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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d) **Abbreviations:** AGEs, advanced glycation end products ; BSA, bovine serum albumin ; COX, cyclooxygenase; cAMP, cyclic adenosine monophosphate ;

dbcAMP, CsA; cyclosporine A, dibutyryl cAMP; ELISA, enzyme-linked immunosorbent assay ; FITC, fluorescein isothiocyanate ; ICAM, intercellular adhesion molecule ; IFN, interferon; m, monoclonal ; IL, interleukin, LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; PBMC, peripheral blood
mononuclear cells; PE, phycoerythrin; PGE2, prostaglandin E2; PKA, protein kinase A; RAGE, receptor for advanced glycation end products; 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-9beta-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, 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, 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]benzodiazocine-10-carboxylic acid hexyl ester
c) Inflammation & Immunopharmacology
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 diabetes complications. The enhancement of adhesion molecule expression on monocytes/macrophages activates T-cells, reducing allograft survival. In the previous study, we found that toxic AGEs, AGE-2 and AGE-3 induced 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 lymphocyte proliferation during human mixed lymphocyte reaction (MLR). AGE-induced up-regulation of adhesion molecule expression was involved in cytokine production and lymphocyte proliferation. 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, and an EP4-receptor agonist, ONO-AE1-329. An EP2-receptor antagonist, AH6809 and an EP4-receptor antagonist, AH23848, inhibited the actions of PGE2. The stimulation of EP2- and EP4-receptors is reported to increase cyclic adenosine monophosphate (cAMP)
levels. The effects of PGE2 were reversed by protein kinase A (PKA) inhibitors, and mimicked by dibutyryl cAMP (dbcAMP) and an adenylate cyclase activator, forskolin. These results as a whole indicated 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 non-enzymatic 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-Mruthinti et al., 2002). Direct immunochemical evidence for the existence of four distinct AGE structures, including 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, it is 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 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., 2007). Cyclosporine A (CsA) and tacrolimus are reported to prevent the proliferation but not transendothelial migration of alloreactive lymphocytes into donor organs (Blaheta et al., 2001). The cause of impaired graft survival in PTDM patients 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 AGEs-induced actions should be a target for clinical use in PTDM patients.

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 (Greenfield et al., 1998; Dustin and Springer, 1989). 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 (Zhu et al., 2000; Shimizu 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 the 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 lymphocyte proliferation during human MLR (Ohashi et al., 2010). Together with these results, we suggested that toxic AGEs-dependent responses, including the enhancement of adhesion molecule expression on monocytes, might partially facilitate rejection in PTDM patients.

PGE2, one of the major products of cyclooxygenase (COX)-initiated arachidonic acid metabolite released from monocytes, primes naive human T-cells for enhanced production of anti-inflammatory cytokines and the inhibition of pro-inflammatory
cytokines through COX-2 (Hempel et al., 1994; Coleman et al., 1994). There are four subtypes of PGE2 receptors: prostanoid EP1-, EP2-, EP3- and EP4-receptors (Coleman et al., 1994). Activation of EP2- and EP4-receptors leads to an increase in cAMP levels (Coleman et al., 1994). In the previous study, we found that PGE2 inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation in PBMC 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 expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation during MLR.
MATERIALS AND METHODS

Reagents

PGE2, AH6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid), AH23848

((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 Chemical (St. Louis, MO). Protein kinase inhibitor (PKI) (14–22) was purchased from Calbiochem (La Jolla, 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


(16-(3-methoxymethyl)phenyl-omega-tetranor-3,7-dithia prostaglandin E1) and
11-deoxy-PGE₁ were kindly provided by Ono Pharmaceutical Co. Ltd. (Tokyo, Japan). Tacrolimus and CsA were purchased from Astellas Pharma US (North Deerfield, IL, USA). AGE-modified bovine serum albumin (BSA) (Sigma Aldrich, St Louis, MO) was prepared as previously described (Takahashi et al., 2009b; Ohashi et al., 2010). Briefly, each protein was incubated under sterile conditions with glyceraldehyde 3-phosphate (AGE-2) (Sigma Aldrich) or glycolaldehyde (AGE-3) (Sigma Aldrich) in 0.2 M phosphate buffer (pH 7.4) at 37 °C for 7 days. AGE-BSA was dialyzed for 2 days at 4°C. The endotoxin concentration of AGEs at 100 μg/ml described above was measured at SRL, 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 PBMC were obtained from ten 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 (Takahashi et al.,
2009b; Ohashi et al., 2010). PBMC at 1x10^6 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 2.0x10^6 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), and they were incubated under various conditions for 48 h at 37°C in a humidified atmosphere of 5% CO2 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 FITC (fluorescein isothiocyanate)-conjugated mouse IgG1 monoclonal (m) Ab against ICAM-1/CD54 (DAKO, Glostrup, Denmark), B7.1 (IMMUNOTECH, Marseille, France), B7.2 and CD40 (Pharmpingen, San Diego, CA) and IgG1 isotype-matched control (Sigma Chemical) with phycoerythrin-conjugated anti-CD14 Ab (monocyte) (DAKO). PBMC and mixed cells at 2x10^6 cells/ml were
incubated for 48 h. Cultured cells at 5x10^5 cells/ml were prepared for flow cytometric analysis as previously described (Takahashi et al., 2009b; Ohashi et al., 2010) and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA). Data were processed using the CELL QUEST program.

**ELISA assays**

PBMC at 1x10^6 cells/ml were used for analyzing IFN-γ and TNF-α production. After culturing for 24 h at 37 °C in a 5%CO2/air mixture, the cell-free supernatant was assayed for IFN-γ, and TNF-α protein by ELISA employing the multiple Abs sandwich principle (R&D Systems, Minneapolis, MN). The detection limit of ELISA for IFN-γ and TNF-α was 10 pg/ml.

**Proliferation assay**

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

**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 ± S.E.M. of triplicate findings from five donors.
RESULTS

The effects of PGE2 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 and lymphocyte proliferation during MLR

In the 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 expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and the proliferation during MLR at 16 h and thereafter up to 24 and 48 h, whereas AGE-4, AGE-5 and BSA at 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
expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and proliferation during MLR (Ohashi et al., 2010).

As shown in Fig. 1, we established the effect of PGE2 at concentrations ranging from 1 nM to 1 μM on the expressions of ICAM-1, B7.1, B7.2 and CD40 and its impact on the production of IFN-γ and TNF-α and the 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 the adhesion molecule expression, cytokine production and lymphocyte proliferation. IC50 values for the inhibitory effect of PGE2 on the expressions of ICAM-1, B7.1, B7.2 and CD40, the 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 expressions 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 the adhesion molecule expression, cytokine production and lymphocyte proliferation in the presence of AGE-4 and AGE-5 (data not shown).
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 expressions of ICAM-1, B7.1, B7.2 and CD40, the 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 the 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). IC50 values for the inhibitory effect of ONO-AE1-259-01 and ONO-AE1-329 on the expressions of ICAM-1, B7.1, B7.2 and CD40, the 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 inhibited 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 μM on adhesion molecule expression, cytokine production and lymphocyte proliferation were examined in the presence of PGE2 at 1 μM (Fig. 4). AH6809 and AH23848 reversed the inhibitory effect of PGE2 on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation in a concentration-dependent manner. On the other hand, AH6809 and AH23848 had no effect on the actions of AGE-2 and AGE-3 in the absence of PGE2. In the absence of AGE-2 and AGE-3, EP2- and EP4-receptor antagonists reversed the inhibitory effects of PGE2 on the basal expressions of adhesion molecule, the 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 expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation, the effect of PKA inhibitor, H89, protein kinase inhibitor (PKI) (14–22) and KT5720, at concentrations ranging from 0.1 to 100 μM on the actions of PGE2 in the presence of AGE-2 and AGE-3 at 100 μg/ml during MLR was determined (Fig. 5). H89, PKI and KT5720 reversed the inhibitory effect of PGE2 on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation. 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, H89, PKI and KT5720 reversed the inhibitory effects of PGE2 on the basal expressions of adhesion molecule, cytokine production and lymphocyte proliferation in the absence of AGE-2 and AGE-3 (data not shown).

As shown in Fig. 6, the effects of a membrane-permeable cAMP analog, dbcAMP, and an adenylate cyclase activator, forskolin, at concentrations ranging from 0.1 to 100 μM
on the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and lymphocyte proliferation during MLR in the presence of AGE-2 and AGE-3 at 100 μg/ml were examined. Both dbcAMP and forskolin inhibited AGE-2 and AGE-3-induced adhesion molecule expressions, cytokine production and lymphocyte proliferation in a concentration-dependent manner. Moreover, dbcAMP and forskolin inhibited basal expressions of adhesion molecule, the 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 expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation during MLR

As shown in Fig. 7, we examined the effects of tacrolimus and CsA at increasing concentration ranging from 1 nM to 1 μM 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 the presence or absence of AGE-2 and AGE-3 at 100 μg/ml during MLR. Tacrolimus and CsA had no effect on adhesion molecule expression in the presence or
absence of AGE-2 and AGE-3, however, the calcineurin inhibitors inhibited the cytokine
production and the lymphocyte proliferation.
DISCUSSION

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

In the present study, we found, for the first time, that PGE2 inhibited the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation during MLR in the presence or absence of AGE-2 and AGE-3 (Fig. 1). Nataraj et al. (Nataraj et al., 2001) reported that the antiproliferative action of PGE2 on
mouse MLR was due to the stimulation of EP2- and EP4-receptors using EP1-EP4-deficient splenocytes from knock-out mice. It is 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 cDNA (Suzawa et al., 2000). The EP2-receptor agonist, ONO-AE1-259, had at least 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 the adhesion molecule expression, cytokine production and lymphocyte proliferation. In the present study, IC50 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 were similar, respectively (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. Since the IC50 values of PGE2 to prevent the up-regulation of adhesion molecule expressions, cytokine production and lymphocyte proliferation were consistent with the affinity of those agonists to typical EP2- and EP4-receptors (Table 1; Morichika et al., 2003; Takahashi et al., 2009a). 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 the 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 the 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, H89, 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. Further work is necessary on this issue.

In the previous study, we found that AGE-2 and AGE-3 had higher affinity for RAGE than AGE-4 and AGE-5 using an in vitro binding assay (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 PBMC 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 the 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 non-selective 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 expressions of adhesion molecule, cytokine production and the 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 in patients with higher frequency among patients with PTDM than nondiabetic patients (Sezer et al., 2006), and atherosclerosis plays roles in the development of chronic allograft nephropathy and graft loss (Carvalho and Soares, 2001). It is 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 is 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 expressions of ICAM-1, B7.1, B7.2 and CD40, the 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


Coleman RA, Smith WL and Narumiya S (1994) VIII. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and


Hartog JW, de Vries AP, Bakker SJ, Graaff R, van Son WJ, van der Heide JJ, Gans RO,
dysfunction and cardiovascular disease are related to accumulation of advanced glycation

Hempel SL, Monick MM, and Hunninghake GW (1994) Lipopolysaccharide induces
prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and

Jespersen B, Thiesson HC, Henriksen C, Therland K, Falk C, Poulsen T, Fogh B, Madsen
COX-2 activity in vitro and in kidney transplant patients in vivo. *Nephrol Dial
Transplant* **24**:1644-1655.

Kamei T, Callery MP and Flye M (1991) Intragraft delivery of 16,16-dimethyl PGE2
induces donor-specific tolerance in rat cardiac allograft recipients. *Transplantation* **51**:
242-546.


Tamura R, Takahashi HK, Iwagaki H, Yagi T, Mori S, Yoshino T, Nishibori M, and Tanaka N (2004) Effect of beta2-adrenergic receptor agonists on intercellular adhesion molecule (ICAM)-1, B7, and CD40 expression in mixed lymphocyte reaction,


Footnotes

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LEGENDS FOR FIGURES

Figure 1 The effects of PGE2 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 and lymphocyte proliferation during MLR

Mixed cells at 2x10^6 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 expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. Isotype-matched control represents FITC-conjugated IgG1. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [3H]-thymidine uptake as described in Methods. Filled circles (●) represent the effect of PGE2 in the absence of AGEs. Open circles (○) represent 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.
Figure 2 The effect of prostanoid receptor agonists on AGE2- and AGE3-induced expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and lymphocyte proliferation during MLR

Mixed cells at 2x10^6 cells/ml were incubated with the EP1-receptor agonist, ONO-D1-004 (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 (filled circles;●) and AGE-3 (open circles;○) at 100 μg/ml for 48 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [3H]-thymidine uptake as described in 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.
Figure 3 The effects of 11-deoxy-PGE1 on AGE2- and AGE3-induced ICAM-1, B7.1, B7.2 and CD40 expressions on human monocytes during MLR.

Mixed cells at 2x10^6 cells/ml were incubated with increasing concentrations of the EP2/EP4-receptor agonist, 11-deoxy-PGE1, at increasing concentrations from 1 nM to 1 μM in the presence of AGE-2 (A) and AGE-3 (B) at 100 μg/ml for 48 h. The 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 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.

Figure 4 The effects of prostanoid receptor antagonists on the inhibitory effect of PGE2 on the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and lymphocyte proliferation during MLR

Mixed cells at 2x10^6 cells/ml treated with PGE2 at 1 μM were incubated with the EP2-receptor antagonist, AH6809 (A), and the EP4-receptor antagonist, AH23848 (B), at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 at 100 μg/ml for 48 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes
were determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [³H]-thymidine uptake as described in Methods. Filled circles (●) represent the effect of antagonists on PGE2-inhibited adhesion molecule expression in the presence of AGE-2. Open circles (○) represent the effect of antagonists on PGE2-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 PGE2. Open squares (□) represent the effect of antagonists on the actions of AGE-3 in the absence of PGE2. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **P<0.01 compared with the values for PGE2 in the presence of AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

Figure 5 The effects of PKA inhibitors on PGE2-inhibited ICAM-1, B7.1, B7.2 and CD40 expressions monocytes, the production of IFN-γ and TNF-α and lymphocyte proliferation during MLR

The effect of PKA inhibitors, H89 (A), KT5720 (B) and PKI (14–22) (C), at increasing
concentrations from 0.1 to 100 μM, on the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes treated with PGE2 at 10 nM in the presence of the AGE-2 and AGE-3 at 100 μg/ml was determined by flow cytometry. The production of TNF-α and IFN-γ was determined by ELISA. Lymphocyte proliferation was determined by [3H]-thymidine uptake as described in Methods. Filled circles (●) represent the effects of PKA inhibitors on the PGE2-induced inhibition of responses in the presence of AGE-2. Open circles (○) represent the effects of PKA inhibitors on the PGE2-induced inhibition of responses in the presence of AGE-3. The results are expressed as the means ± S.E.M. of triplicate findings from five donors. **P< 0.01 compared with the value for PGE2 in the presence of AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

Figure 6 The effects of forskolin and dbcAMP on AGEs-induced ICAM-1, B7.1, B7.2 and CD40 expressions on human monocytes, the production of IFN-γ and TNF-α and lymphocyte proliferation during MLR.

Mixed cells at 2x10⁶ cells/ml were incubated with an adenylate cyclase activator, forskolin (A), and a cAMP analog, dbcAMP (B), at increasing concentrations from 0.1 to
100 μM in the presence of AGE-2 (filled circles;●) and AGE-3 (open circles;○) at 100 μg/ml for 48 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. Lymphocyte proliferation was determined by [³H]-thymidine uptake as described in Methods. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **P < 0.01 compared with the values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

Fig. 7 The effect of tacrolimus and CsA on the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation during MLR

Mixed cells at 2x10⁶ cells/ml were incubated with tacrolimus (A) and CsA (B) in the presence or absence of AGE-2 and AGE-3 at 100 μg/ml for 48 h, and the expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes was determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA.
Lymphocyte proliferation was determined by $[^3]H$-thymidine uptake as described in Methods. Open circles (○) represent the effect of tacrolimus and CsA on the actions of AGE-2, and open squares (□) represent the effect of tacrolimus and CsA on the actions of AGE-3 during MLR. Filled circles (●) represent the effect of tacrolimus and CsA in the absence of AGEs. The results are expressed as the means ± S.E.M. of triplicate findings from five distinct responder stimulator pairs. **P< 0.01 compared with the value for medium. #P< 0.05, ##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.
Table 1 The IC50 values for the inhibitory effect of PGE2 and EP-2/4 receptor agonists in the presence of AGE-2 and AGE-3

A) AGE-2

<table>
<thead>
<tr>
<th>(nM)</th>
<th>ICAM-1</th>
<th>B7.1</th>
<th>B7.2</th>
<th>CD40</th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE2</td>
<td>15 ± 0.5</td>
<td>10 ± 0.5</td>
<td>10 ± 0.4</td>
<td>11 ± 0.5</td>
<td>11 ± 0.4</td>
<td>15 ± 0.6</td>
<td>12 ± 0.8</td>
</tr>
<tr>
<td>ONO-AE1-259-01</td>
<td>9 ± 0.3</td>
<td>8 ± 0.2</td>
<td>9 ± 0.3</td>
<td>8 ± 0.4</td>
<td>7 ± 0.2</td>
<td>7 ± 0.3</td>
<td>10 ± 0.4</td>
</tr>
<tr>
<td>ONO-AE1-329</td>
<td>10 ± 0.5</td>
<td>9 ± 0.2</td>
<td>10 ± 0.1</td>
<td>9 ± 0.2</td>
<td>7 ± 0.2</td>
<td>8 ± 0.5</td>
<td>7 ± 0.4</td>
</tr>
</tbody>
</table>

B) AGE-3

<table>
<thead>
<tr>
<th>(nM)</th>
<th>ICAM-1</th>
<th>B7.1</th>
<th>B7.2</th>
<th>CD40</th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE2</td>
<td>12 ± 0.2</td>
<td>9 ± 0.4</td>
<td>10 ± 0.5</td>
<td>10 ± 0.5</td>
<td>9 ± 0.4</td>
<td>10 ± 0.6</td>
<td>9 ± 0.7</td>
</tr>
<tr>
<td>ONO-AE1-259-01</td>
<td>8 ± 0.3</td>
<td>9 ± 0.2</td>
<td>8 ± 0.4</td>
<td>6 ± 0.4</td>
<td>7 ± 0.2</td>
<td>8 ± 0.3</td>
<td>8 ± 0.5</td>
</tr>
<tr>
<td>ONO-AE1-329</td>
<td>9 ± 0.5</td>
<td>9 ± 0.2</td>
<td>9 ± 0.5</td>
<td>8 ± 0.2</td>
<td>8 ± 0.2</td>
<td>9 ± 0.4</td>
<td>7 ± 0.6</td>
</tr>
</tbody>
</table>

The results are expressed as the means ± S.E.M. of five donors with triplicate determinations.
Figure 1

A) Fluorescence intensity (arbitrary units) vs. log (M) for ICAM-1, B7.1, B7.2, CD40, and IgG1. Data points marked with # indicate significant differences.

B) Fluorescence intensity (arbitrary units) vs. log (M) for TNF-α, IFN-γ, and proliferation. Data points marked with # indicate significant differences.

Proliferation data shows a decrease as the log (M) decreases, indicating a negative correlation. Fluorescence intensity decreases as log (M) increases, indicating a positive correlation.

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Figure 3

A) ICAM-1 B7.1 B7.2 CD40

Fluorescence intensity (arbitrary units)

log (M) $\infty$ -9 -8 -7 -6

B) ICAM-1 B7.1 B7.2 CD40

Fluorescence intensity (arbitrary units)

log (M) $\infty$ -9 -8 -7 -6
Figure 4

A) 

ICAM-1

B7.1

B7.2

CD40

Fluorescence intensity (arbitrary units)

log (M) -7 -6 -5 -4

0 40 80 120 160

B) 

ICAM-1

B7.1

B7.2

CD40

Fluorescence intensity (arbitrary units)

log (M) -7 -6 -5 -4

0 40 80 120 160

TNF-α

IFN-γ

[3H]-thymidine (cpm)

proliferation

0 2000 4000 6000 8000

IFN-γ

TNF-α

[3H]-thymidine (cpm)

proliferation

0 2000 4000 6000 8000
Figure 5
Figure 6

A) ICAM-1 B7.1 B7.2 CD40

Fluorescence intensity (arbitrary units)

log (M) $\infty -7 -6 -5 -4$

B) ICAM-1 B7.1 B7.2 CD40

Fluorescence intensity (arbitrary units)

log (M) $\infty -7 -6 -5 -4$

proliferation

log (M) $\infty -7 -6 -5 -4$

$[\text{H}]$-thymidine (cpm)
Figure 7

A) Fluorescence intensity (arbitrary units) vs. log (M) for ICAM-1, B7.1, B7.2, and CD40.

B) Fluorescence intensity (arbitrary units) vs. log (M) for ICAM-1, B7.1, B7.2, and CD40.

Proliferation levels measured in [3H]-thymidine (cpm) for IFN-γ and TNF-α.