histamine (0.1–100  $\mu\text{M}$ ) for 48 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. FITC-conjugated IgG<sub>1</sub> was used as an isotype-matched control Ab. IFN- $\gamma$  concentration in conditioned media was determined by ELISA. Lymphocyte proliferation was determined by [<sup>3</sup>H]-thymidine uptake. The results are expressed as the means  $\pm$  SEM of five donors with triplicate determinations. \*\* $P < 0.01$  compared with the value for AGE-2 and AGE-3 in PBMCs. # $P < 0.05$ ; ## $P < 0.01$  compared with the value for AGE-2 and AGE-3 during MLR. When an error bar is within a symbol, the bar is omitted.