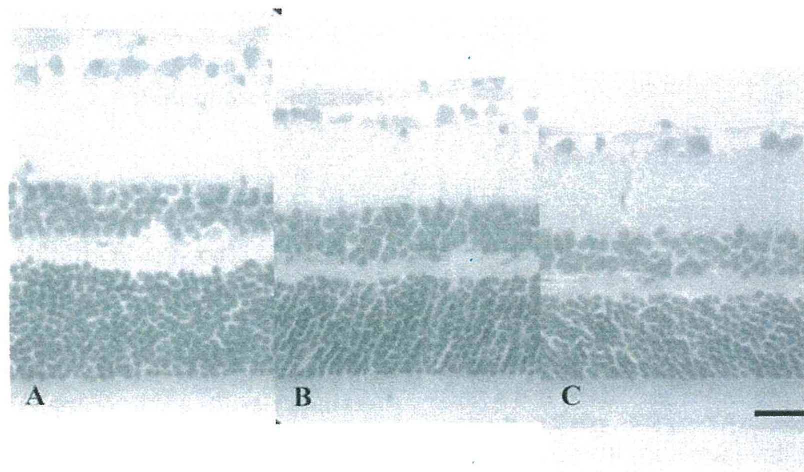

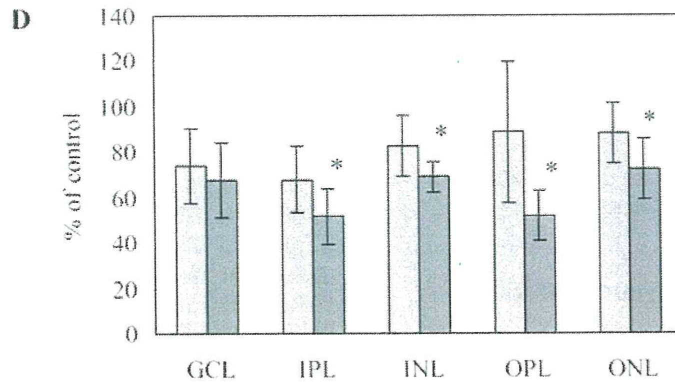

FIGURE 1 Light micrographs of a cross-section through normal rat retina (A) and at 7 days after ischemia when treated with control class-matched mAb (anti-*Keyhole Limpet* hemocyanin mAb, IgG2a) (B) or anti-HMGB1 mAb (C). Percentages indicate change relative to control values for the number of GCL cells and for the IPL, INL, ONL, and OPL thicknesses 7 days after ischemia when treated with IgG2a ( ) or anti-HMGB1 ( ). Results are expressed as the mean ± SD (*$p < 0.05$). Scale bar = 20 μm.

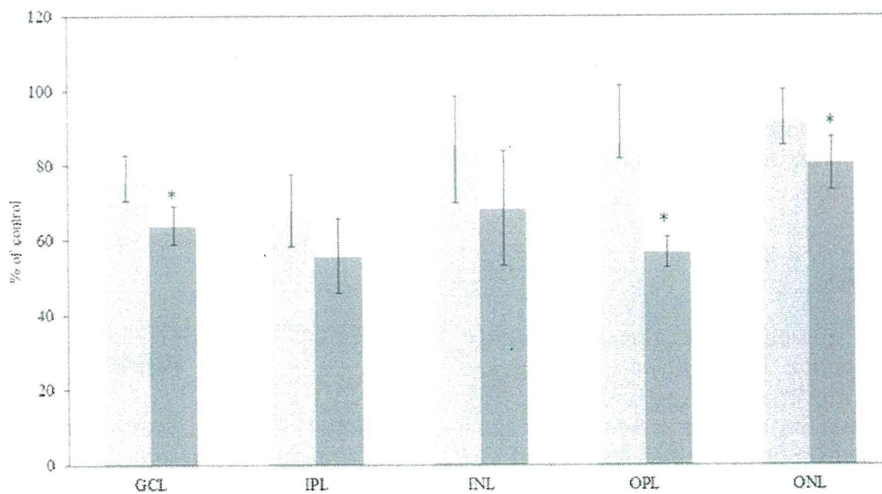

FIGURE 2 Percentages indicate change relative to control values for the number of RGC cells and for IPL, INL, ONL, and OPL thickness 7 days after ischemia when treated with intravenous injections of IgG2a ( ) or anti-HMGB1 ( ). Results are expressed as the mean ± SD (*$p < 0.05$).

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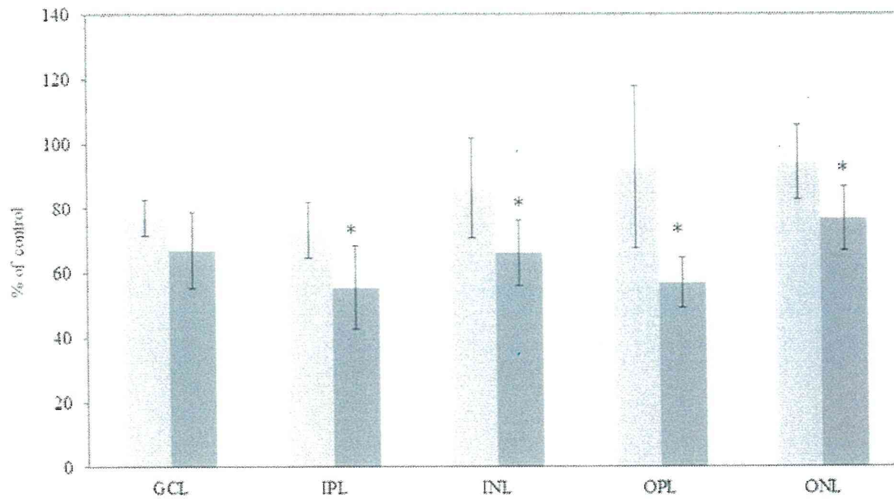

FIGURE 3 Percentages indicate change relative to control values for the number of RGC cells and for IPL, INL, ONL, and OPL thickness 7 days after ischemia when treated local administration of IgG2a ( ) or anti-HMGB1 ( ). Results are expressed as the mean ± SD (* $p < 0.05$).

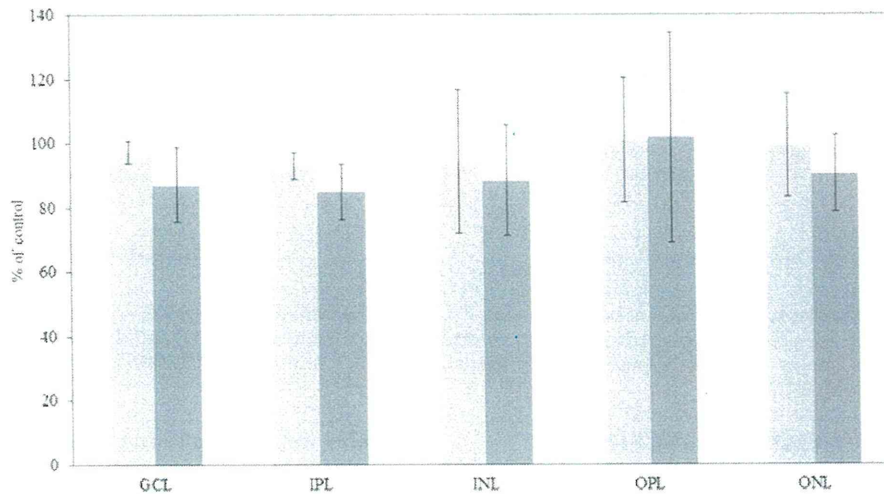

FIGURE 4 Percentages indicate change relative to control values for the number of RGC cells and for IPL, INL, ONL, and OPL thickness 7 days after treatment with IgG2a ( ) or anti-HMGB1 ( ). Results are expressed as the mean ± SD.

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Scotopic ERG was measured 7 days after the intraperitoneal injection of anti-HMGB1 mAb. There was no significant difference between the two groups (Figure 6A, B) ($n=5$, each group). Treatment with anti-HMGB1 did not affect the retinal function in normal rat.

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We examined the expression of HMGB1 in the retina at 6, 12, and 24h after 45min of ischemia (Figure 7). Figure 3A shows the localization of HMGB1 in the normal retina. Immunostaining for HMGB1 was noted in the ONL in the normal retina. However, immunostaining for HMGB1 in the post-ischemic retina (Figure 7B–D) was detected in not only the ONL but also in the INL and GCL. A high degree of edema was noted in the post-ischemic retina.

We examined the effect of anti-HMGB1 mAb on endogenous HMGB1 expression in the normal retina and the retina at 6, 12, and 24h after 45min of ischemia (Figure 8). Intraperitoneal injection of anti-HMGB1 mAb was administered 30min before ischemia. Normal eye balls were enucleated after 30min administration of anti-HMGB1. Administration of anti-HMGB1 suppressed the expression of endogenous HMGB1.

We also examined the direct effect of anti-HMGB1 mAb on retinal HMBG1 expression (Figure 9). Normal eye balls were enucleated at 12h after intravitreous injection of anti-HMGB1. Immunostaining for HMGB1 was not detected.

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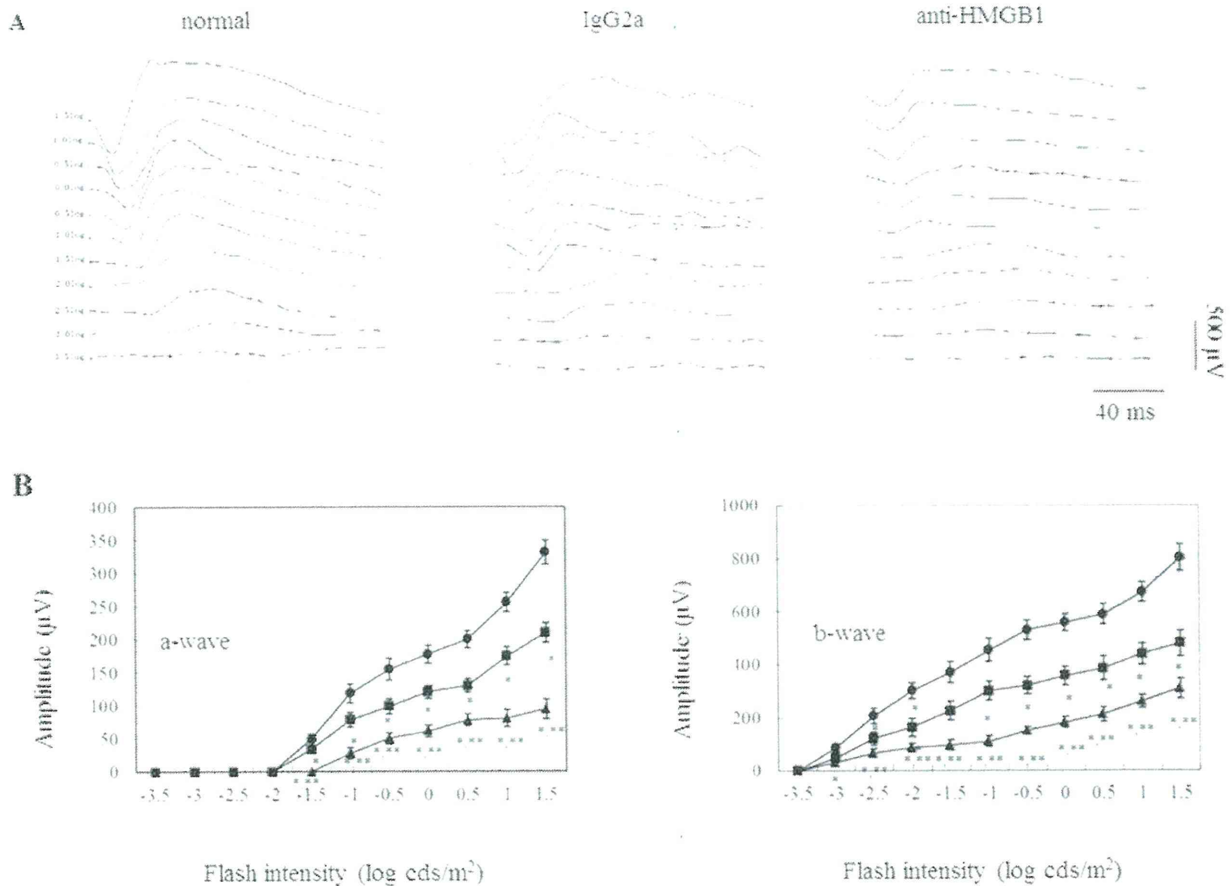

FIGURE 5 (A) Representative scotopic ERGs at baseline and at 7 days after ischemia when treated with control mAb or anti-HMGB1 mAb. (B) Amplitudes for a- and b-waves plotted as a function of flash intensity. Pretreatment with anti-HMGB1 mAb markedly reduced the amplitudes. Results are expressed as the mean ± SD. •: normal, ▪: IgG2a, ▴: anti-HMGB1. *$p<0.05$ versus normal retina. **$p<0.05$ versus ischemic retina with IgG2a.

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ROS ACTIVATION BY ISCHEMIA

We used DHE staining to test whether ROS were enhanced by treatment with 200 μg anti-HMGB1 mAb. DHE specifically reacts with intracellular O_2^-, a ROS, and is converted to the red fluorescent compound ethidium in nuclei. In the post-ischemic retina, DHE fluorescence was clearly up-regulated in the retinal neuronal cells, and this up-regulation was efficiently enhanced by anti-HMGB1 mAb (Figure 10A–C). Figure 9D shows the quantified specific retinal DHE fluorescence. The mean ROS activation was significantly increased by treatment with anti-HMGB1 mAb ($n=4$, each group).

DISCUSSION

This study shows that, compared to the IgG2a treatment, pretreatment with anti-HMGB1 mAb significantly enhanced the ischemic injury of the retina. The results also showed that there was expression of HMGB1 mAb in the retina after ischemia-reperfusion injury.

A recent study showed that HMGB1 inhibited glial glutamate transport by GLAST in mouse gliosomes and suggested that HMGB1 can increase extracellular glutamate levels in ischemic brain.[21] We previously reported that anti-HMGB1 mAb suppressed ischemia-reperfusion-induced brain injury in a transient middle cerebral artery occlusion model in rats.[17] Based on these findings, we predicted that neutralizing mAb could be used to inhibit HMGB1 activity, and thus to significantly decrease the progression of the retinal ischemia-reperfusion injury. However, use of the neutralizing anti-HMGB1 mAb treatment in the present study remarkably increased the retinal damage following ischemia-reperfusion. This was due to an increased production of ROS caused by the anti-HMGB1 mAb treatment. Therefore, it might be possible that elevated levels of HMGB1 had neuroprotective effects against retinal ischemia- reperfusion injury. It has also been reported that treatment with anti-HMGB1 mAb increased ischemia-reperfusion

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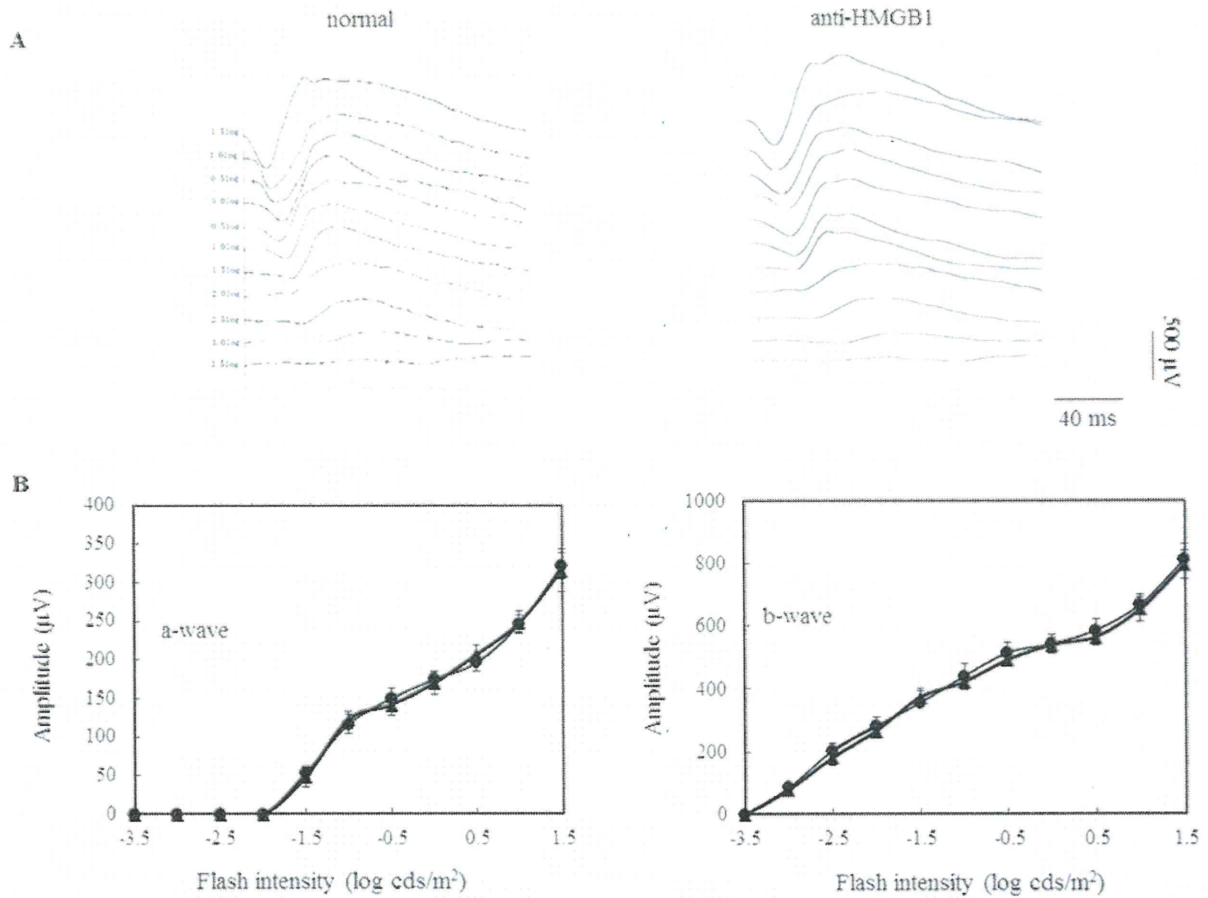

FIGURE 6 (A) Representative scotopic ERGs 7 days after treatment with anti-HMGB1. (B) Amplitudes for a- and b-waves plotted as a function of flash intensity. Results are expressed as the mean ± SD. •: normal, ▲: anti-HMGB1.

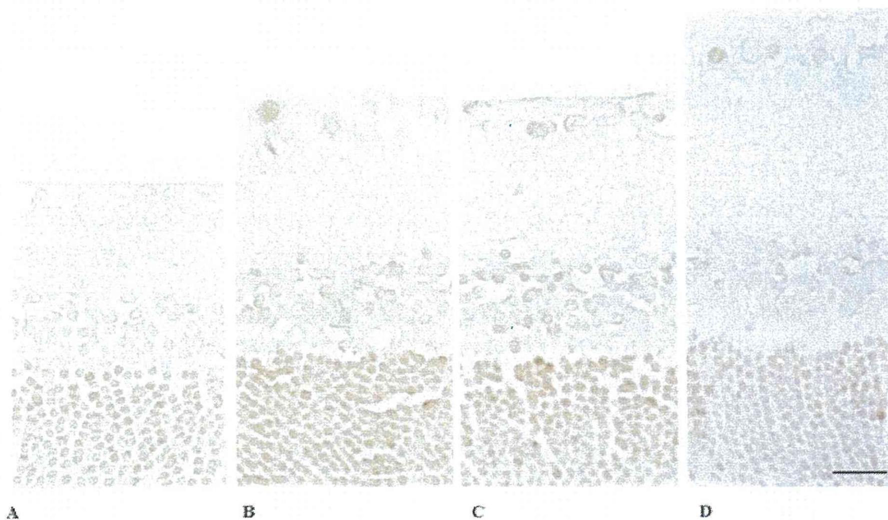

FIGURE 7 Immunohistochemical staining of HMGB1 expression in the retina. Retinal sections from normal animals (A) or 6h (B), 12h (C), or 24h (D) after ischemia. Scale bar = 20 μm.

injury in the rat heart.[22] Therefore, it appears that the effect of anti-HMGB-1 mAb depends on the organ involved.

When the IOP is increased, glutamate is released from the retina during and after the ischemia.[3,23,24] The major causes of the cell death that occur after activation

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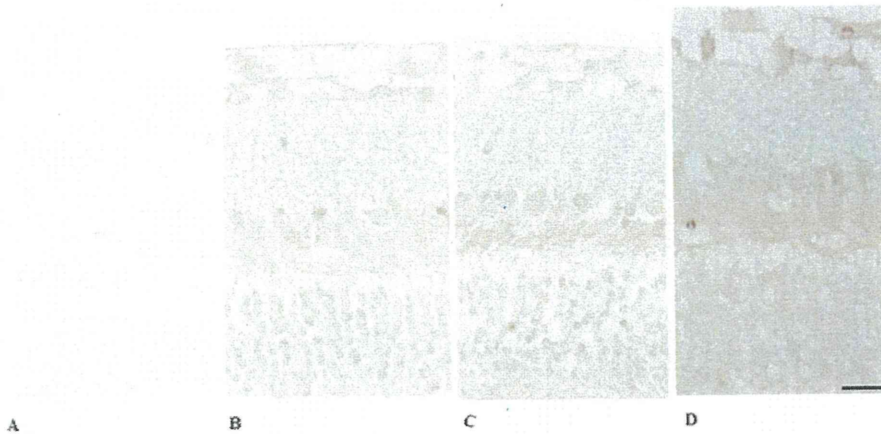

FIGURE 8 The effect of anti-HMGB1 mAb on endogenous HMGB1 expression. Intraperitoneal injection of anti-HMGB1 mAb was administered 30 min before ischemia. Retinal sections from normal animals (A) or 6 h (B), 12 h (C), or 24 h (D) after ischemia. Scale bar = 20 μm.

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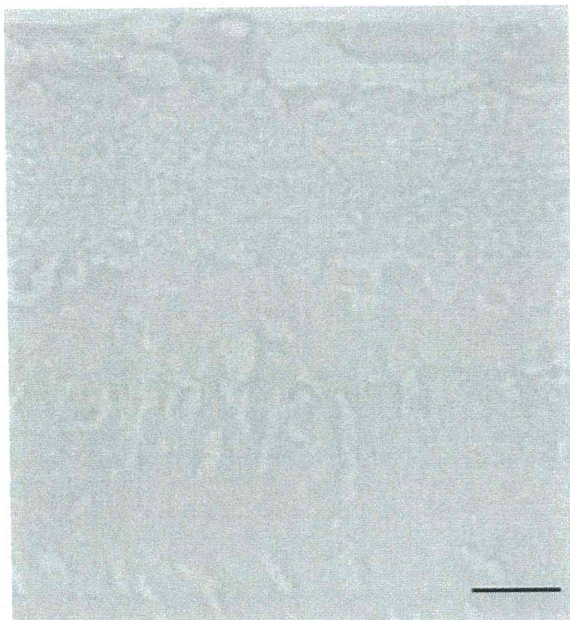

FIGURE 9 The direct effect of anti-HMGB1 mAb on endogenous HMGB1 expression in the normal retina. Scale bar = 20 μm.

of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors are related to the influx of calcium into the cells and the generation of free radicals.[25] Excessive accumulation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) can have a wide range of detrimental effects, including inhibition of mitochondrial function, reduction of cellular ATP levels, enhancement of ROS production, and activation of cellular proteases and nitric oxide (NO) synthase.[26] Since production of ROS was increased by anti-HMGB1 mAb treatment in the current study, anti-HMGB1 mAb played a large deleterious role in the resultant ischemia-reperfusion injury. In the present study, there was an increase in the HMGB1 level in the retina after the ischemia-reperfusion injury. These results suggest that endogenous HMGB1 released from retinal cells may modulate ischemia-reperfusion injury in the retina. Therefore, the anti-HMGB1 mAb treatment increased the delayed neuronal death.

We evaluated the functional retinal damage after ischemia-reperfusion injury by measuring the ERG a- and b-wave amplitudes. The b-wave of the ERG has been identified as a particularly sensitive index of retinal ischemia both in humans[25] and in experimental models of retinal ischemia in vitro.[27] After the anti-HMGB1 mAb treatment, there was a decrease in the thickness of ONL following ischemia-reperfusion, with the a-wave of the ERG also lower than that noted in the eyes treated with IgG2a. There was a good correlation between the ERG for both a- and b-waves and the histological results. It has been reported that administration of pentobarbital enhance the a- and b-wave of the ERG.[28,29] Under the anesthesia, many factors indirectly affecting the retinal activity could not be completely excluded.

It has been reported in previous studies that HMGB1 is expressed in GCL, INL, ONL, the inner and outer segments of photoreceptors, and in the retinal pigment epithelial cells in normal retina.[30,31] However, the current immunohistochemical study showed that HMGB1 was present in the ONL in normal retinas, which may be due to the use of different antibodies in the various studies (monoclonal antibody vs. polyclonal antibody).

HMGB1 passively released from necrotic cells.[32] Cell death was frequently observed in both the GCL and the INL after 3 h of ischemia-reperfusion.[33] In the present study, we demonstrated that immunostaining for HMGB1 in the post-ischemic retina was detected in not only the ONL but also in the INL and GCL. HMGB1 may play a key role in the protection of retinal injury after ischemia-reperfusion.

The current study showed, for the first time, that treatment with anti-HMGB1 mAb increased ischemia-reperfusion injury in the rat retina. Further investigations are needed to clarify the mechanism of anti-HMGB1 mAb in retinal ischemia-reperfusion

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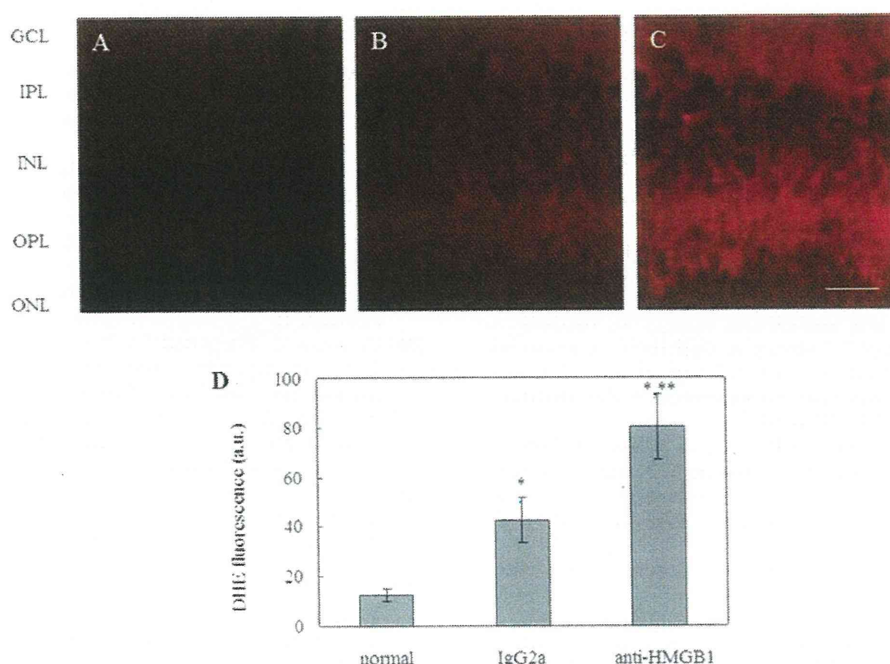

FIGURE 10 Effect of anti-HMGB1 mAb pretreatment on the release of ROS. The use of DHE to detect ROS indicated up-regulation of retinal neuronal cells in the retina after ischemia (IgG2a (B) as compared to normal retina (A)). Pretreatment with anti-HMGB1 mAb enhanced the level of ROS (C). (D) Quantified specific retinal DHE fluorescence is expressed for sections in arbitrary units (AU) for each group. Data express the mean ± SD; *$p<0.05$ normal retina. **$p<0.05$ versus ischemic retina with IgG2a. Scale bar = 20 μm.

injury. Additionally, anti-HMGB1 mAb function needs be further explored, as this could potentially lead to the development of neuroprotective therapeutic strategies for acute retinal ischemic disorders.

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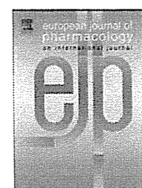

Immunopharmacology and Inflammation

β_2-adrenoceptor stimulation inhibits advanced glycation end products-induced adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells

Hideo Kohka Takahashi [a,*], Shuji Mori [b], Keyue Liu [a], Hidenori Wake [a], Jiyong Zhang [a], Rui Liu [a], Tadashi Yoshino [c], Masahiro Nishibori [a]

[a] Department of Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama, Japan
[b] Department of Pharmacy, Shujitsu University, 1-6-1 Nishikawahara, Okayama, Japan
[c] Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama, Japan

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ABSTRACT

Cell-to-cell interaction through binding of intercellular adhesion molecule-1 (ICAM-1) and CD40 on monocytes to their ligands on T-cells plays crucial roles in cytokine production. Advanced glycation end products (AGEs) subtypes induce complications in diabetes. In a previous study, we found that glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3) at 100 μg/ml induced the expressions of ICAM-1 and CD40 on monocytes and the production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α in human peripheral blood mononuclear cells. β_2-adrenoceptor stimulation has been demonstrated to modulate the production of inflammatory mediators. In the present study, we found that norepinephrine, epinephrine and isoproterenol inhibited AGE-2- and AGE-3-induced adhesion expression and cytokine production in a concentration-dependent manner. The action of these catecholamines was antagonized by β_2-adrenoceptor antagonist, but not by α_1-, α_2- and β_1-adrenoceptor antagonist. β_2-adrenoceptor agonists, salbutanol and terbutaline inhibited AGE-2- and AGE-3-induced adhesion expression and cytokine production, but α_1-, α_2- and β_1-adrenoceptor agonist had no effect, indicating that the stimulation of β_2-adrenoceptor might improve AGEs-initiated complications in diabetes.

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1. Introduction

Acute myocardial infarction is believed to result from the acute rupture of a lipid-laden coronary atherosclerotic plaque, which in turn leads to acute thrombosis, cardiac ischemia, and subsequent myocardial necrosis (Shah, 2003). Hypertension and diabetes mellitus are well-known coronary risk factors (Guzik et al., 2000;Sampson et al., 2002). β-blockade inhibits the progression of ischemic heart disease complicated with diabetes (Shinozaki et al., 1999; Warnholtz et al., 1999; Hink et al., 2001). β_2-adrenoceptor stimulation influences blood pressure and cardiovascular function by regulating vasomotor tone in the peripheral vasculature as well as chronotropic and inotropic responses in the myocardium (Guimaraes and Moura, 2001).

AGEs and their intermediates have been implicated in pathophysiological dysfunction associated with atherosclerosis and cardiac alteration in diabetic patients (Ramasamy et al., 2005). Direct immunochemical evidence for the existence of four distinct AGE structures, including AGE-2, AGE-3, AGE-4, and AGE-5, was provided from the analysis of AGEs within modified proteins and peptides (Takeuchi and Yamagishi, 2004). Recently, it was demonstrated that toxic AGE structures, AGE-2 and AGE-3 had diverse biological activities on vascular endothelial cells, vascular smooth muscle cells, mesangial cells, Schwann cells, malignant melanoma cells and cortical neurons (Okamoto et al., 2002;Yamagishi et al., 2002). AGEs have also been suggested to have profound effects on inflammatory and immune cells (Imani et al., 1993; Ding et al., 2007; Figarola et al., 2007).

Microinflammation is a common major mechanism in the pathogenesis of diabetic vascular complications. It is reported that diabetes has greater macrophage and T-cell infiltration in atherosclerotic plaques (Burke et al., 2004). Monocyte/macrophage and T-cell activation induce the progression of inflammatory atherosclerotic plaques (Stoll and Bendszus, 2006; Figarola et al., 2007). It is known that the enhanced expression of adhesion molecules, including ICAM-1, B7 and CD40, on monocytes results in the activation of T-cells (Durie, et al., 1994; Ranger et al., 1996; Camacho et al., 2001). In a previous study, we found that, among the AGEs examined, AGE-2 and AGE-3 selectively induced the expressions of ICAM-1, B7.1, B7.2 and CD40 and the production of IFN-γ and TNF-α in peripheral blood

Abbreviations: AGEs, advanced glycation end products; RAGE, receptor for AGE.

* Corresponding author. Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. Tel./fax: +81 86 235 7140.
E-mail address: mbori@md.okayama-u.ac.jp (M. Nishibori).

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mononuclear cells (Takahashi et al., 2009). Antagonism experiments using antibodies against adhesion molecules demonstrated that cell-to-cell interaction between monocytes and T/NK-cells was involved in AGE-2- and AGE-3-induced cytokine production (Takahashi et al., 2009). The receptor for AGEs (RAGE) is a cell-surface receptor for AGEs (Neeper et al., 1992; Schmidt et al., 1992). In the previous study, we found that expression of RAGE was detected on monocytes, and that AGE-2 and AGE-3 up-regulated the expression of RAGE (Takahashi et al., 2009).

In the present study, we examined the effect of adrenoceptor agonist stimulation on AGE-2- and AGE-3-induced expressions of ICAM-1 and CD40 on monocytes and the production of IFN-γ and TNF-α in peripheral blood mononuclear cells.

2. Materials and methods

2.1. Reagents and drugs

Epinephrine, norepinephrine, isoproterenol, salbutamol, terbutaline, butoxamine, SR59230A and BRL37344 were purchased from Sigma Chemical (St. Louis, MO). AGE-modified bovine serum albumin (BSA) (Sigma Aldrich, St Louis, MO) was prepared as previously described (Schmidt et al., 1992; Takeuchi et al., 2000). Briefly, each protein was incubated under sterile conditions with glyceraldehyde 3-phosphate (AGE-2) (Sigma Chemical) or glycolaldehyde (AGE-3) (Sigma Chemical) at 50 mg/ml in 0.2 M phosphate buffer (pH 7.4) at 37 °C for 7 days. All incubations were performed under sterile conditions in the dark. After incubation, unbound material was removed by extensive dialysis against phosphate-buffered saline (PBS) or by gel filtration over Sephadex G-10 (Pharmacia, Uppsala, Sweden) for 2 days at 4 °C. The endotoxin concentration of AGEs at 100 μg/ml described above was measured at Special Reference Laboratory (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). For flow cytometric analysis, an FITC-conjugated mouse IgG1 monoclonal (m) Ab against ICAM-1/CD54 was purchased from DAKO (Glostrup, Denmark). An FITC-conjugated mouse IgG1 mAb against CD40 was purchased from Pharmingen (San Diego, CA).

2.2. Isolation of peripheral blood mononuclear cells

Normal human peripheral blood mononuclear cells were obtained from ten healthy volunteers after acquiring IRB approval (Okayama Univ. IRB No.106). Twenty to fifty milliters of peripheral blood were withdrawn from a forearm vein. Peripheral blood mononuclear cells were isolated from the buffy coat of ten healthy volunteers by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden), then washed three times in RPMI 1640 medium (Nissui Co. Ltd., Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated FCS, 20 μg/ml of kanamycin and 100 μg/ml of streptomycin and penicillin (Sigma). Peripheral blood mononuclear cells were suspended at a final concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum.

2.3. Flow cytometric analysis

Changes in the expressions of ICAM-1 and CD40 on monocytes were examined by multi-color flow cytometry using a combination of anti-CD14 Ab with anti-ICAM-1 or anti-CD40 Ab. Peripheral blood mononuclear cells at 1×10^6 cells/ml were incubated with AGEs, adrenoceptor agonists and antagonists for 24 h. Cultured cells at 5×10^5 cells/ml were washed once with washing buffer (PBS supplemented with 2.5% normal horse serum, 0.1% NaN3, and 0.01 M HEPES, pH7.3). Then, the cells were incubated with 1 μg of FITC-conjugated anti-ICAM-1Ab, anti-B7.1 Ab, anti-B7.2 Ab or anti-CD40 Ab, and PE-conjugated anti-CD14 Ab for 20 min at 4 °C. After washing, the cells were fixed with 2% paraformaldehyde and analyzed with a FACSCalibur (Becton Dickinson, Biosciences, San Jose, CA), and the data were processed using the CELL QUEST program (Becton Dickinson Biosciences).

2.4. Cytokine assay

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

2.5. Statistical examination

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

3. Results

3.1. The effects of adrenoceptor agonists on AGE-2 and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes and the production of IFN-γ and TNF-α by peripheral blood mononuclear cells

In a 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., 2009). AGE-2 and AGE-3 showed relatively high affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed moderate affinity for sRAGE. The appropriate incubation time and concentration of AGEs were determined according to the study (Takahashi et al., 2009). AGE-2 and AGE-3 at 100 μg/ml significantly induced the expressions of ICAM-1 and CD40 and the production of IFN-γ and TNF-α at 16 h and thereafter up to 24 and 48 h.

We investigated the effects of adrenoceptor agonists, including norepinephrine, epinephrine and isoproterenol, at concentrations ranging from 0.1 to 100 μM on the expressions of ICAM-1 and CD40 and the production of IFN-γ and TNF-α in the presence of AGE-2 and AGE-3 at 100 μg/ml at 24 h (Fig. 1). Adrenoceptor agonists concentration-dependently inhibited AGE-2- and AGE-3-induced expressions of ICAM-1 and CD40 and the production of IFN-γ and TNF-α.

3.2. The effects of adrenoceptor antagonists on the actions of epinephrine, norepinephrine and isoproterenol

To determine the adrenoceptor subtypes involved in the effects of epinephrine, norepinephrine and isoproterenol on the expressions of ICAM-1 and CD40 and the production of IFN-γ and TNF-α in the presence of AGE-2 and AGE-3, the blocking effects of an α_1-adrenoceptor antagonist, prazosin, an α_2-adrenoceptor antagonist, yohimbine, a β_1-adrenoceptor antagonist, atenolol, a β_2-adrenoceptor antagonist, butoxamine, and a β_3-AR antagonist, SR59230A, at concentrations ranging from 0.1 to 100 μM on the action of norepinephrine, epinephrine and isoproterenol at 100 μM were examined (Fig. 2). Butoxamine antagonized the inhibitory effects of norepinephrine, epinephrine and isoproterenol on the expressions of ICAM-1 and CD40 and the production of IFN-γ and TNF-α in a concentration-dependent manner. However, prazosin, yohimbine, atenolol and SR59230 had no effect on the actions of norepinephrine, epinephrine and isoproterenol (data not shown).

3.3. The effects of selective adrenoceptor agonists on the actions of epinephrine, norepinephrine and isoproterenol

As shown in Fig. 3, the effects of an α_1-adrenoceptor agonist, methoxamine, an α_2-adrenoceptor agonist, clonidine, a β_1-adrenoceptor agonist, dobutamine, β_2-adrenoceptor agonists, salbutamol and terbutaline, and a β_3-adrenoceptor agonist, BRL37344, at concentrations ranging from 0.1 to 100 µM in the presence of AGE-2 and AGE-3 at 100 µg/ml were determined. Salbutamol and terbutaline mimicked the modulatory effects of isoproterenol on the expressions of ICAM-1 and CD40 and the production of IFN-γ and TNF-α. The potency and efficacy of the effects of two agonists were quite similar to those of isoproterenol. However, meth oxamine, clonidine, dobutamine and BRL37344 had no effect (data not shown).

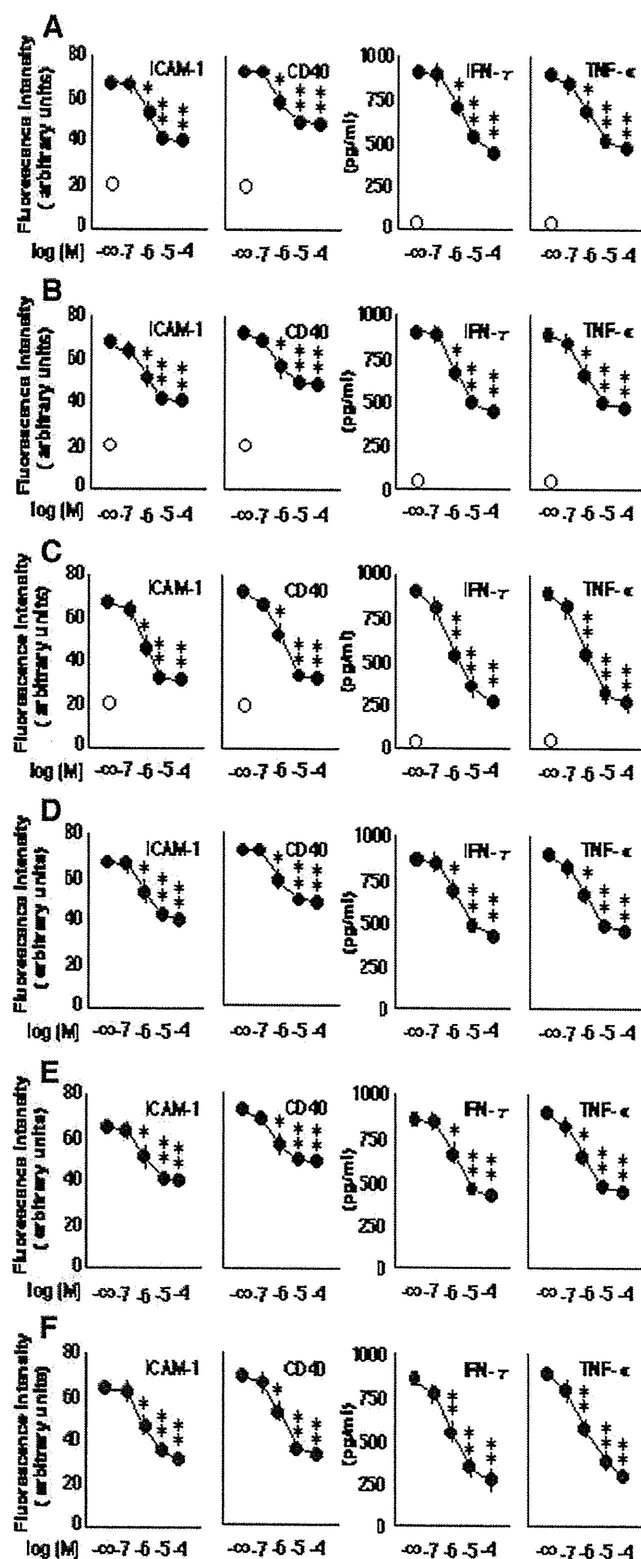

Fig. 1. The effects of epinephrine, norepinephrine and isoproterenol on AGE-2- and AGE-3-induced ICAM-1 and CD40 expressions on monocytes and the production of IFN-γ and TNF-α in peripheral blood mononuclear cells. Peripheral blood mononuclear cells at 1×10^6 cells/ml were incubated with AGE-2 and AGE-3 at 100 µg/ml and norepinephrine, epinephrine and isoproterenol at increasing concentrations from 0.1 to 100 mM for 24 h. The expressions of ICAM-1 and CD40 were determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. The effects of norepinephrine, epinephrine and isoproterenol on the actions of AGE-2 at 100 µg/ml are shown in A, B and C, and those on the actions of AGE-3 at 100 µg/ml are shown in D, E and F, respectively. The X-axis represents the concentrations of norepinephrine, epinephrine and isoproterenol. Filled circles (●) represent the effect of norepinephrine, epinephrine and isoproterenol on adhesion molecule expression and cytokine production in the presence of AGE-2 and AGE-3. Open circles (○) represent adhesion molecule expression and cytokine production in peripheral blood mononuclear cells with medium alone. 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 values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

4. Discussion

In the present study, we clearly demonstrated that the endogenous catecholamines norepinephrine and epinephrine down-regulated AGE-2- and AGE-3-induced production of TNF-α and IFN-γ as well as ICAM-1 and CD40 expression on the cell-surface of monocytes (Fig. 1). IC50 values of the inhibitory effect of adrenoceptor agonists on ICAM-1 expression and TNF-α production in the presence of AGE-2 were 1 and 0.8 µM, and those in the presence of AGE-3 were 1 and 0.7 µM, respectively. Norepinephrine, epinephrine and isoproterenol had no effect on B7.1 and B7.2 expressions in the presence and absence of AGE-2 and AGE-3 (data not shown). Moreover, we found that adrenoceptor agonists had no effect on adhesion molecule expressions and cytokine production in the presence of AGE-4 and AGE-5 (data not shown).

AGEs abolish the cardioprotection induced by stimulation of β_1-adrenoceptor (Robinet et al., 2007), suggesting an adverse interaction between RAGE and cardioprotective signal stimulation on the actions of AGEs in monocytes. To investigate the receptor subtypes involved in the action of norepinephrine and epinephrine, we used subtype-selective adrenoceptor antagonists and agonists. The effects of norepinephrine, epinephrine and isoproterenol on ICAM-1 and CD40 expression and cytokine production induced by AGE-2- and AGE-3 were blocked by β_2-adrenoceptor antagonist, butoxamine (Fig. 2). Selective β_2-adrenoceptor agonists salbutamol and terbutaline were potent inhibitors of AGE-2- and AGE-3-induced ICAM-1 and CD40 expression and cytokine production in human peripheral blood mononuclear cells (Fig. 3). IC50 values for the inhibitory effect of salbutamol and terbutaline on the expression of ICAM-1 induced by AGE-2 were estimated to be 0.7 and 0.8 µM, respectively. Salbutamol and terbutaline did not show any influence on B7.1 and B7.2 expressions in the presence and absence of AGE-2 and AGE-3 (data not shown). Since IC50 values of norepinephrine, epinephrine, isoproterenol and β_2-adrenoceptor agonist to prevent the up-regulation of adhesion molecule expression and cytokine production were consistent with the affinity of those agonists to typical β_2-adrenoceptor (Takahashi et al., 2003; Kuroki et al., 2004), it was concluded that the inhibitory effect of norepinephrine, epinephrine and isoproterenol was mediated by the stimulation of β_2-adrenoceptor but not α_1-, α_2- and β_1-adrenoceptor.

It was investigated that β_3-adrenoceptor, has 49 and 51% overall homology at the amino acid level with β_2- and β_1-adrenoceptor in humans, respectively (Emorine et al., 1989; Granneman, and Lahners, 1994). The affinity of norepinephrine and epinephrine for human β_3-adrenoceptor expressed on Chinese hamster ovary cells is 20- and 5-fold higher than that of isoproterenol, respectively (Isogawa et al., 2002). On the other hand, isoproterenol is more potent than norepinephrine and epinephrine at inducing β_2-adrenoceptor-mediated effects. The relative potency of the action of isoproterenol and endogenous catecholamines excluded the possibility of a major role of β_3-adrenoceptor in these responses. It is reported that the β_3-adrenoceptor agonist, BRL37344 has a marked selectivity for β_3-adrenoceptors (Muzzin et al., 1988; Kullmann et al., 2009), and that SR59230A shows potent antagonistic effects at β_3-adrenoceptors

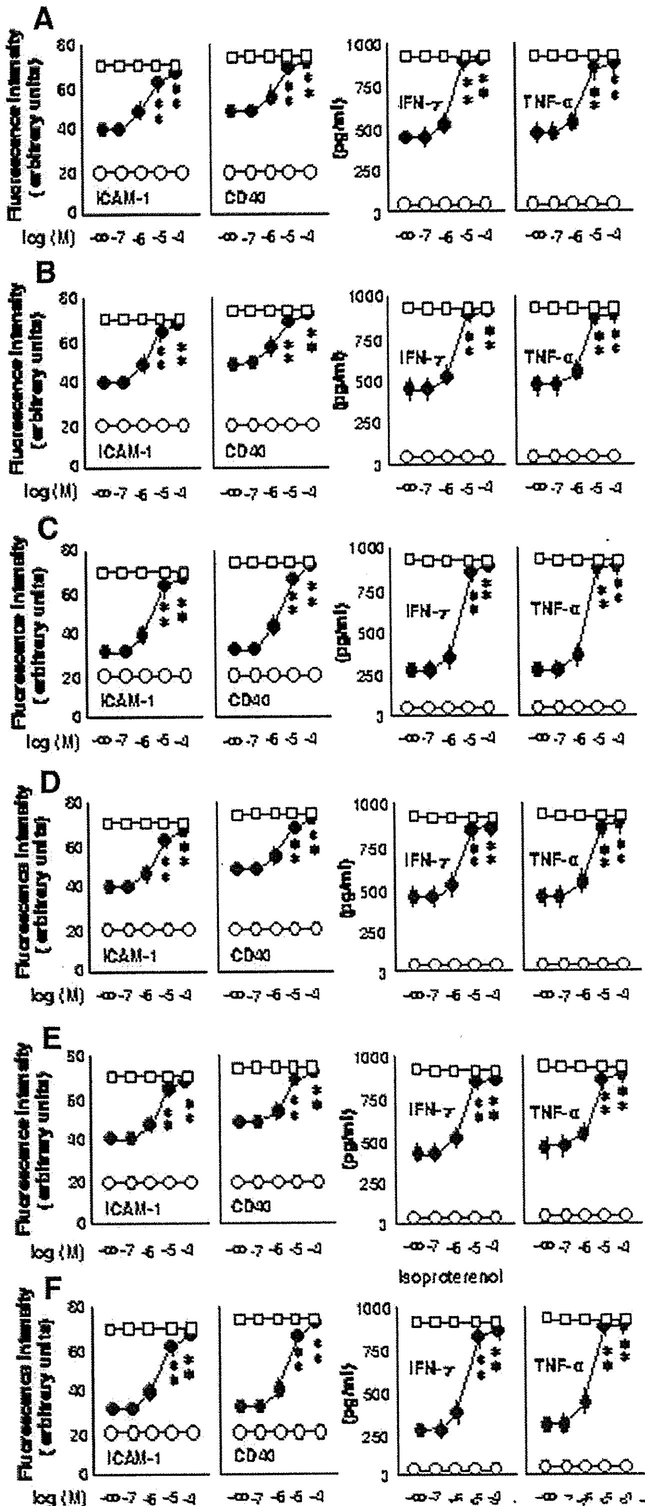

Fig. 2. The effects of selective β_2-adrenoceptor antagonist on the actions of epinephrine, norepinephrine and isoproterenol. Peripheral blood mononuclear cells at 1×10^6 cells/ml were incubated with selective β_2-adrenoceptor antagonist, butoxamine, at increasing concentrations from 0.1 to 100 μM. The effects of butoxamine on the actions of norepinephrine, epinephrine and isoproterenol in the presence of AGE-2 at 100 μg/ml are shown in A, B and C, and those in the presence of AGE-3 at 100 μg/ml are shown in D, E and F, respectively. The X-axis represents the concentrations of butoxamine. Filled circles (●) represent the effect of butoxamine on 100 μM norepinephrine-, epinephrine- and isoproterenol-inhibited adhesion molecule expression and cytokine production in the presence of AGE-2 and AGE-3. Open squares (□) represent the effect of butoxamine in the presence of AGE-2 and AGE-3 without norepinephrine, epinephrine and isoproterenol stimulation. Open circles (○) represent the effect of butoxamine on adhesion molecule expression and cytokine production in the absence of norepinephrine, epinephrine, isoproterenol, 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 norepinephrine, epinephrine and isoproterenol alone.

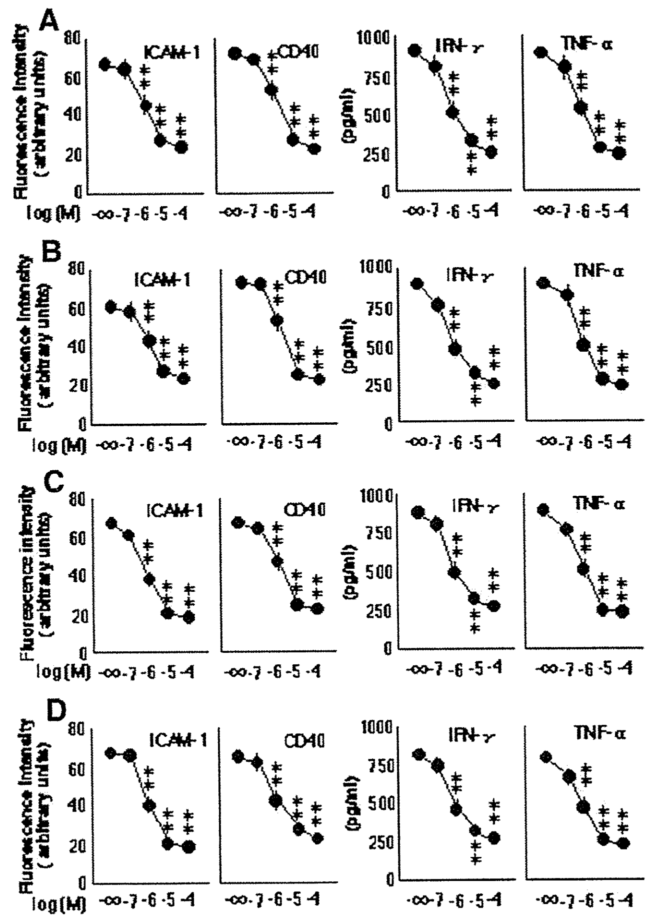

Fig. 3. The effects of selective β_2-adrenoceptor agonists on the action of AGE-2 and AGE-3. Peripheral blood mononuclear cells at 1×10^6 cells/ml were incubated with β_2-adrenoceptor agonists, salbutamol and terbutaline, at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 at 100 μg/ml for 24 h. The effects of salbutamol and terbutaline on the action of AGE-2 are shown in A and B, and those on the action of AGE-3 are shown in C and D, respectively. The X-axis represents the concentrations of salbutamol and terbutaline. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. $^{**}P < 0.01$ compared with the value for AGE-2 or AGE-3 alone.

(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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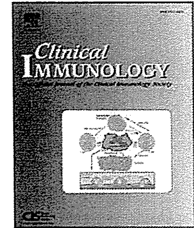

Advanced glycation end products enhance monocyte activation during human mixed lymphocyte reaction

Katsuhisa Ohashi [a], Hideo Kohka Takahashi [b,*], Shuji Mori [c], Keyue Liu [b], Hidenori Wake [b], Hiroshi Sadamori [a], Hiroaki Matsuda [a], Takahito Yagi [a], Tadashi Yoshino [d], Masahiro Nishibori [b], Noriaki Tanaka [a]

[a] *Department of Gastroenterological Surgery, Transplant, and Surgical Oncology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama, Japan*
[b] *Department of Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama, Japan*
[c] *Department of Pharmacy, Shujitsu University, 1-6-1 Nishikawahara, Okayama, Japan*
[d] *Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama, Japan*

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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 intercellular 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.
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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-

* Corresponding author. Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. Fax: +81 86 235 7140.
E-mail address: kohka611@md.okayama-u.ac.jp (H.K. Takahashi).

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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′-CTGACCTATGCGGCCGCTGCTCAAAACATCACAGC-3′ and 5′-GACTGAATTCATCAGTGATGATGGTGATGGTGAGTTCCCAGCCCTGATCC-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-

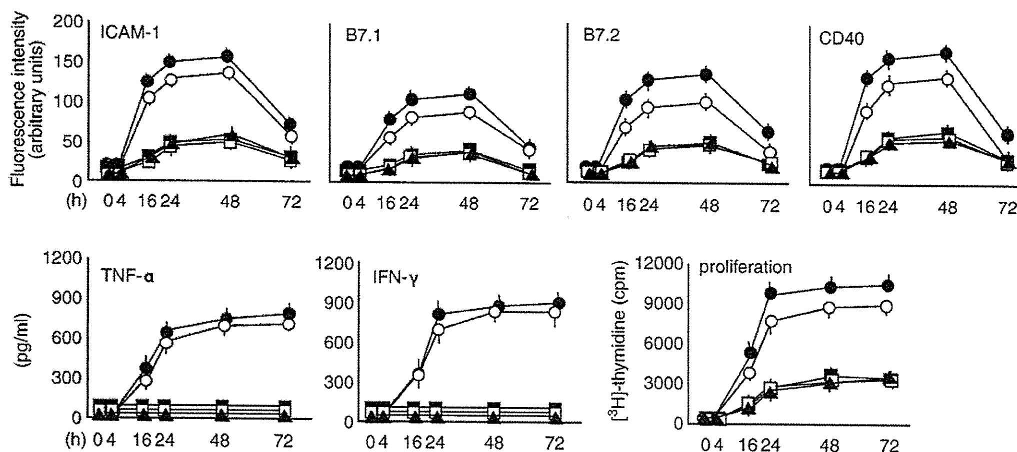

Figure 1 The time-course effects of AGEs on the expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the proliferation of T-cells during human MLR. Mixed cells at 2 × 10^6 cells/ml were incubated with AGE-2 (●), AGE-3 (○), AGE-4 (■), AGE-5 (□) or BSA (▲) at 100 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.

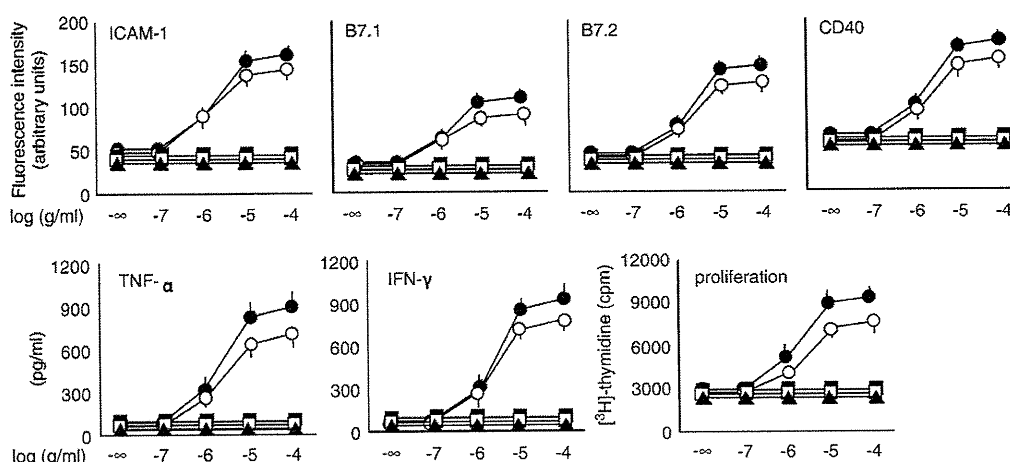

Figure 2 The dose–response relationship of AGEs with the expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the proliferation of T-cells during human MLR. Mixed cells at 2×10^6 cells/ml were incubated with AGE-2 (●), AGE-3 (○), AGE-4 (■), AGE-5 (□) or BSA (▲) 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

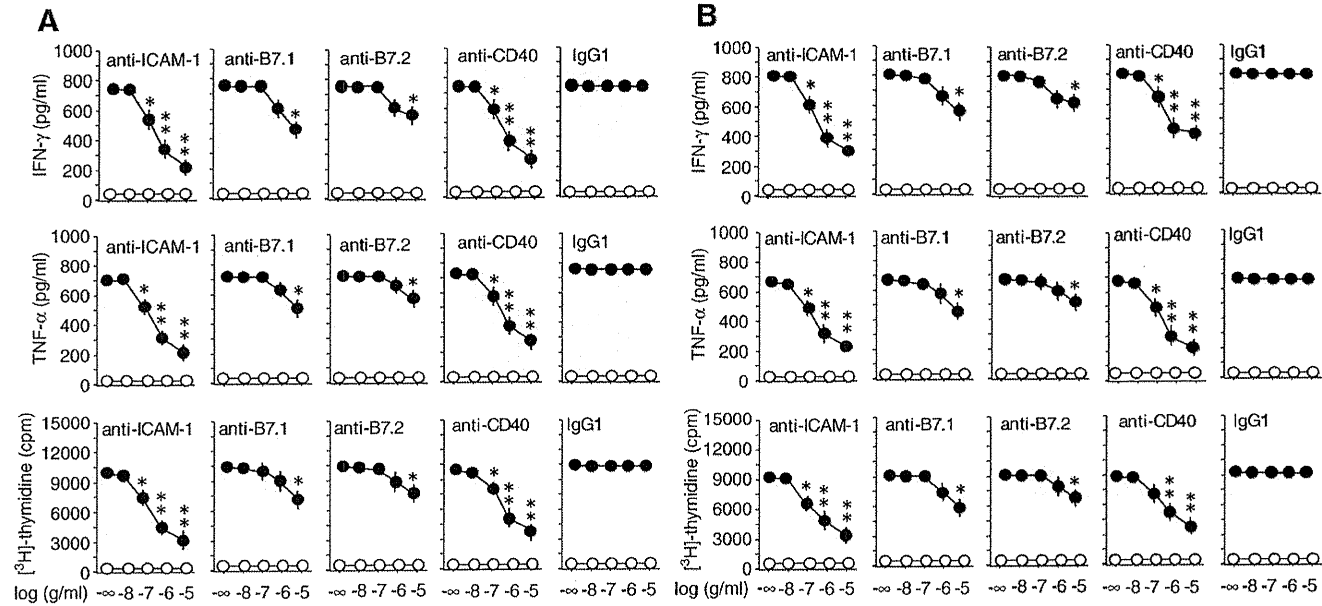

Figure 3 The involvement of adhesion molecule in the effect of AGE-2 and AGE-3 on cytokine production and T-cell proliferation. Mixed cells at 2×10^6 cells/ml were incubated with anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs at increasing concentrations from 0.1 to 100 ng/ml in the absence (○) 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.

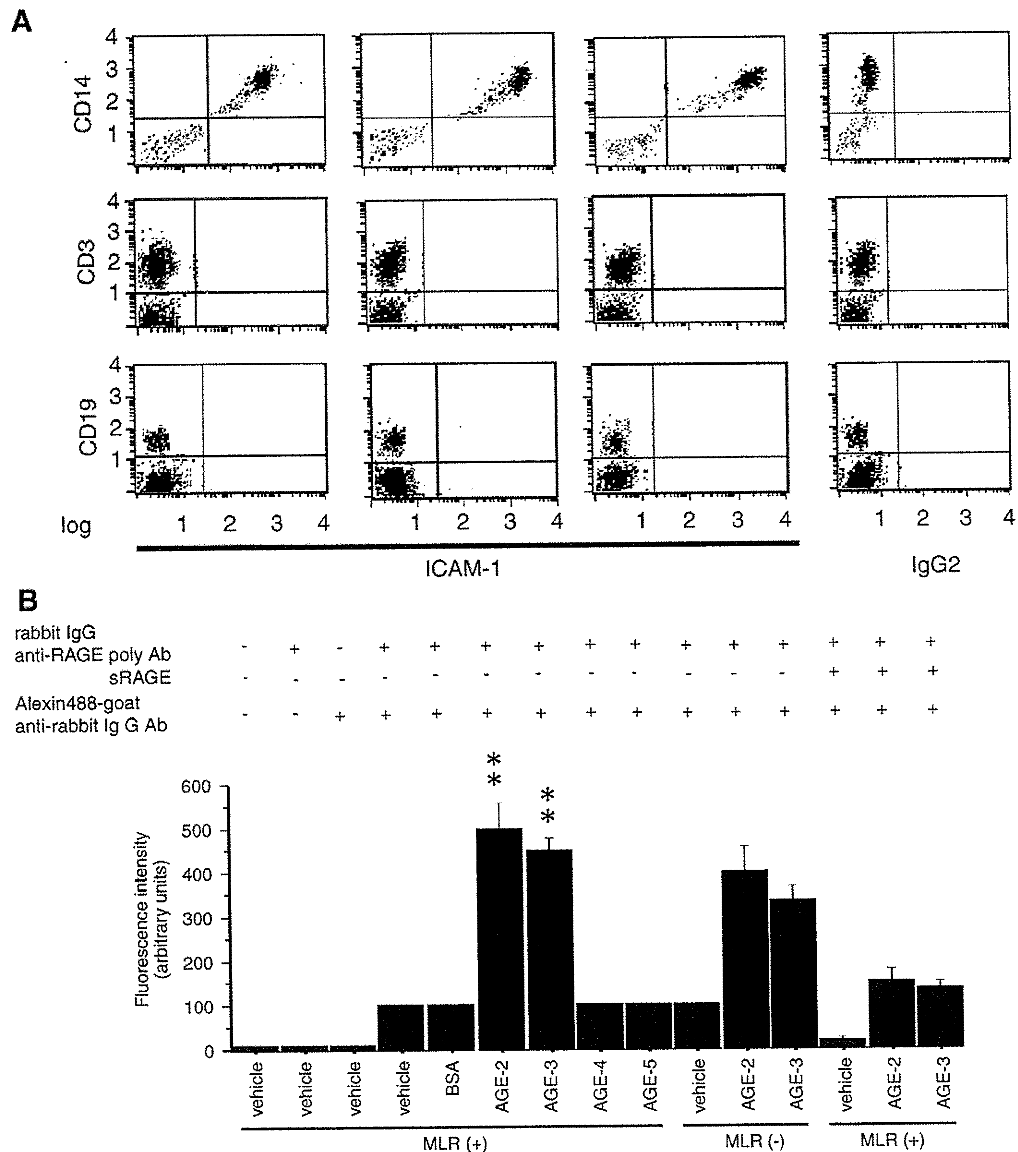

Figure 4 The expression of RAGE on monocytes. The expression of RAGE on monocytes in the presence or absence of AGE-2, AGE-3, AGE4, AGE5 and BSA at 100 μg/ml 48 h after incubation was examined by flow cytometry (A and B). Mixed cells were double-stained with cell-specific antigen (CD14, CD3 or CD 19) 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.

Acknowledgments

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