(Manara et al., 1996). In the present study, we found that SR59230A had no effect on the actions of norepinephrine, epinephrine and isoproterenol in the presence of AGE-2 and AGE-3, and that BRL37344 had no effect on AGE-2- and AGE-3-induced expressions of adhesion molecules and the cytokine production (data not shown).

Abnormal vascular endothelial function and intimal thickening are common in patients with diabetes mellitus, and increased superoxide production accounts for a significant proportion of the NO reduction associated with diabetes (Vasquez-Vivar et al., 1998; Meininger et al., 2000; Du et al., 2001). β_2-adrenoceptor stimulation is reported to improve endothelial function by increasing NO release (Liao et al., 2004). It is reported that celiprolol, a β_1 inhibitor with an agonistic effect at β_2-adrenoceptors, but not atenolol, a β_1-selective inhibitor, improves endothelial function and prevents the atherosclerotic morphological changes seen in diabetic rats regardless of whether or not they were subjected to NOS inhibition (Hayashi et al., 2007). Therefore, β_2-adrenoceptor-induced inhibition of toxic AGE-dependent responses in monocytes may partially contribute to regulation of the development of atherosclerotic plaques in diabetes. The present study explored the therapeutic potential of β_2-adrenoceptor agonist on the systemic inflammatory response evoked by diabetes.

5. Conclusion

The stimulation of β_2-adrenoceptor inhibited AGE-2- and AGE-3-induced expressions of ICAM-1 and CD40 and the production of IFN-γ and TNF-α.

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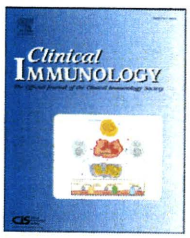

Advanced glycation end products enhance monocyte activation during human mixed lymphocyte reaction

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KEYWORDS
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Abstract Posttransplant diabetes mellitus (PTDM) is a frequent complication among transplant recipients. Ligation of advanced glycation end products (AGEs) with their receptor (RAGE) on monocytes/macrophages plays roles in the diabetes complications. The enhancement of adhesion molecule expression on monocytes/macrophages activates T-cells, leading to reduced allograft survival. We investigated the effect of four distinct AGE subtypes (AGE-2/AGE-3/AGE-4/AGE-5) on the expressions of intracellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes, the production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α and the proliferation of T-cells during human mixed lymphocyte reaction (MLR). AGE-2 and AGE-3 selectively induced the adhesion molecule expression, cytokine production and T-cell proliferation. The AGE-induced up-regulation of adhesion molecule expression was involved in the cytokine production and T-cell proliferation. AGE-2 and AGE-3 up-regulated the expression of RAGE on monocytes; therefore, the AGEs may activate monocytes, leading to the up-regulation of adhesion molecule expression, cytokine production and T-cell proliferation.

Introduction

Diabetes mellitus (DM) is characterized by hyperglycemia, which facilitates the formation of AGEs both in blood and intracellularly [1,2]. AGEs are formed by a non-enzymatic reaction between a carbonyl group of reducing sugars and free amino groups from macromolecules such as proteins, lipopro-

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teins and nucleic acids. AGEs accumulate in the plasma and tissues of patients with diabetes, leading to the pathogenesis of diabetes complications [3,4]. Tissue deposition of AGEs contributes to macrophage-mediated injury in diabetic complications that correlate with the severity and duration of hyperglycemia [5,6]. Interaction of AGEs with the receptor for AGEs (RAGE) on monocytes/macrophages at sites of chronic inflammation stimulates macrophage activation and cytokine production [7,8]. Direct immunochemical evidence for the existence of six distinct AGE structures, including AGE-2, AGE-3, AGE-4, AGE-5 and AGE-6, was provided from the analysis of AGEs within modified proteins and peptides [9]. Recently, it was examined whether toxic AGE structures, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3), have diverse biological activities on vascular endothelial cells, vascular smooth muscle cells, mesangial cells, Schwann cells, malignant melanoma cells and cortical neurons [10,11]. Among the various subtypes of AGE, it has been shown that AGE-2 and AGE-3 are the main structures of AGEs detectable in the serum of diabetic patients [9].

Monocyte-derived costimulatory signals plays roles 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 [12,13]. 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 [14,15]. Therefore, it is suggested that the regulation of adhesion molecule expression on monocytes might decrease lymphocyte proliferation and cytokine production during MLR [16–18].

Good glycemic control inhibits the vascular complications of diabetes, resulting in reduced allograft loss [19]. PTDM affects approximately 20–40% of orthotopic liver transplant (OLT) recipients [20–22]. Sumrani et al. [23] and Miles et al. [24] reported a high incidence of graft loss in PTDM compared with nondiabetic patients. Atherosclerotic disease is documented in 12% patients with higher frequency among patients with PTDM than nondiabetic patients [25]. Many risk factors for atherosclerosis, including hypertension and hyperlipidemia, play important roles in the development of chronic allograft nephropathy and graft loss [26]. Hyperglycemia is also a marker for insulin resistance, which is characterized by hypertension, dyslipidemia, hyperinsulinemia, and increased levels of inflammatory cytokines [27,28]. However, the mechanism of impaired graft survival in PTDM patients is uncertain. In the present study, we examined the effect of AGE-2, AGE-3, AGE-4 and AGE-5 on the expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes and its impact on the production of IFN-γ and TNF-α and the proliferation of T-cells during human MLR.

Materials and methods

Reagents

AGE-modified bovine serum albumin (BSA) was prepared as previously described [29]. Briefly, BSA at 50 mg/ml (Sigma) in $NaPO_4$ buffer (0.2 M, pH 7.4) was incubated with D-glyceraldehyde (AGE-2) at 0.2 M, D-glycoaldehyde (AGE-3) at 0.2 M, methylglyoxal (AGE-4) at 0.2 M or glyoxal (AGE-5) at 0.2 M (Wako, Tokyo, Japan) at 37 °C for 7 days in the presence of 1.5 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 1.0×10^5 U/l penicillin under endotoxin-free conditions. BSA was incubated under the same conditions. AGE-BSA and BSA were dialyzed for 2 days at 4 °C. The endotoxin concentration of AGEs at 100 μg/ml described above was measured at SRL (Okayama, Japan) and it was found to be 1.2 pg/ml. AGE-specific fluorescence was measured at 450 nm after excitation at 390 nm with a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). sRAGE was prepared as previously described [30]. Briefly, sRAGE was cloned into the *E. coli* expression vector pASK-IBA32; some modifications to the pASK-IBA32 polylinker region were made for cloning purposes. sRAGE, from amino acid number 23 to 340 [31], was amplified by PCR using the following oligonucleotides (MWG Biotech, High Point, SC) (the underlined bases designate restriction sites): 5′-CTGACC-TATGCGGCCGCTGCTCAAAACATCACAGC-3′ and 5′-GACTGAAT-TCATCAGTGATGATGGTGATGGTGAGTTCCCAGCCCTGATCC-3′.

Culture conditions during MLR

Normal human PBMC were obtained from 10 healthy volunteers after acquiring IRB approval (Okayama Univ. IRB No.106). Samples of 20–50 ml peripheral blood were withdrawn from a forearm vein, after which PBMC were prepared [32,33]. PBMC at 1×10^6 cells/ml from an individual volunteer were mixed with cells from an unrelated person (mixed cells), and the final concentration of cells was adjusted to 2.0×10^6 cells/ml. The mixed cells were subsequently suspended in RPMI 1640 medium (Nissui, Co. Ltd., Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 20 μg/ml kanamycin and 100 μg/ml streptomycin and penicillin (Sigma-Aldrich, St. Louis, MO), and they were incubated under various conditions for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. All reagents were added to the media at the start of MLR.

Flow cytometric analysis for adhesion molecule expression

For flow cytometric analysis, FITC-conjugated mouse IgG1 monoclonal (m) Ab against ICAM-1/CD54 and 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), and FITC-conjugated IgG1 isotype-matched control was obtained from Sigma-Aldrich. Changes in the expression of human leukocyte antigens, ICAM-1, B7.1, B7.2 and CD40 were determined by anti-ICAM-1, anti-B7.1, anti-B7.2 or anti-CD40 Ab with anti-CD14 Ab. In addition, to analyze changes in the expression of human RAGE on monocytes were examined by multi-color flow cytometry using a combination of PE-conjugated anti-CD3 (T-cell), anti-CD14 (monocyte), and anti-CD19 (B cell) (HD37) mouse IgG (DAKO) and monoclonal anti-human RAGE mouse IgG2 (R&D Systems, Minneapolis, MN) followed by FITC-conjugated anti-mouse IgG goat IgG (DAKO), respec-

tively. FITC-conjugated mouse IgG2 (DAKO) is used as an isotype control of anti-human RAGE. The mixed cells at 2 × 10^6 cells/ml were incubated for 48 h. Cultured cells at 5 × 10^5 cells/ml were prepared for flow cytometric analysis as previously described [32,33] and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA). Data were processed using the CELL QUEST program.

ELISA assays

The mixed cells at 2 × 10^6 cells/ml were used for analyzing IFN-γ and TNF-α production. After culturing for 48 h at 37 °C in a 5%CO_2/air mixture, the cell-free supernatant was assayed for IFN-γ, and TNF-α protein by ELISA employing the multiple Ab sandwich principle (R&D Systems, Minneapolis, MN). The detection limit of ELISA for IFN-γ and TNF-α was 10 pg/ml.

Proliferation assay

The mixed cells were treated with various conditions. Cultures were incubated for 48 h, during which they were pulsed with [^{3}H]thymidine (3·3 Ci/well) for the final 16 h. Cells were then divided into 96-well microplates, 200 μl/well, resulting in 1 μCi [^{3}H]thymidine per well, and harvested by the Micro-Mate 196 Cell Harvester (Perkin Elmer Life Science, Inc., Boston, MA). 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 to indicate statistical significance. The results are expressed as the means ± SEM of triplicate findings from five donors.

Results

Time-course effects of AGEs on the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the proliferation of T-cells during human MLR

To determine the appropriate incubation time, we examined the kinetics as shown in Fig. 1. AGE-2 and AGE-3 at 100 μg/ml significantly enhanced the expressions of ICAM-1, B7.1, B7.2 and CD40 at 16 h and thereafter up to 24 and 48 h, whereas AGE-4, AGE-5 and BSA at 100 μg/ml moderately increased at 16 h and thereafter up to 24 and 48 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 at 72 h were inhibited and were the same level as at 0 h. In the absence of AGEs, the expressions of ICAM-1 and CD40, the production of IFN-γ and TNF-α and the proliferation of T-cells were elevated at 16 h and thereafter up to 24 and 48 h [34]. AGE-2 and AGE-3 at 100 μg/ml also time-dependently induced the production of IFN-γ and TNF-α and the proliferation of T-cells. AGE-4, AGE-5 and BSA did not induce cytokine production, whereas AGE-4, AGE-5 and BSA moderately increased the proliferation of T-cells at 16 h and thereafter up to 24 and 48 h. The viability of MLR-cultured monocytes at 72 h was 10–15% that at 0 h.

Dose–response relationship of AGEs on the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the proliferation of T-cells during human MLR

The effects of AGE-2, AGE-3, AGE-4, AGE-5 and BSA at concentrations ranging from 100 ng/ml to 100 μg/ml on the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the proliferation of T-cells were determined at 48 h (Fig. 2). AGE-2 and AGE-3 concentration-

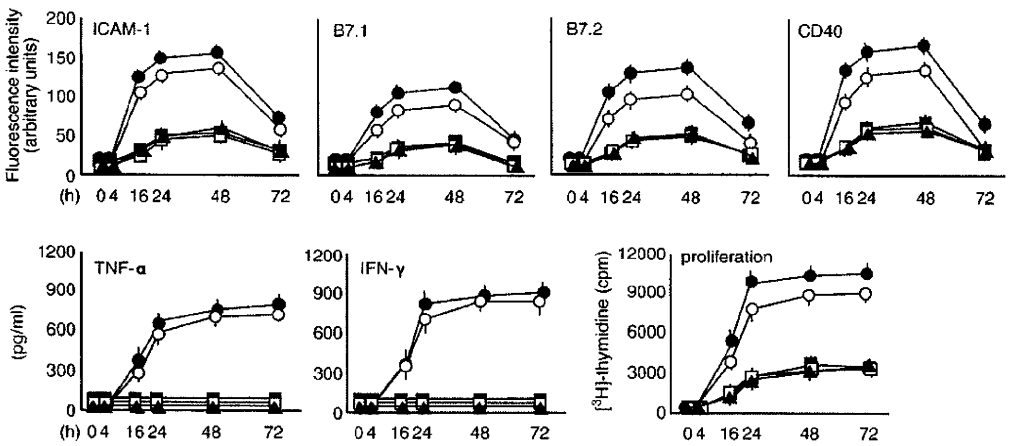

Figure 1 The time-course effects of AGEs on the expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the proliferation of T-cells during human MLR. Mixed cells at 2 × 10^6 cells/ml were incubated with AGE-2 (●), AGE-3 (○), AGE-4 (■), AGE-5 (□) or BSA (▲) at 100 μg/ml for the indicated periods. The expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes was determined by flow cytometry. The production of IFN-γ and TNF-α was determined by ELISA, and the proliferation of T-cells was determined by [^{3}H]thymidine uptake. The results are expressed as the means ± SEM of triplicate findings from five distinct responder stimulator pairs.

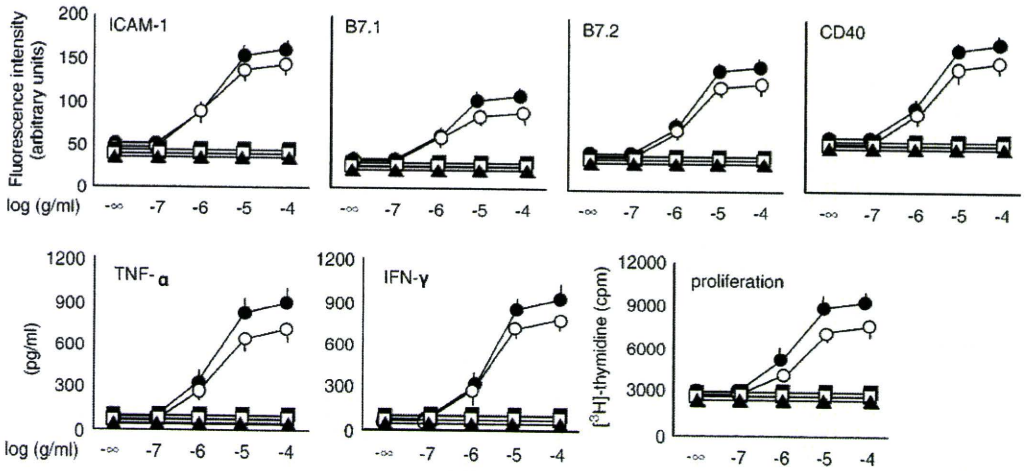

Figure 2 The dose–response relationship of AGEs with the expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the proliferation of T-cells during human MLR. Mixed cells at 2×10^6 cells/ml were incubated with AGE-2 (●), AGE-3 (○), AGE-4 (■), AGE-5 (□) or BSA (▲) at increasing concentrations from 0.1 to 100 μg/ml for 48 h. The expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes was determined by flow cytometry. The production of IFN-γ and TNF-α was determined by ELISA, and the proliferation of T-cells was determined by [^{3}H]thymidine uptake. The results are expressed as the means ± SEM of triplicate findings from five distinct responder stimulator pairs.

dependently induced adhesion molecule expression with similar potency, whereas AGE-4, AGE-5 and BSA showed no effect. Consistent with the up-regulation of adhesion molecules, AGE-2 and AGE-3 concentration-dependently induced the production of IFN-γ and TNF-α and the proliferation of T-cells. When we assumed that the effects of AGE-2 and AGE-3 were maximal at the concentration of 100 μg/ml, the ED50 values of AGE-2 for the induction of ICAM-1 expression and TNF-α production were calculated to be 3 and 5 μg/ml, and those of AGE-3 were 3 and 6 μg/ml, respectively. However, AGE-4, AGE-5 and BSA had no effect on cytokine production.

Involvement of adhesion molecule in the effect of AGE-2 and AGE-3 on cytokine production and T-cell proliferation

We examined the involvement of ICAM-1, B7.1, B7.2 and CD40 in the AGE-2- and AGE-3-induced production of IFN-γ and TNF-α and proliferation of T-cells during MLR. As shown in Fig. 3, anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs inhibited the AGE-2 and AGE-3-induced production of IFN-γ and TNF-α and proliferation of T-cells in a concentration-dependent manner. The maximal inhibitory effect obtained by each Ab at 10 μg/ml varied and the rank order of inhibition was as follows: ICAM-1 > CD40 > B7.1 > B7.2.

Expression of RAGE on monocytes

The expression of RAGE on monocytes, T-cells and B-cells was determined by double-stained flow cytometry with anti-CD14, anti-CD3, anti-CD19 and anti-RAGE Abs (Fig. 4A). The expression of RAGE on T-cells and B-cells was not detected regardless of the presence of AGEs. AGE-2 and AGE-3 at 100 μg/ml remarkably enhanced the expression of RAGE on monocytes 48 h after stimulation, but AGE-4 and AGE-5 had no effect (Figs. 4A and B). To confirm the binding specificity of anti-RAGE Ab to RAGE, we used a combination of anti-RAGE Ab with a soluble form of RAGE (sRAGE) for flow cytometry in the presence or absence of AGE-2 and AGE-3 (Fig. 4B). The detection of RAGE in the presence or absence of AGE-2 and AGE-3 was inhibited completely by the addition of sRAGE, indicating that anti-RAGE Ab recognized the extracellular domain of RAGE.

Discussion

In a previous study, to evaluate the binding of AGE subtypes to RAGE, we established the *in vitro* assay using immobilized AGE subspecies and His-tagged sRAGE protein [34]. AGE-2 and AGE-3 showed relatively high affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed moderate affinity for sRAGE. In addition, we found that, among AGEs, AGE-2 and AGE-3 induced the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes and the production of IFN-γ and TNF-α in PBMC [35]. AGE-2 and AGE-3 induced the production of IFN-γ and TNF-α in monocytes isolated from PBMC, but not in T-cells isolated from PBMC. Blockade of interaction of monocytes and T-cells by anti-ICAM-1, B7.1, B7.2 or CD40 Ab inhibited AGE-2- and AGE-3-induced IFN-γ and TNF-α in PBMC. Anti-IFN-γ and anti-TNF-α Abs up to 100 ng/ml, which blocked the effect of exogenous IFN-γ and TNF-α at 100 ng/ml on adhesion molecule expression, had no effect on the actions of AGE-2 and AGE-3 at 100 μg/ml. Therefore, while adhesion molecule expression on monocytes was independent of endogenous cytokine production in the presence of AGE-2 and AGE-3, the induction of IFN-γ and TNF-α depended on the engagement of monocytes and T-cells through the up-regulation of ICAM-1, B7.1, B7.2 and CD40.

The present study suggests the mechanism of association between PTDM and reduced graft survival. To determine the appropriate incubation time, when investigating whether

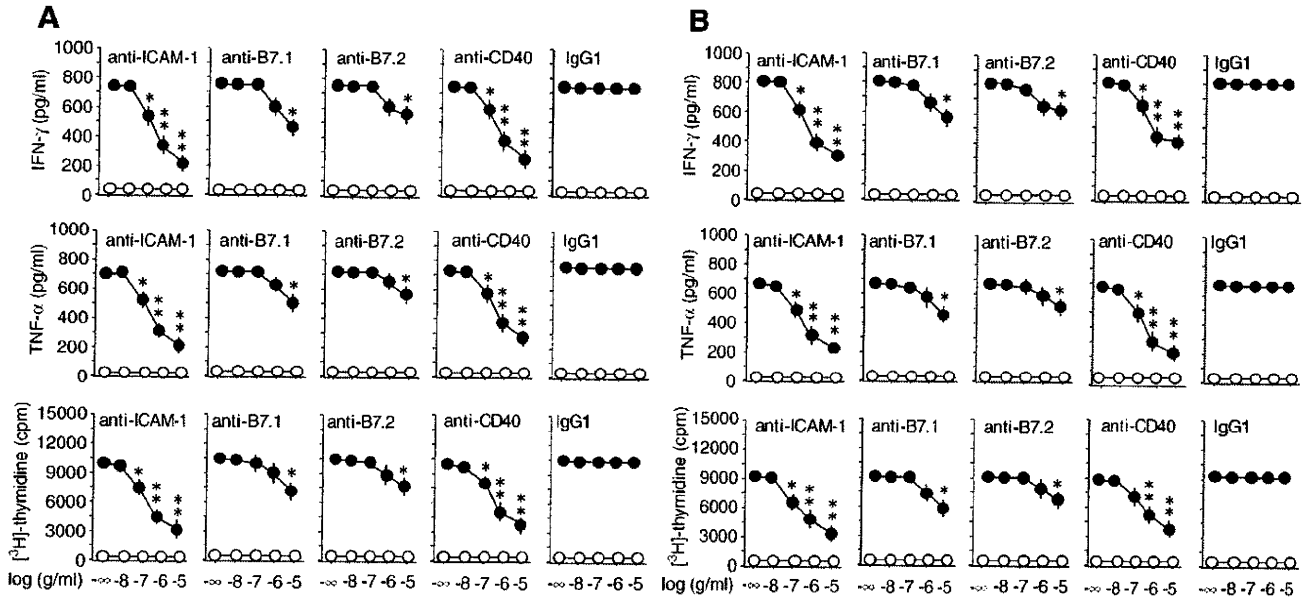

Figure 3 The involvement of adhesion molecule in the effect of AGE-2 and AGE-3 on cytokine production and T-cell proliferation. Mixed cells at 2×10^6 cells/ml were incubated with anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs at increasing concentrations from 0.1 to 100 ng/ml in the absence (○) or presence (●) of AGE-2 (A) or AGE-3 (B) at 100 μg/ml for 48 h. The production of IFN-γ and TNF-α was determined by ELISA, and the proliferation of T-cells was determined by [^{3}H]thymidine uptake. The results are expressed as the means ± SEM of triplicate findings from five distinct responder stimulator pairs. *$P<0.05$, **$P<0.01$ compared with the value for AGE-2 or AGE-3 alone. When an error bar was within a symbol, the bar was omitted.

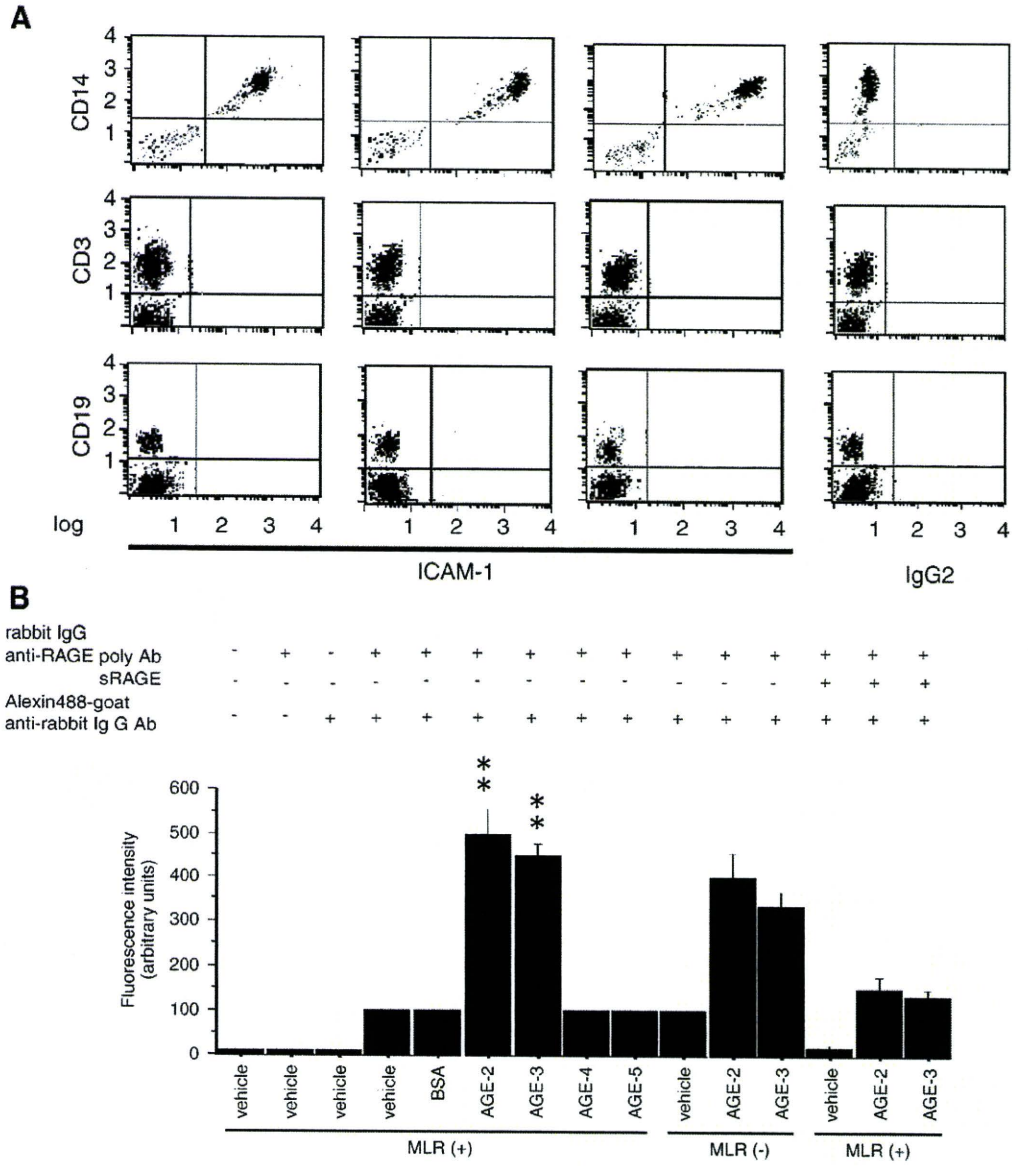

Figure 4 The expression of RAGE on monocytes. The expression of RAGE on monocytes in the presence or absence of AGE-2, AGE-3, AGE4, AGE5 and BSA at 100 μg/ml 48 h after incubation was examined by flow cytometry (A and B). Mixed cells were double-stained with cell-specific antigen (CD14, CD3 or CD 19) and RAGE. Mouse IgG2 is used as an isotype control. The results of flow cytometry obtained under different conditions were quantified. To block the engagement of anti-RAGE Ab with RAGE, anti-RAGE Ab was mixed with sRAGE for 24 h prior to use. The results are expressed as the means ± SEM of triplicate findings from five distinct responder stimulator pairs. $^{**}P<0.01$ compared with the corresponding value for medium alone.

AGEs affect the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the proliferation of T-cells during MLR, we examined the kinetics as shown in Fig. 1, and investigated the effects of AGEs after 48-h incubation. In the present study, we examined, for the first time, the effect of AGE subtypes on the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the proliferation of T-cells AGE-2 and AGE-3, but not AGE-4 and AGE-5, increased the expression of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the proliferation of T-cells (Figs. 1 and 2). Our results may support the idea that AGE-2 and AGE-3 function as toxic AGEs and play a central role in the pathophysiological processes associated with AGE formation.

It is reported that RAGE are expressed on monocytes [36,37]. The interaction of AGE-2 with RAGE alters intracellular signaling, gene expression and the release of pro-inflammatory molecules in monocytes and macrophages [38]. The interaction of AGEs and RAGE was reported to result in the up-regulation of RAGE through the activation of nuclear factor-kappa B in monocytes [39]. In the previous study, we also found the up-regulation of RAGE expression on monocytes by AGE-2 and AGE-3 [34]. As shown in Fig. 4, we found the AGE-2- and AGE-3-enhanced expression of RAGE on

monocytes during MLR. It is reported that blockade of RAGE reduced rate of recurrent diabetes and prolonged allograft survival by RAGE blockade and in RAGE-deficient mice, suggesting that RAGE may be an important new target for therapeutic strategies to prevent adaptive immune responses [40].

The plasma interleukin (IL)-18 level is elevated during acute rejection [41] and graft-versus-host disease [42], suggesting that IL-18 might be involved in the pathogenesis of rejection. Recently, we reported that IL-18 induced the production of IL-12 and IFN-γ during MLR [16,17]. We found that the levels of IL-18, IL-12, IL-2 and IL-10 were under the detection limit, 10 pg/ml, in the presence of AGE-2 and AGE-3 (data not shown). Anti-IL-18, IL-12, IL-2 or IL-10 Abs had no effect on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40 and the production of IFN-γ and TNF-α (data not shown), indicating that the actions of AGE-2 and AGE-3 might also be independent of IL-18 and IL-12. As shown in Fig. 3, AGE-2- and AGE-3-enhanced production of IFN-γ and TNF-α may require the enhancement of cell-to-cell interaction between monocytes and T-cells through the induction of plural adhesion molecule expressions on monocytes.

Immunosuppressive medications used after transplantation are risk factors for aggravating PTDM among recipients who have insulin resistance before transplantation [43,44]. Cyclosporin A reduces HCV replication *in vitro* [45], whereas PTDM is a common side effect of cyclosporin and tacrolimus [46], suggesting that calcineurin inhibitors impact on HCV, leading to PTDM. Evidence of direct islet toxicity is observed in pancreas allograft biopsies from patients receiving calcineurin inhibitors [47]. Many patients develop DM early after transplantation, when exposure to tacrolimus and steroids is highest [48,49]. The cause of impaired graft survival in PTDM patients depends on the use of lower dosages of immunosuppressive agents in diabetic patients. It is reported that the accumulation of AGEs is elevated in recipients with chronic renal dysfunction and cardiovascular disease after renal transplantion [50]. Therefore, we suggested that the inhibition of AGE-induced actions should be a target for clinical use in PTDM patients. In conclusion, among AGEs, AGE-2 and AGE-3 induced the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the proliferation of T-cells during MLR. The production of IFN-γ and TNF-α depended on the engagement of monocytes and T-cells through the up-regulation of ICAM-1, B7.1, B7.2 and CD40. The actions of AGE-2 and AGE-3 might be through the stimulation and up-regulation of RAGE. Together with these results, toxic AGE-dependent responses, including the enhancement of adhesion molecule expression on monocytes, may partially facilitate rejection in PTDM patients.

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Effect of Nicotine on Advanced Glycation End Product-Induced Immune Response in Human Monocytes

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ABSTRACT

The up-regulation of adhesion molecule expressions on monocytes enhances cell-to-cell interactions with T cells, leading to cytokine production. Advanced glycation end products (AGEs) are modifications of proteins/lipids that become nonenzymatically glycated after contact with aldose sugars. Among various subtypes of AGEs, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3) induce the expressions of intercellular adhesion molecule-1, B7.1, B7.2, and CD40 on monocytes, the production of interferon-γ and tumor necrosis factor-α, and the lymphocyte proliferation in human peripheral blood mononuclear cells. Nicotine is reported to inhibit the activation of monocytes via nicotinic acetylcholine receptor α7 subunit (α7-nAChR). In the present study, we found that nicotine inhibited the actions of AGE-2 and AGE-3. A nonselective and selective α7-nAChR antagonist, mecamylamine and α-bungarotoxin, reversed the inhibitory effects of nicotine, suggesting the involvement of α7-nAChR stimulation. Nicotine induced the expression of cyclooxygenase-2, prostaglandin E_2 (PGE_2), and cAMP in the presence and absence of AGE-2 and AGE-3. PGE_2 is known to activate the EP_2/EP_4 receptor, increasing the cAMP level and protein kinase A (PKA) activity. The actions of nicotine were reversed in part by an EP_2-receptor antagonist, AH6809, an EP_4-receptor antagonist, AH23848, and a PKA inhibitor, *N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89). These results indicate that the mechanism of action of nicotine may be partially via endogenous PGE_2 production.

Advanced glycation end products (AGEs), products of the nonenzymatic glycation/oxidation of proteins/lipids, accumulate during natural aging and are also greatly augmented in disorders, such as diabetes, renal failure, and Alzheimer's disease (Schmidt et al., 1994; Brownlee, 1995; Takedo et al., 1996). Direct immunochemical evidence for the existence of four distinct AGE structures, including AGE-2, AGE-3, AGE-4, and AGE-5, has been identified within AGE-modified proteins and peptides (Takeuchi and Yamagishi, 2004). Among various subtypes of AGE, toxic AGE structures, AGE-2 and AGE-3, are the main structures of AGEs that are detectable in the serum of diabetic patients (Takeuchi and Yamagishi, 2004). AGE-2 and AGE-3 also have diverse biological activities on vascular wall cells, mesangial cells, Schwann cells, malignant melanoma cells, and cortical neurons (Okamoto et al., 2002; Yamagishi and Imaizumi, 2005). It is reported that AGEs ligate the cell-surface receptor for AGE (RAGE) on the vascular endothelium, monocytes, vascular smooth muscle, and neurons to activate cell-signaling pathways, such as p44/p42 mitogen-activated protein kinase and nuclear factor-κB (Yan et al., 1994; Lander et al., 1997), leading to the progression of pathogenesis of diabetic microvascular disease (Schmidt et al., 1994). It is noteworthy that AGEs up-regulate RAGE expression in various tissues, facilitating the AGE-RAGE response by forming a positive feed-

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ABBREVIATIONS: AGE, advanced glycation end product; BSA, bovine serum albumin; COX, cyclooxygenase; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; ICAM, intercellular adhesion molecule; IFN, interferon; m, monoclonal; IL, interleukin, LPS, lipopolysaccharide; α7-nAChR, nicotinic acetylcholine receptor α7 subunit; PBMC, peripheral blood mononuclear cell; PGE_2; prostaglandin E_2; PKA, protein kinase A; RAGE, receptor for advanced glycation end product; Ab, antibody; mAb, monoclonal antibody; SMC, smooth muscle cell; TNF, tumor necrosis factor; H89, *N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; NS398, *N*-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide; AH6809, 6-isopropoxy-9-xanthone-2-carboxylic acid; AH23848, (4*Z*)-7-[(rel-1*S*,2*S*,5*R*)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid hemicalcium salt hydrate; ONO-AE1–259-01, 11,15-O-dimethyl prostaglandin E_2; ONO-AE1–329, 16-(3-methoxymethyl)phenyl-ω-tetranor-3,7-dithia prostaglandin E_1.

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-γ and TNF-α, 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-γ and TNF-α 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 α7-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_2, 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_2 receptor subtypes EP_1, EP_2, EP_3, and EP_4, activation of EP_2 and EP_4 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_2 inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.2, and CD40, the production of IFN-γ and TNF-α, and the lymphocyte proliferation via the EP_2 and EP_4 receptors (Takahashi et al., 2009b). The cAMP/PKA pathway was involved in the actions of PGE_2. Nicotine is reported to induce the expressions of COX-2 and PGE_2 in whole blood and microgria through α7-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-γ and TNF-α, and the lymphocyte proliferation in human PBMCs; we also investigated the involvement of PGE_2 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 μg/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), α-bungarotoxin, mecamylamine, 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 1×10^6 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 1×10^6 cells/ml were incubated for 24 h. Cultured cells at 5×10^5 cells/ml were prepared for flow cytometric analysis as described previously (Takahashi et al., 2003) and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA). The data were processed by use of the CellQuest program.

ELISA Assays. PBMCs at 1×10^6 cells/ml were used to analyze IFN-γ and TNF-α production, and monocytes at 1×10^6 cells/ml were used to analyze PGE_2 production. After culturing for 24 h at 37°C in a 5% CO_2/air mixture, cell-free supernatants were assayed for IFN-γ, TNF-α (R&D Systems, Minneapolis, MN) and PGE_2 protein (Cayman Chemical) by ELISA using the multiple Abs sandwich principle. The detection limits of ELISA for IFN-γ, TNF-α, and PGE_2 were 10 pg/ml.

Proliferation Assay. PBMCs were divided into 96-well microplates, 200 μl/well, and were incubated with various conditions for 48 h, during which they were pulsed with 1 μCi of [^{3}H]thymidine per well for the final 16 h. Then, the cells were harvested by the MicroMate 196 Cell Harvester (PerkinElmer Life and Analytical Sciences, Waltham, MA). Thymidine incorporation was measured by a β-counter (Matrix 9600; PerkinElmer Life and Analytical Sciences).

Western Immunoblotting. Monocytes at 1×10^6 cells/ml were incubated with nicotine in the presence or absence of AGE-2 and AGE-3 at 37°C in a 5% CO_2-air mixture for 30 min. After incubation, the cells were washed twice in phosphate-buffered saline before the addition of 60 μl 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 μl 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 β-actin (Sigma-Aldrich).

Measurement of cAMP Production in Monocytes. Monocytes at 1×10^6 cells/ml were incubated at 37°C in a 5% CO_2/air mixture. After 30 min, cells at 2×10^5 cells/200 μl/well were supplemented with trichloroacetic acid to a final concentration of 5% and 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase at 100 μM and frozen at −80°C. Frozen samples were subsequently sonicated and assayed for cAMP 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 ± 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-γ and TNF-α 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 μg/ml significantly induced the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN-γ and TNF-α 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 μg/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 μg/ml for 24 h were examined. AGE-2 and AGE-3 at 10 and 100 μg/ml significantly induced the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN-γ and TNF-α 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 μM on 100 μg/ml AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN-γ and TNF-α 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-γ and TNF-α, and the lymphocyte proliferation (Fig. 2, A and B). IC_{50} values for the inhibitory effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40, the production of IFN-γ and TNF-α, and the lymphocyte proliferation in the presence of AGE-2 were 2, 1, 1, 2, 0.9, 1, and 0.9 μM, and those in the presence of AGE-3 were 1, 1, 2, 1, 0.9, 1, and 1 μM, respectively. In the presence of BSA at 100 μg/ml, nicotine had no effect on the expressions of adhesion molecule (Fig. 1C), cytokine production, and lymphocyte proliferation (Fig. 2C).

Effects of α7-nAChR Antagonists on the Actions of Nicotine. To determine the involvement of α7-nAChR in nicotine activity, we examined the effect of a nonselective α7-nAChR antagonist, mecamylamine, and a selective α7-nAChR antagonist, α-bungarotoxin, ranging from 0.1 to 100 μM on 100 μM nicotine-induced inhibition of ICAM-1, B7.1,

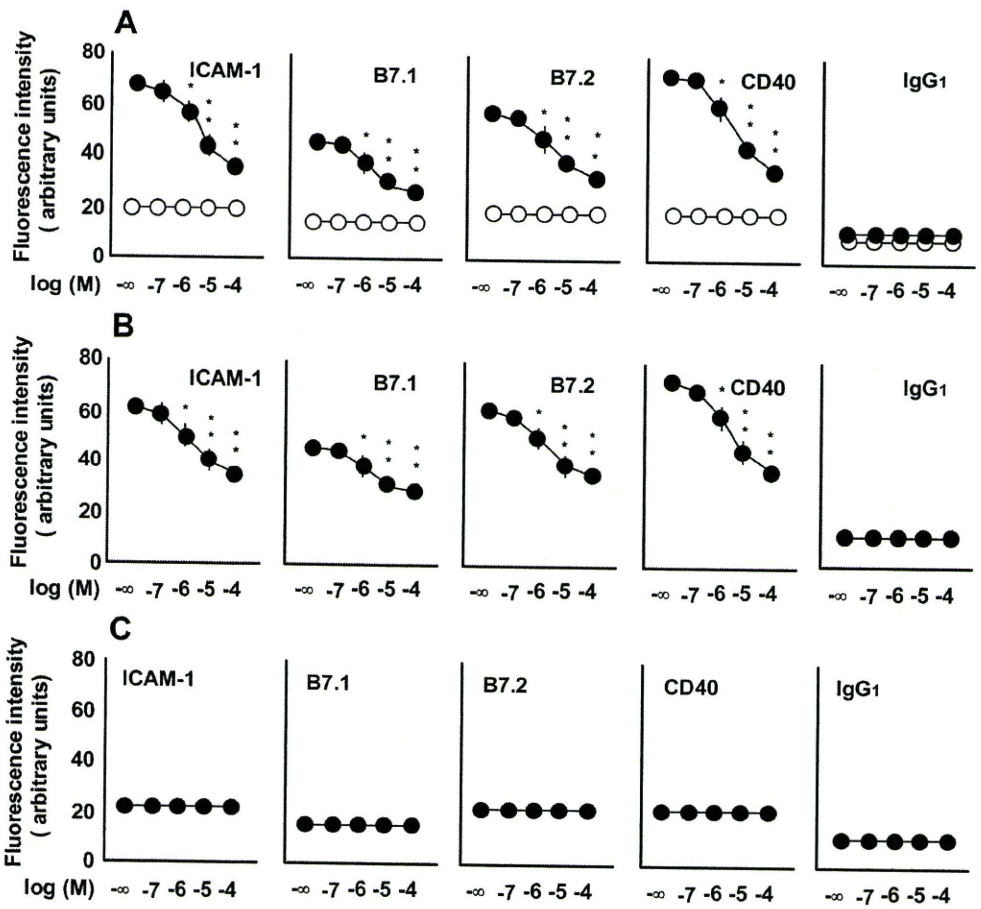

Fig. 1. The effects of nicotine on AGE-2-, AGE-3-, and BSA-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes. PBMCs at 1×10^6 cells/ml were incubated with AGE-2 (A), AGE-3 (B), and BSA (C) at 100 μg/ml and nicotine at increasing concentrations from 0.1 to 100 μM for 24 h. The expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes were determined by flow cytometry. FITC-conjugated IgG1 was used as an isotype-matched control Ab. 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 ± 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.

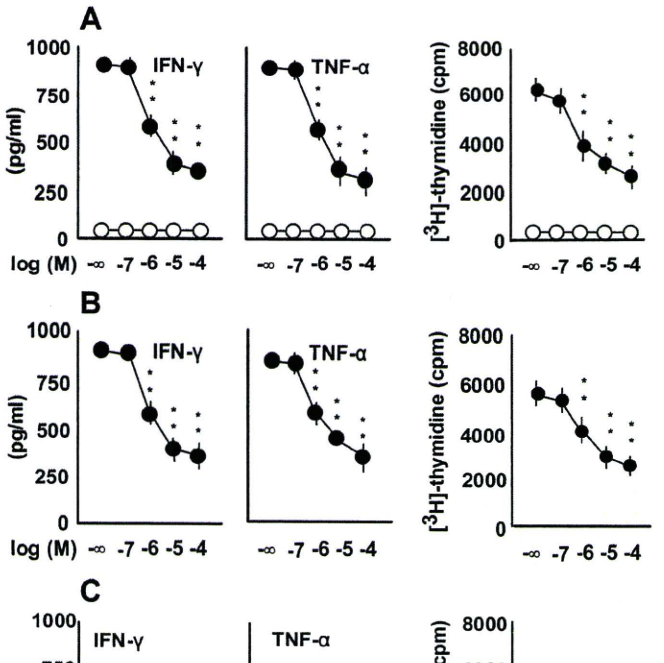

Fig. 2. The effects of nicotine on AGE-2-, AGE-3-, and BSA-induced production of IFN-γ and TNF-α and the lymphocyte proliferation in PBMCs. PBMCs at 1×10^6 cells/ml were incubated with AGE-2 (A), AGE-3 (B), and BSA (C) at 100 μg/ml and nicotine at increasing concentrations from 0.1 to 100 μM for 24 h. IFN-γ and TNF-α 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 ± 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-α and IFN-γ, and the lymphocyte proliferation in PBMCs (Fig. 4) in the presence or absence of AGE-2 and AGE-3 at 100 μg/ml. The α7-nAChR antagonists reversed the inhibitory effects of nicotine. Mecamylamine and α-bungarotoxin had no effect in the absence of nicotine (data not shown).

Effects of Nicotine on Expression of COX-2 and Production of PGE_2 in Monocytes. As shown in Fig. 5A, nicotine at 100 μM induced the expression of COX-2 in monocytes in the presence or absence of AGE-2 and AGE-3 at 100 μg/ml, but AGE-2 or AGE-3 alone had no effect. As shown in Fig. 5, B and C, nicotine induced PGE_2 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 α-bungarotoxin (Fig. 5E) prevented nicotine-stimulated PGE_2 production in the presence or absence of AGE-2 and AGE-3. Without nicotine, α7-nAChR antagonist inhibitors had no effect on PGE_2 production (data not shown). A nonselective or a selective COX-2 inhibitor, indomethacin

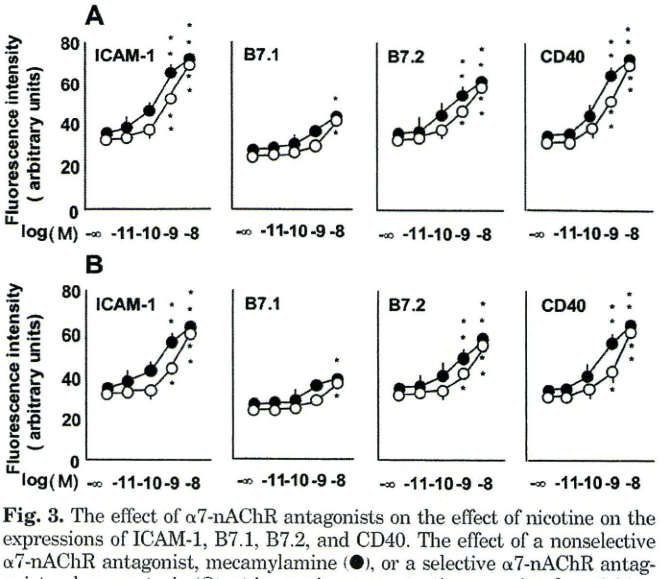

Fig. 3. The effect of α7-nAChR antagonists on the effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40. The effect of a nonselective α7-nAChR antagonist, mecamylamine (●), or a selective α7-nAChR antagonist, α-bungarotoxin (○), at increasing concentrations ranging from 0.01 to 10 nM on the actions of nicotine at 100 μM in the presence of AGE-2 (A) and AGE-3 (B) at 100 μg/ml. The expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes were determined by flow cytometry. The results are expressed as the means ± 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.

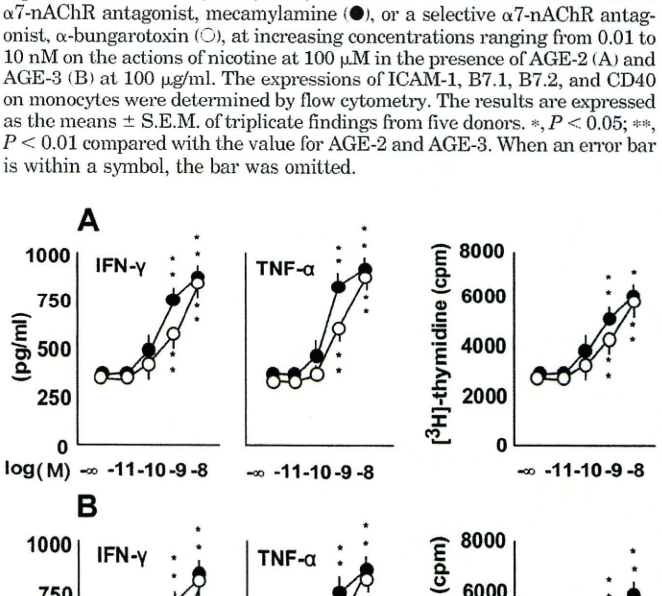

Fig. 4. The effect of α7-nAChR antagonists on the effect of nicotine on the production of IFN-γ and TNF-α and the lymphocyte proliferation. The effect of a nonselective α7-nAChR antagonist, mecamylamine (●), or a selective α7-nAChR antagonist, α-bungarotoxin (○), at increasing concentrations ranging from 0.01 to 10 nM on the actions of nicotine at 100 μM in the presence of AGE-2 (A) and AGE-3 (B) at 100 μg/ml. IFN-γ and TNF-α 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 ± 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_2 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_2 production (data not shown).

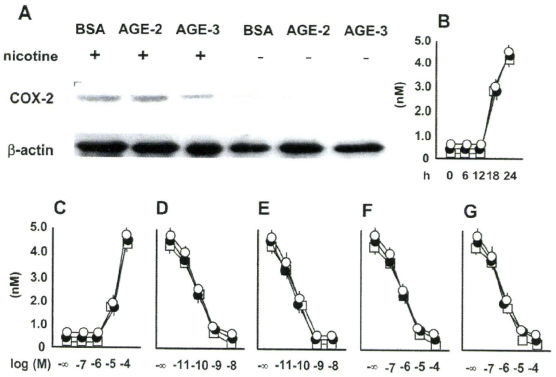

Fig. 5. The effect of nicotine on the expression of COX-2 and the production of PGE_2 in monocytes. A, monocytes at 1×10^6 cells/ml were incubated with nicotine at 100 μM in the presence or absence of AGE-2, AGE-3, and BSA at 100 μg/ml for 30 min. The expression of COX-2 protein was determined by Western immunoblotting as described under *Materials and Methods*. β-Actin was used as control to correct for loading. The concentration of PGE_2 was determined by ELISA. B, the time course effect of nicotine on PGE_2 production was determined. Monocytes at 1×10^6 cells/ml were incubated with nicotine at 100 μM in the presence of AGE-2, AGE-3, and BSA at 100 μg/ml for the indicated periods. C, the effect of nicotine at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2, AGE-3, and BSA at 100 μg/ml for 24 h was determined. D–G, the effect of a nonselective α7-nAChR antagonist, mecamylamine (D); a selective α7-nAChR antagonist, α-bungarotoxin (E); a nonselective COX-2 inhibitor, indomethacin (F); and a selective COX-2 inhibitor, NS398 (G), on the actions of nicotine at 100 μM was determined. Filled circles represent the effect of nicotine on PGE_2 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 ± 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 μg/ml, nicotine at 100 μM significantly activated intracellular cAMP in monocytes at 15 min and thereafter up to 30 and 60 min (Fig. 6A). As shown in Fig. 6B, α7-nAChR antagonists, mecamylamine and α-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, α7-nAChR antagonists and COX-2 inhibitors had no effect on the activation of cAMP (data not shown).

Involvement of Prostanoid EP_2 and EP_4 Receptors in the Actions of Nicotine. To determine the involvement of PGE_2 receptor subtypes in the effects of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation, the effect of an EP_2-receptor antagonist, AH6809 (Kay et al., 2006; Takahashi et al., 2009b) and an EP_4-receptor antagonist, AH23848 (Kay et al., 2006; Takahashi et al., 2009b) at concentrations ranging from 0.1 to 100 μM in the presence of nicotine at 100 μM 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-γ and TNF-α, 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

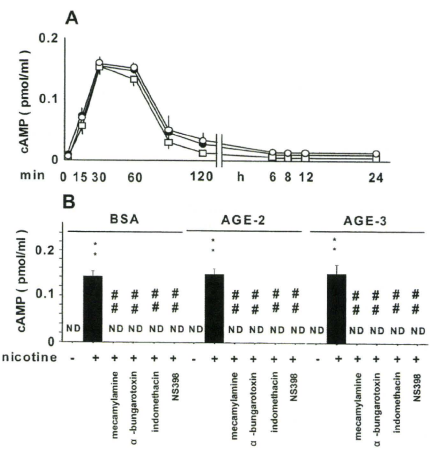

Fig. 6. The effect of nicotine on the activation of cAMP in monocytes. A, the time course effect of nicotine at 100 μM on production of cAMP was determined by ELISA. Monocytes at 1×10^6 cells/ml were incubated with AGE-2 (●), AGE-3 (○), and BSA (□) at 100 μg/ml and nicotine at 100 μM for 24 h. B, the effect of α7-nAChR antagonists, mecamylamine or α-bungarotoxin, at 10 nM and the COX-2 inhibitors, indomethacin and NS398, at 100 μM on 100 μM nicotine-induced production of cAMP in the presence of AGE-2, AGE-3, and BSA at 100 μg/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 ± S.E.M. of triplicate findings from five donors. ND, not detected.

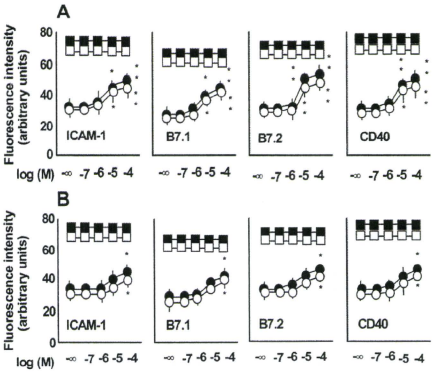

Fig. 7. The effects of prostanoid receptor antagonists on the inhibitory effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40. PBMCs at 1×10^6 cells/ml treated with nicotine at 100 μM were incubated with the EP_2-receptor antagonist AH6809 (A) and the EP_4-receptor antagonist AH23848 (B) at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 at 100 μg/ml. 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 ± 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.

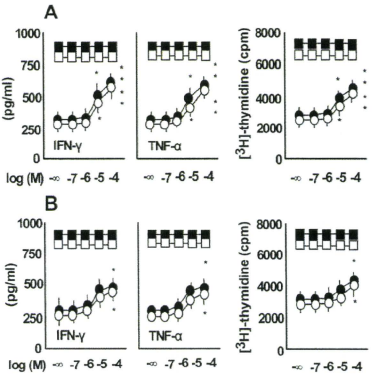

Fig. 8. The effects of prostanoid receptor antagonists on the inhibitory effect of nicotine on the production of IFN-γ and TNF-α and the lymphocyte proliferation. PBMCs at 1×10^6 cells/ml treated with nicotine at 100 μM were incubated with the EP_2-receptor antagonist AH6809 (A) and the EP_4-receptor antagonist AH23848 (B) at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 at 100 μg/ml. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [^{3}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 ± 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-γ and TNF-α, 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-γ and TNF-α, 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 μg/ml in the serum of patient with diabetes (Enomoto et al., 2006; Nakamura et al., 2007). It is reported that AGEs at the concentrations ranging from 50 to 200 μg/ml remarkably induce human monocyte adhesion to bovine retinal endothelial cells (Mamputu and Renier, 2004). AGEs at 200 μg/ml induce the expression of CD40, CD80 and CD86 and the production of IFN-γ in dendritic cells (Ge et al., 2005). In the previous study, we found that AGE-2 and AGE-3 at 10 and 100 μg/ml significantly up-regulated the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation (Takahashi et al., 2009a; Wake et al., 2009). Therefore, the concentration (100 μg/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-γ and TNF-α 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-γ and TNF-α 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-γ and TNF-α 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-γ and TNF-α in PBMCs, and the lymphocyte proliferation in PBMCs.

As shown in Figs. 1 and 2, we found that nicotine prevented 100 μg/ml AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN-γ and TNF-α, and the lymphocyte proliferation in PBMCs. In the presence of AGE-2 and AGE-3 at 10 μg/ml, nicotine also inhibited expressions of ICAM-1, B7.1, B7.2, and CD40, the produc-

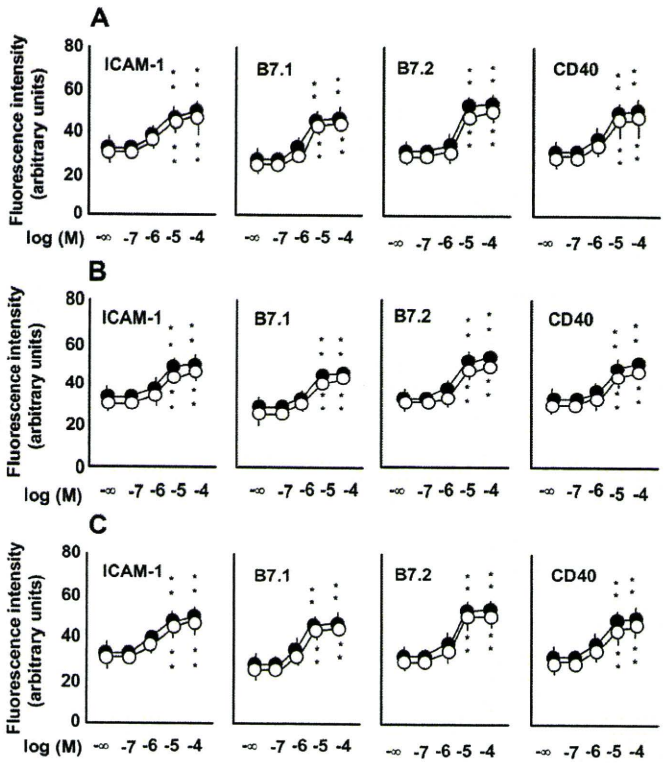

Fig. 9. The involvement of COX-2 and PKA in the effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40. The effect of COX-2 inhibitors, indomethacin (A) and NS398 (B), and a PKA inhibitor, H89 (C), at increasing concentrations ranging from 0.1 to 100 μM on the actions of nicotine at 100 μM in the presence of AGE-2 (●) and AGE-3 (○) at 100 μg/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 ± 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.

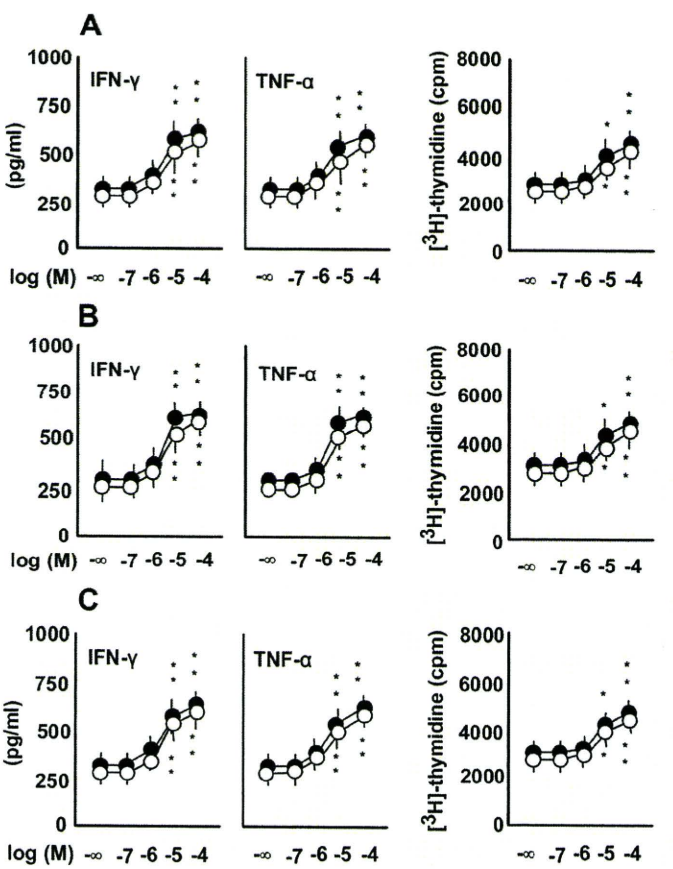

Fig. 10. The involvement of COX-2 and PKA in the effect of nicotine on the production of IFN-γ and TNF-α and the lymphocyte proliferation. The effect of COX-2 inhibitors, indomethacin (A) and NS398 (B), and a PKA inhibitor, H89 (C), at increasing concentrations ranging from 0.1 to 100 μM on the actions of nicotine at 100 μM in the presence of AGE-2 (●) and AGE-3 (○) at 100 μg/ml. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [^{3}H]thymidine uptake as described under *Materials and Methods*. The results are expressed as the means ± S.E.M. of 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.

tion of IFN-γ and TNF-α, 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 α7-nAChR (de Jonge et al., 2005; Saeed et al., 2005). α7-nAChR is required for acetylcholine inhibition of lipopolysaccharide-induced TNF-α production in human macrophages (Wang et al., 2003). In the present study, we found that α7-nAChR antagonists reversed the inhibitory effect of nicotine on the expressions of ICAM-1, B7.1, B7.2, and CD40, the production of IFN-γ and TNF-α 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 α7-nAChR.

We further investigated the action mechanism of nicotine. PGE_2, 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_2, 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_2 in the presence or absence of AGE-2 and AGE-3. The α7-nAChR and COX-2 inhibitors prevented nicotine-initiated PGE_2 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_2 depends on the stimulation of α7-nAChR. We also determined the levels of other COX-2 metabolites, including PGE_1, PGD2, PGF_2, PGI_2, PGJ_2, 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. α7-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 α7-nAChR stimulation and COX-2 production in nicotine-induced elevation of cAMP.

In the previous study, we reported that PGE_2 inhibited AGE-2- and AGE-3-enhanced expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes, the production of IFN-γ and TNF-α, and the lymphocyte proliferation in PBMCs (Taka-

hashi et al., 2009b). Among the four subtypes of receptors: prostanoid EP_1, EP_2, EP_3, and EP_4 receptors, the EP_2 and EP_4 receptors were involved in the actions of PGE_2. In the previous study, we found that the EP_2-receptor antagonist, AH6809 and the EP_4-receptor antagonist, AH23848, inhibited the actions of PGE_2 (Takahashi et al., 2009b). An EP_2-receptor agonist, ONO-AE1-259-01 and an EP_4-receptor agonist, ONO-AE1-329 mimicked the actions of PGE_2. In the presence of AGE-2 and AGE-3, PGE_2 induced the elevation of cAMP via EP_2 and EP_4 receptor. Moreover, the PKA inhibitor, H89, inhibited the action of PGE_2, and a membrane-permeable cAMP analog, dibutyryl cAMP, and an adenylate cyclase activator, forskolin, mimicked the effect of PGE_2. These results suggested the involvement of the EP_2/EP_4-receptors-cAMP/PKA pathway in the actions of PGE_2.

In the present study, we found that the EP_2-receptor antagonist, AH6809 and the EP_4-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-γ and TNF-α, and the lymphocyte proliferation in PBMCs in the presence of AGE-2 and AGE-3. These results imply that the additional increase in PGE_2 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 α7-nAChR (Hamano et al., 2006; Takahashi et al., 2006). Nicotine induced PGE_2 production in monocytes treated with LPS and IL-18 via α7-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 α7-nAChR-PGE_2-cAMP/PKA system. Further work is necessary on this issue.

Nicotine by it own did not inhibit basal production of IFN-γ and TNF-α in the absence of AGE-2 and AGE-3 (Fig. 2). As shown in Fig. 5, nicotine at 100 μM induced production of PGE_2 at 4.7 nM in the absence of AGE-2 and AGE-3. Exogenous PGE_2 at 5 nM had no effect on the production of IFN-γ and TNF-α in the absence of AGE-2 and AGE-3 (data not shown). Therefore, 100 μM nicotine-induced PGE_2 might not effect on IFN-γ and TNF-α 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_2 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). α7-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 α7-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, α7-nAChR stimulation may inhibit the activation of monocytes. Further study of the role of α7-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 α7-nAChR. The COX-2-PGE_2-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 α7-nAChR may partially contribute to regulation of the development of atherosclerotic plaques in diabetes.

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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-γ and tumor necrosis factor α, 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 [(4*Z*)-7-[(rel-1*S*,2*S*,5*R*)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid], 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 dibutyryl 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 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-

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ABBREVIATIONS: DM, diabetes mellitus; PTDM, posttransplant diabetes mellitus; AGE, advanced glycation end product; BSA, bovine serum albumin; COX, cyclooxygenase; CsA, cyclosporine A; dbcAMP, dibutyryl 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, 17*S*-2,5-ethano-6-oxo-17,20-dimethyl prostaglandin E1; ONO-AE1-259-01, 11,15-*O*-dimethyl prostaglandin E2; ONO-AE-248, 16*S*-9-deoxy-9β-chloro-15-deoxy-16-hyfroxy-17,17-trimethylene-19,20-didehydro prostaglandin F2; ONO-AE1-329, 16-(3-methoxymethyl)phenyl-omega-tetranor-3,7dithia prostaglandin E1; AH6809, 6-isopropoxy-9-oxaxanthene-2-carboxylic acid; AH23848, (4*Z*)-7-[(rel-1*S*,2*S*,5*R*)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid; H-89, *N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride); PKI, protein kinase inhibitor; KT5720, (9*S*,10*S*,12*R*)-2,3,9,10,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg: 3′,2′,1′-kl]pyrrolo[3,4-i][1,6]-benzo-diazocine-10-carboxylic acid hexyl ester.