kindly provided by Bayer Yakuhin, Ltd. (Osaka, Japan). N-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-89), an EP_2 receptor antagonist (Alexander *et al.*, 2008), 6-isopropoxy-9-oxaxanthene-2-carboxylic acid (AH6809) and an EP_4 receptor antagonist, (4Z)-7-[(rel-1S,2S,5R)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid (AH23848) were purchased from Sigma-Aldrich. NS398 and indomethacin were from Cayman Chemical. For flow cytometric analysis, fluorescein isothiocyanate (FITC)-conjugated mouse IgG_1 mAb against ICAM-1/CD54 and phycoerythrin-conjugated anti-CD14 mAb were purchased from DAKO (Glostrup, Denmark). FITC-conjugated mouse IgG_1 mAb against B7.2 and CD40 was obtained from Pharmingen (San Diego, CA, USA), and FITC-conjugated IgG_1, an isotype-matched control, was obtained from Sigma Chemical.

Results

The effect of CIP on the expression of COX-2 protein and the production of PGE_2 in monocytes

In a previous study, we established an *in vitro* binding assay using immobilized AGE subspecies and the His-tagged soluble form of RAGE (sRAGE) protein to evaluate the binding of AGE subtypes to RAGE (Takahashi *et al.*, 2009a). AGE-2 and AGE-3 showed relatively high affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed moderate affinity for sRAGE. The appropriate incubation time and concentration of AGEs were determined according to these previous studies (Wake *et al.*, 2009; Takahashi *et al.*, 2009a).

The effect of CIP 100 μg mL^{-1} on COX-2 protein expression in monocytes in the presence or absence of AGE-2 and AGE-3 at 100 μg mL^{-1} was determined by Western blot analysis 30 min after the addition of CIP (Figure 1A). COX-2 expression in monocytes treated with BSA was marginal, but the addition of CIP markedly increased the expression of COX-2 irrespective of the presence of AGE-2 and AGE-3.

Whereas AGE-2 and AGE-3 had no effect on the production of PGE_2, CIP at 100 μg·mL^{-1} increased the production of PGE_2 in a time-dependent manner, with a maximum level at 24 h (Figure 1B). CIP concentration-dependently increased the production of PGE_2 both in the presence and absence of AGE-2 and AGE-3 at 24 h (Figure 1C). At 100 μg·mL^{-1}, CIP induced the production of 20 nM PGE_2 irrespective of the presence of AGE-2 and AGE-3.

The effects of a non-selective COX-2 inhibitor, indomethacin, and a selective COX-2 inhibitor, NS398, at concentrations ranging from 0.01 to 100 μM on CIP-enhanced production of PGE_2 in monocytes were determined after the 24 h incubation (Figure 1D,E). Indomethacin and NS398 inhibited the production of PGE_2, irrespective of the presence of AGE-2 and AGE-3, in a concentration-dependent manner.

The effect of CIP on cAMP production in monocytes

The effect of CIP 100 μg·mL^{-1} on intracellular cAMP in monocytes was determined in the presence and absence of AGE-2 and AGE-3 at 100 μg·mL^{-1} (Figure 2). AGE-2 and AGE-3 did not induce the production of cAMP, whereas CIP and PGE_2 elicited the production of cAMP irrespective of the presence of AGE-2 and AGE-3. NS398 100 μM blocked the production of cAMP induced by CIP.

The effects of CIP on AGE-2- and AGE-3-induced adhesion molecule expression, cytokine production and lymphocyte proliferation

To determine an appropriate incubation time for investigating the effects of AGE-2 and AGE-2 on these cells, we examined the kinetics at 0, 4, 16, 24 and 48 h as reported previously (Wake *et al.*, 2009; Takahashi *et al.*, 2009a). In the absence of AGEs, the expression of ICAM-1, B7.1, B7.2 and CD40 moderately increased at 16 h and, thereafter, up to 24 and 48 h. AGE-2 and AGE-3 at 100 μg·mL^{-1} significantly increased the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α in PBMC and their proliferation at 16 h and, thereafter, up to 24 and 48 h, whereas AGE-4, AGE-5 and BSA at 100 μg·mL^{-1} had no effect at all (Wake *et al.*, 2009; Takahashi *et al.*, 2009a). Moreover, to determine the appropriate concentration of AGEs, the effects of AGE-2 and AGE-3 at concentrations ranging from 100 ng·mL^{-1} to 100 μg·mL^{-1} were examined after 24 h. AGE-2 and AGE-3, 10 and 100 μg·mL^{-1}, significantly enhanced the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α in PBMC and the proliferation in PBMC (Wake *et al.*, 2009; Takahashi *et al.*, 2009a). Therefore, the effects of CIP, at concentrations ranging from 0.1 to 100 μg·mL^{-1}, were determined on the responses induced by AGE-2 and AGE-3 100 μg·mL^{-1} (Figure 3). CIP concentration-dependently inhibited the expressions of adhesion molecules, cytokine production and lymphocyte proliferation induced by AGE-2 and AGE-3 at

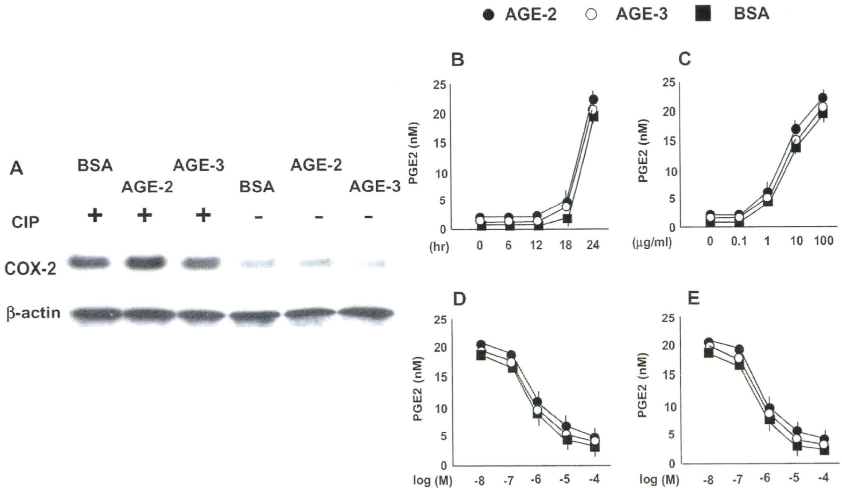


Figure 1

Effect of CIP on the expression of COX-2 and the production of prostaglandins E_2 (PGE_2) in monocytes. (A) Monocytes at 1×10^6 cells·mL^{-1} were incubated with CIP 100 μg·mL^{-1} in the presence or absence of advanced glycation end product (AGE)-2, AGE-3 or bovine serum albumin (BSA) 100 μg·mL^{-1} for 1 h. The expression of COX-2 protein was determined by Western immunoblotting as described in Methods. β-Actin was used as a control to correct for loading. (B) Time course for effect of CIP on PGE_2 production. Monocytes at 1×10^6 cells·mL^{-1} were incubated with CIP 100 μg·mL^{-1} in the presence of AGE-2, AGE-3 or BSA at 100 μg·mL^{-1} for the indicated times. PGE_2 levels in the supernatant were determined by enzyme-linked immunosorbent assay. (C) Effect of CIP at increasing concentrations from 0.1 to 100 μg·mL^{-1} was determined in the presence of AGE-2, AGE-3 or BSA 100 μg·mL^{-1} for 24 h. The effects of a non-selective COX-2 inhibitor, indomethacin (D) and a selective COX-2 inhibitor, NS398 (E) on the actions of CIP at 100 μg·mL^{-1} were determined in the presence of AGE-2, AGE-3 or BSA. The results are expressed as the means ± SEM of five donors with triplicate determinations. When an error bar was within a symbol, the bar was omitted.

100 μg·mL^{-1}. The IC_{50} values for the inhibitory effect of CIP on the expressions of ICAM-1, B7.1, B7.2 and CD40 in the presence of AGE-2 were estimated to be 4, 5, 4 and 5 μg·mL^{-1}, and those in the presence of AGE-3 were 3, 3, 5 and 4 μg·mL^{-1}, respectively. Moreover, the IC_{50} values for the effect of CIP on cytokine production and lymphocyte proliferation in the presence of AGE-2 were estimated to be 3, 3 and 2 μg·mL^{-1}, and those in the presence of AGE-3 were 3, 3 and 2 μg·mL^{-1}, respectively. In the absence of AGEs, CIP at 10 and 100 μg·mL^{-1} also inhibited the basal expression levels of adhesion molecule, but had no effect on cytokine production and lymphocyte proliferation.

The involvement of prostanoid EP_2 and EP_4 receptors in the actions of CIP

To determine the involvement of PGE_2 receptor subtypes in the effects of CIP 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 an EP_2 receptor antagonist, AH6809 (Takahashi *et al.*, 2009b) and an EP_4 receptor antagonist, AH23848 (Takahashi *et al.*, 2009b) at concentrations ranging from 0.01 to 100 μM were examined in the presence of CIP 100 μg·mL^{-1} (Figure 4). AH6809 and AH23848 reversed the inhibitory effect of CIP on the increased expressions of adhesion molecules, cytokine production and lymphocyte proliferation induced by AGE-2 and AGE-3 in a concentration-dependent manner. AH6809 and AH23848 had no effect on the actions of AGE-2 and AGE-3 in the absence of CIP.

The effect of indomethacin, NS398 and H-89 on the actions of CIP

The effects of indomethacin, NS398 and a PKA inhibitor, H-89 at concentrations ranging from 0.01

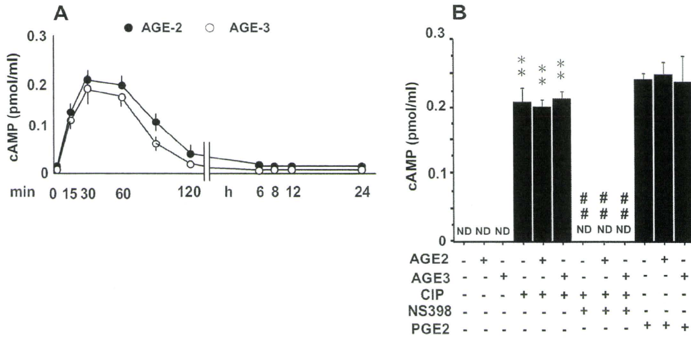


Figure 2

Effect of CIP on the activation of cAMP in monocytes. (A) Time course for effect of CIP 100 μg·mL^{-1} on cAMP production was determined by enzyme-linked immunosorbent assay. Monocytes at 1×10^6 cells·mL^{-1} were incubated with CIP in the presence of advanced glycation end product (AGE)-2 and AGE-3 at 100 μg·mL^{-1}, and the time course changes in the levels of cAMP in monocytes were determined at the indicated time points. (B) Effect of COX-2 inhibitor, NS398 100 μM on CIP-induced production of cAMP in the presence or absence of AGE-2 and AGE-3. $**P < 0.01$ compared with the value for bovine serum albumin alone. $\#\#P < 0.01$ compared with the value for CIP. The results are the means ± SEM of triplicate findings from five donors. ND, not detected; PGE_2, prostaglandins E_2.

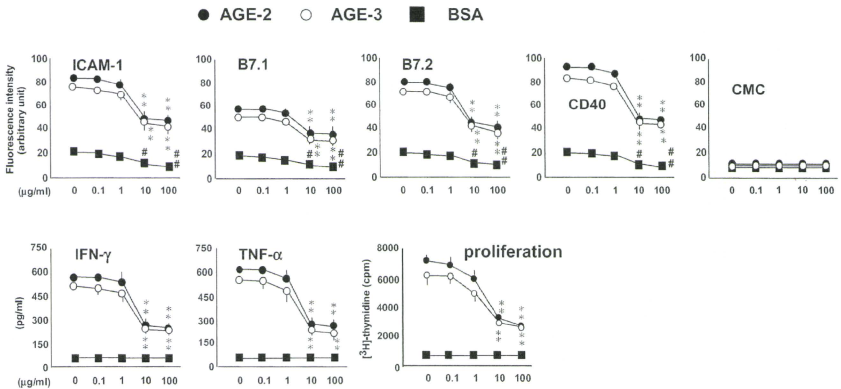


Figure 3

Effects of CIP on advanced glycation end product (AGE)-2- and AGE-3-induced expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes, production of interferon (IFN)-γ and tumour necrosis factor (TNF)-α in peripheral blood mononuclear cells (PBMC) and lymphocyte proliferation in PBMC. PBMC at 1×10^6 cells mL^{-1} were incubated with AGE-2, AGE-3 and bovine serum albumin (BSA) at 100 μg·mL^{-1} in the presence of CIP at increasing concentrations from 0.1 to 100 μg·mL^{-1} for 24 h. Expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. Fluorescein isothiocyanate-conjugated IgG_1 was used as an isotype-matched control Ab. IFN-γ and TNF-α concentrations in conditioned media were determined by enzyme-linked immunosorbent assay. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake as described in Methods. The results are expressed as the means ± SEM of five donors with triplicate determinations. $**P < 0.01$ compared with the value in the presence of AGE-2 and AGE-3 alone. $\#P < 0.05$, $\#\#P < 0.01$ compared with the value for medium alone. When an error bar was within a symbol, the bar was omitted.

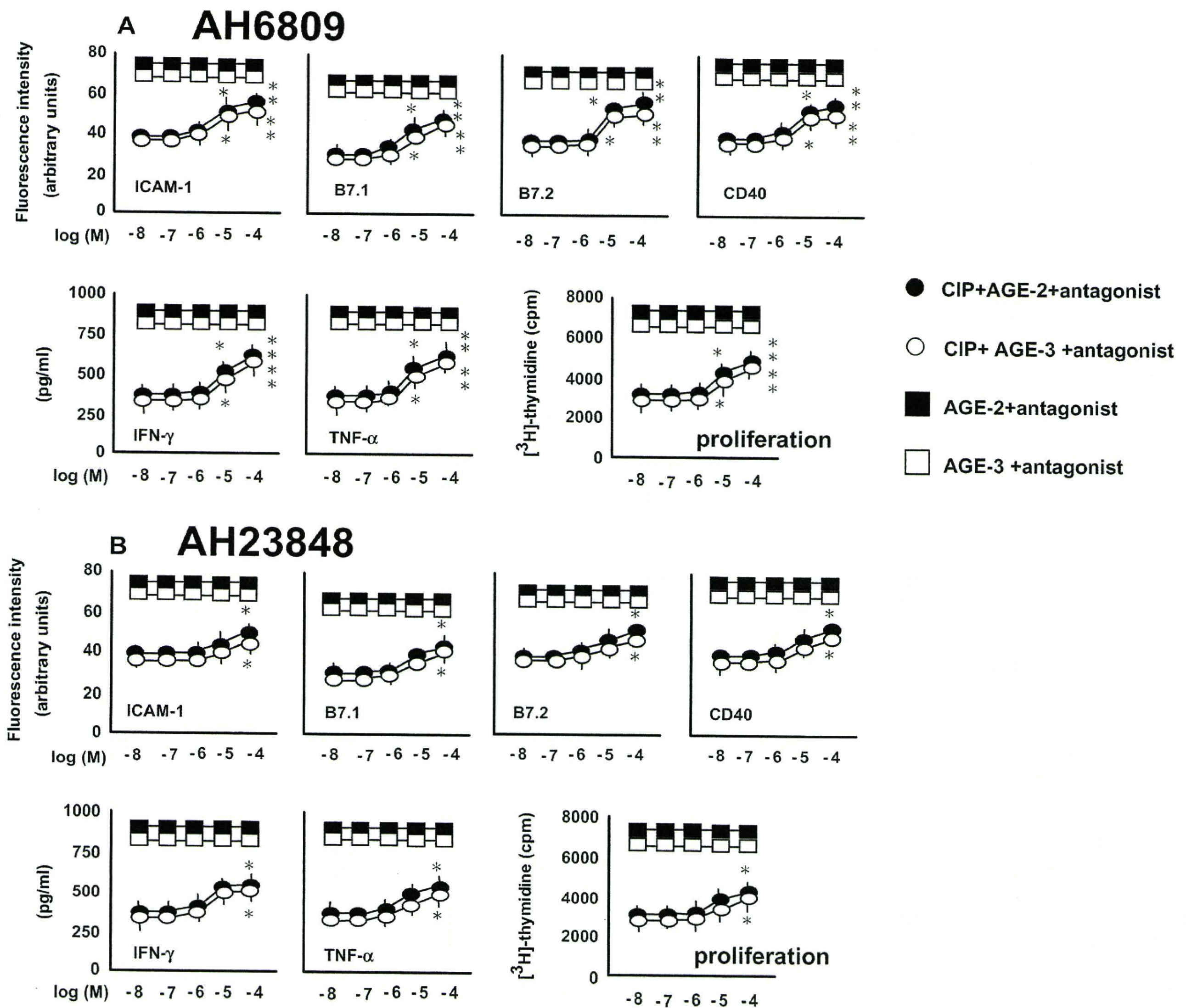


Figure 4

Effects of prostanoid receptor antagonists on the inhibitory effects of CIP on the expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40, the production of interferon (IFN)-γ and tumour necrosis factor (TNF)-α and lymphocyte proliferation. PBMC at 1×10^6 cells·mL^{-1} treated with an E-prostanoid (EP)$_2$ receptor antagonist, 6-isopropoxy-9-oxaxanthene-2-carboxylic acid (A) or an EP$_4$ receptor antagonist, (4Z)-7-[(rel-1S,2S,5R)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid (B), at increasing concentrations from 0.01 to 100 μM were incubated with CIP 100 μg·mL^{-1} in the presence of advanced glycation end product (AGE)-2 and AGE-3 at 100 μg·mL^{-1}. 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 enzyme-linked immunosorbent assay. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake as described in Methods. The results are expressed as the means ± SEM of five donors with triplicate determinations. *$P < 0.05$, **$P < 0.01$ compared with the value for CIP in the presence of AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

to 100 μM on CIP-inhibited expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and lymphocyte proliferation in PBMC were determined in the presence of AGE-2 and AGE-3, 100 μg·mL^{-1}, at 24 h (Figure 5). NS398, indomethacin and H-89 reversed the inhibitory effect of CIP on adhesion molecule expressions, cytokine production and lymphocyte proliferation. In the absence of CIP, these inhibitors did not affect the actions of AGE-2 and AGE-3 (data not shown).

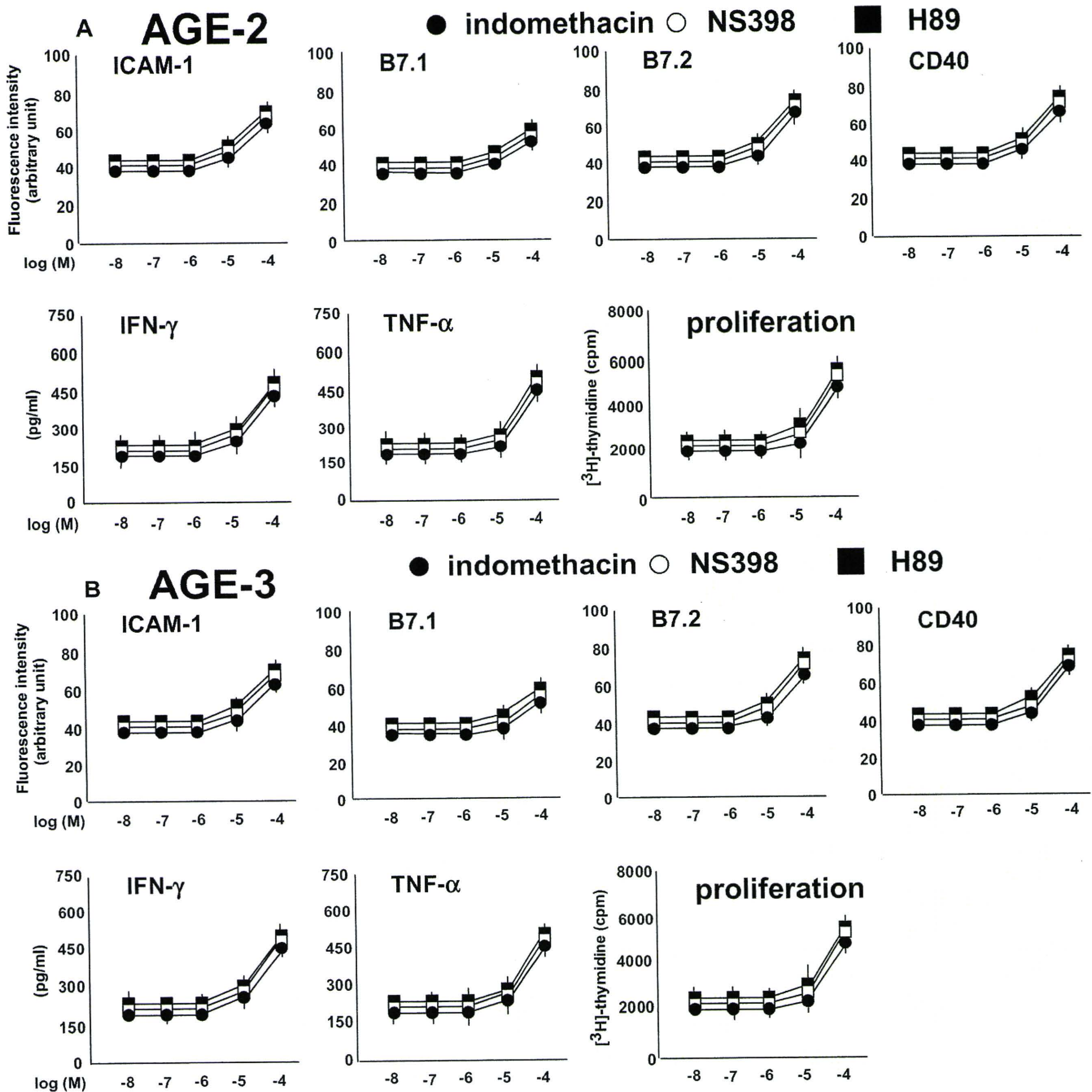


Figure 5

Effects of indomethacin, NS398 and N-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-89) on the actions of CIP. The effect of indomethacin, NS398 and H-89 at increasing concentrations ranging from 0.01 to 100 μM on the actions of CIP 100 $\mu g \cdot mL^{-1}$ were examined in the presence of , advanced glycation end product (AGE)-2 (A) and AGE-3 (B) at 100 $\mu g \cdot mL^{-1}$. Expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. Interferon (IFN)-γ and tumour necrosis factor (TNF)-α concentrations in conditioned media were determined by enzyme-linked immunosorbent assay. Lymphocyte proliferation was determined by [^{3}H]-thymidine uptake as described in Methods. The results are expressed as the means ± SEM of triplicate findings from five donors. When an error bar was within a symbol, the bar was omitted.

Discussion and conclusions

The level of AGE-2 in the serum of patients with diabetes has been reported to be 17 $\mu g \cdot mL^{-1}$ (Enomoto *et al.*, 2006; Nakamura *et al.*, 2007). AGEs at concentrations ranging from 50 to 200 $\mu g \cdot mL^{-1}$ have been shown to significantly increase human monocyte adhesion to bovine retinal endothelial

cells (Mamputu and Renier, 2004). AGEs at 200 μg·mL^{-1} increase the expressions of CD40, CD80 and CD86 and the production of IFN-γ in dendritic cells (Ge *et al.*, 2005). In a previous study, we found that AGE-2 and AGE-3 at 10 and 100 μg·mL^{-1} significantly up-regulate the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α in PBMC and lymphocyte proliferation (Wake *et al.*, 2009; Takahashi *et al.*, 2009a). Therefore, the concentration of 100 μg·mL^{-1} used in the present study covers the pathological concentration of AGEs in the serum of patients with diabetes reported in previous studies (Enomoto *et al.*, 2006; Nakamura *et al.*, 2007).

We found that AGE-2 and AGE-3 increased the production of IFN-γ and TNF-α in monocytes isolated from PBMC, exhibiting 20% of the amount obtained in PBMC (Takahashi *et al.*, 2009a). AGE-2 and AGE-3 had no effect on the production of IFN-γ and TNF-α in T-cells isolated from PBMC. Anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs inhibited the AGE-2- and AGE-3-induced production of IFN-γ and TNF-α in PBMC, indicating that an enhancement of the cell-to-cell interaction between monocytes and T-cells, through an increase in plural adhesion molecule expression on monocytes, is required for the effects of AGE-2- and AGE-3 on cytokine production. Together with these results, we examined the effect of CIP on the increased expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α in PBMC and lymphocyte proliferation in PBMC induced by AGE-2 and AGE-3.

In a randomized crossover study, the concentration of ciprofloxacin was found to be 3 μg·mL^{-1} (C max) or 14 μg·h^{-1}·mL (area under the serum concentration time curve from 0 to 12 h) in the serum of a patient who had been given a single oral dose of 500 mg (Issa *et al.*, 2007; van Zanten *et al.*, 2008), which is within the range of the concentrations used in the present study. Recently, we reported that CIP increased the expression of COX-2 and the production of PGE_2 in human monocytes (Takahashi *et al.*, 2005). In the present study, we examined, for the first time, the effects of CIP on the immune response of monocytes treated with AGE-2 and AGE-3. CIP increased the expression of COX-2 and the production of PGE_2 in the presence or absence of AGE-2 and AGE-3 (Figure 1); 20 nM PGE_2 was detected in the medium of monocytes treated with 100 μg·mL^{-1} CIP in the absence or presence of these AGEs. We also determined the levels of other COX-2 metabolites, including PGE_1, PGD_2, PGF_2, PGI_2, PGJ_2 and thromboxane, in the medium of monocytes treated with CIP in the presence or absence of AGE-2 and AGE-3, but all were under the detection limits (data not shown). This increase in endogenous PGE_2 production induced by CIP was inhibited by the non-selective COX-2 inhibitor, indomethacin, and the selective COX-2 inhibitor, NS398 (Figure 1C,D), indicating that this increase in endogenous PGE_2 production might depend on the enhancement of COX-2 expression. CIP also elevated the intracellular level of cAMP in monocytes irrespective of the presence of AGE-2 and AGE-3 (Figure 2) and this effect was abolished by NS398 (Figure 2C). These results suggest that the endogenously produced PGE_2 and elevation of cAMP are associated with the CIP-induced enhancement of COX-2 expression.

In a previous study, we found that PGE_2 inhibited the enhanced expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and lymphocyte proliferation in human PBMC induced by AGE-2 and AGE-3 (Takahashi *et al.*, 2009b). An EP_2 receptor agonist, ONO-AE1-259-01, an EP_4 receptor agonist, ONO-AE1-329, and a mixed EP_2/EP_4 receptor agonist, 11-deoxy-PGE_1, mimicked the effects of PGE_2 on adhesion molecule expression, cytokine production and lymphocyte proliferation. Moreover, an EP_2 receptor antagonist, AH6809, and an EP_4 receptor antagonist, AH23848, inhibited the actions of PGE_2. Therefore, it was suggested that the inhibitory effect of PGE_2 was mediated by the stimulation of EP_2 and EP_4 receptors. PGE_2, EP_2 and EP_4 receptor agonists induced the production of cAMP in monocytes irrespective of the presence of AGE-2 and AGE-3. A PKA inhibitor, H89, inhibited the actions of PGE_2. A cAMP analogue, dibutyryl cAMP, and an adenylate cyclase activator, forskolin, mimicked the effect of PGE_2. These results suggested the involvement of EP_2/EP_4 receptor and the cAMP/PKA pathway in the actions of PGE_2.

As shown in Figure 3, CIP suppressed the increased expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation induced by AGE-2 and AGE-3. The IC_{50} values for these effects of CIP in the presence of AGE-2 and AGE-3 were consistent with those in the presence of IL-18 (Takahashi *et al.*, 2005). While CIP on its own inhibited the basal expression levels of adhesion molecule, it had no effect on cytokine production and lymphocyte proliferation. On the other hand, PGE_2 by itself had no effect on the basal levels of adhesion molecule expressions, cytokine production and lymphocyte proliferation (Takahashi *et al.*, 2009a). The COX-2 inhibitors, but not the PKA inhibitor, abolished the effect of CIP on adhesion molecule expressions in the absence of AGE-2 and AGE-3 (Takahashi *et al.*, 2005), suggesting that endogenous PGE_2 is not

involved in the effects of CIP in the absence of AGE-2 and AGE-3.

The EP_2 receptor antagonist, AH6809, and the EP_4 receptor antagonist, AH23848, partially inhibited the actions of CIP (Figure 4). Moreover, inhibitors of COX-2 and PKA partially reversed the inhibitory effect of CIP on the enhanced expressions of adhesion molecule, cytokine production and lymphocyte proliferation induced by AGE-2 and AGE-3 (Figure 5). We observed a similar pattern for the inhibitory effects of CIP on IL-18-induced activation of monocytes in human PBMC via EP_2/EP_4 receptor (Takahashi *et al.*, 2005). Therefore, endogenous mechanisms that are both PGE_2 dependent and PGE_2 independent may be associated with the actions of CIP.

In a previous study, using an *in vitro* binding assay, we found that AGE-2 and AGE-3 had a higher affinity for RAGE than AGE-4 and AGE-5 (Takahashi *et al.*, 2009a). AGE-2 and AGE-3, but not AGE-4 and AGE-5, up-regulated the expression of the RAGE receptor on the cell surface of monocytes. We found that PGE_2 had no effect on the expression of RAGE in the presence and absence of AGE-2 and AGE-3 (Takahashi *et al.*, 2009b). In the present study, we found that CIP also had no effect on the expression of RAGE (data not shown), suggesting that there might be distinct signal transduction pathways for the regulation of expression of RAGE and adhesion molecules, leading to enhanced expression of adhesion molecules and RAGE, which are differentially regulated by the cAMP-PKA system.

Skin ulceration is a very common complication in diabetic patients and is often associated with cutaneous microangiopathy and neuropathy in these patients (Ngo *et al.*, 2005). In addition, AGEs have been shown to accumulate in the skin of diabetic patients (Liao *et al.*, 2009) and bacterial infections frequently occur in the feet of patients with diabetes mellitus and can cause serious complications (Peterson *et al.*, 1989). CIP is the antibiotic that is most frequently used to treat these foot infections (Peterson *et al.*, 1989) and the concentrations of CIP reached at the target site are several-fold higher than those in the serum (Licitra *et al.*, 1987). In addition, PGE_2, which is induced by monocytes, inhibits procollagen secretion by human vascular smooth muscle cells, leading to extracellular matrix remodelling and resistance to rupture during atherosclerosis (Fitzsimmons *et al.*, 1999). An elevation of cAMP in endothelial cells inhibits proliferation, leading to the inhibition of atherosclerosis in patients with diabetes (Lorenowicz *et al.*, 2007). The present data are consistent with the finding that the elevation of cAMP prevents the production of TNF-α in monocytes of diabetic patients (Jain *et al.*, 2002). These findings together with our results indicate that an elevation of intracellular cAMP production may regulate the activation of vascular smooth muscle cells, endothelial cells and monocytes. In conclusion, we found that the anti-microbial agent CIP is able to regulate monocyte responses and that an increased production of PGE_2 is involved in this effect. Hence, the present results suggest that CIP has therapeutic potential for the treatment of the systemic inflammatory response associated with diabetes. However, ciprofloxacin also has the ability to increase blood glucose levels; therefore, this should be taken into consideration when assessing its therapeutic value.

Acknowledgements

The authors also thank Ms Miyuki Shiotani and Mr Yukinari Isomoto for technical assistance. This work was supported in part by grants from the Japanese Society for the Promotion of Science [Grants 18590509, 20590539, 17659159, 19659061, 21659141, 21390071, 215905694], from the Scientific Research from Ministry of Health, Labour and Welfare of Japan and from the Takeda Science Foundation.

Statement of conflicts of interest

None.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edn. Br J Pharmacol 153 (Suppl. 2): S1–S209.

Bastien L, Sawyer N, Grygorczyk R, Metters KM, Adam M (1994). Cloning, functional expression, and characterization of the human prostaglandin E2 receptor EP2 subtype. J Biol Chem 269: 11873–11877.

Bierhaus A, Hofmann MA, Ziegler R, Nawroth PP (1998). AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. Cardiovasc Res 37: 586–600.

Brownlee M (1995). Advanced protein glycosylation in diabetes and aging. Annu Rev Med 46: 223–234.

Burke AP, Kolodgie FD, Zieske A, Fowler DR, Weber DK, Varghese PJ *et al.* (2004). Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. Arterioscler Thromb Vasc Biol 24: 1266–1271.

Camacho SA, Heath WR, Carbone FR, Sarvetnick N, LeBon A, Karlsson L *et al.* (2001). A key role for ICAM-1 in generating effector cells mediating inflammatory responses. Nat Immunol 6: 523–529.

Coleman RA, Smith WL, Narumiya S (1994). VIII. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptor and their subtypes. Pharmacol Rev 46: 205–229.

Cuccurullo C, Iezzi A, Fazia ML, De Cesare D, Di Francesco A, Muraro R *et al.* (2006). Suppression of RAGE as a basis of simvastatin-dependent plaque stabilization in type 2 diabetes. Arterioscler Thromb Vasc Biol 26: 2716–2723.

Durie FH, Foy TM, Masters SR, Laman JD, Noelle RJ (1994). The role of CD40 in the regulation of humoral and cell-mediated immunity. Immunol Today 9: 406–411.

Enomoto M, Adachi H, Yamagishi S, Takeuchi M, Furuki K, Hino A *et al.* (2006). Positive association of serum levels of advanced glycation end products with thrombogenic markers in humans. Metabolism 55: 912–917.

Fitzsimmons C, Proudfoot D, Bowyer DE (1999). Monocyte prostaglandins inhibit procollagen secretion by human vascular smooth muscle cells: implications for plaque stability. Atherosclerosis 142: 287–293.

Fukami K, Ueda S, Yamagishi S, Kato S, Inagaki Y, Takeuchi M *et al.* (2004). AGEs activate mesangial TGF-beta-Smad signaling via an angiotensin II type I receptor interaction. Kidney Int 66: 2137–2147.

Ge J, Jia Q, Liang C, Luo Y, Huang D, Sun A *et al.* (2005). Advanced glycosylation end products might promote atherosclerosis through inducing the immune maturation of dendritic cells. Arterioscler Thromb Vasc Biol 25: 2157–2163.

Issa MM, Nejem RM, El-Abadla NS, El-Naby MK, Roshdy AA, Kheiralla ZA (2007). Effects of paracetamol on the pharmacokinetics of ciprofloxacin in plasma using a microbiological assay. Clin Drug Investig 27: 463–467.

Jain SK, Kannan K, Lim G, McVie R, Bocchini JA Jr (2002). Hyperketonemia increases tumor necrosis factor-alpha secretion in cultured U937 monocytes and Type 1 diabetic patients and is apparently mediated by oxidative stress and cAMP deficiency. Diabetes 51: 2287–2293.

Liao H, Zakhaleva J, Chen W (2009). Cells and tissue interactions with glycated collagen and their relevance to delayed diabetic wound healing. Biomaterials 30: 1689–1696.

Licitra CM, Brooks RG, Sieger BE (1987). Clinical efficacy and levels of ciprofloxacin in tissue in patients with soft tissue infection. Antimicrob Agents Chemother 31: 805–807.

Lorenowicz MJ, Fernandez-Borja M, Hordijk PL (2007). cAMP signaling in leukocyte transendothelial migration. Arterioscler Thromb Vasc Biol 27: 1014–1022.

Mamputu JC, Renier G (2004). Advanced glycation end-products increase monocyte adhesion to retinal endothelial cells through vascular endothelial growth factor-induced ICAM-1 expression: inhibitory effect of antioxidants. J Leukoc Biol 75: 1062–1069.

Nakamura K, Yamagishi SI, Matsui T, Adachi H, Takeuchi M, Imaizumi T (2007). Serum levels of soluble form of receptor for advanced glycation end products (sRAGE) are correlated with AGEs in both diabetic and non-diabetic subjects. Clin Exp Med 7: 188–190.

Ngo BT, Hayes KD, DiMiao DJ, Srinivasan SK, Huerter CJ, Rendell MS (2005). Manifestations of cutaneous diabetic microangiopathy. Am J Clin Dermatol 6: 225–237.

Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS *et al.* (1998). Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. Nat Med 4: 1025–1031.

Peterson LR, Lissack LM, Canter K, Fasching CE, Clabots C, Gerding DN (1989). Therapy of lower extremity infections with ciprofloxacinin patients with diabetes mellitus, peripheral vascular disease, or both. Am J Med 86: 801–808.

Ranger AM, Das MP, Kuchroo VK, Glimcher LH (1996). B7-2 (CD86) is essential for the development of IL-4-producing T cells. Int Immunol 10: 1549–1560.

Riesbeck K (2002). Immunomodulating activity of quinolones: review. J Chemother 14: 3–12.

Riesbeck K, Forsgren A (1990). Selective enhancement of synthesis of interleukin-2 in lymphocytes in the presence of ciprofloxacin. Eur J Clin Microbiol Infect Dis 9: 409–413.

Schmidt AM, Hasu M, Popov D, Zhang JH, Chen J, Yan SD *et al.* (1994). Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins. Proc Natl Acad Sci U S A 91: 8807–8811.

Takahashi HK, Iwagaki H, Tamura R, Xue D, Sano M, Mori S *et al.* (2003). Unique regulation profile of prostaglandin E1 on adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells. J Pharmacol Exp Ther 307: 1188–1195.

Takahashi HK, Iwagaki H, Xue D, Katsuno G, Sugita S, Mizuno K *et al.* (2005). Effect of ciprofloxacin-induced prostaglandin E2 on interleukin-18-treated monocytes. Antimicrob Agents Chemother 49: 3228–3233.

Takahashi HK, Mori S, Wake H, Yoshino T, Ohashi K, Tanaka N *et al.* (2009a). Advanced glycation end products subspecies-selectively induce adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells. J Pharmacol Exp Ther 330: 89–98.

Takahashi HK, Liu K, Wake H, Mori S, Zhang J, Liu R *et al.* (2009b). Prostaglandins E_2 inhibits advanced glycation end products-induced adhesion molecule

expression, cytokine production and lymphocyte proliferation in human peripheral blood mononuclear cells. J Pharmacol Exp Ther 331: 656–670.

Takedo A, Yasuda T, Miyata T, Mizuno K, Li M, Yoneyama S *et al.* (1996). Immunohistochemical study of advanced glycation end products in aging and Alzheimer's disease brain. Neurosci Lett 221: 17–20.

Takeuchi M, Yamagishi S (2004). TAGE (toxic AGEs) hypothesis in various chronic diseases. Med Hypotheses 63: 449–452.

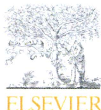
Takeuchi M, Makita Z, Bucala R, Suzuki T, Koike T, Kameda Y (2000). Immunological evidence that non-carboxymethyllysine advanced glycation end-products are produced from short chain sugars and dicarbonyl compounds in vivo. Mol Med 6: 114–125.

van Zanten AR, Polderman KH, van Geijlswijk IM, van der Meer GY, Schouten MA, Girbes AR (2008). Ciprofloxacin pharmacokinetics in critically ill patients: a prospective cohort study. J Crit Care 23: 422–430.

Wake H, Takahashi HK, Mori S, Liu K, Yoshino T, Nishibori M (2009). Histamine inhibits advanced glycation end products-induced adhesion molecule expression on human monocytes. J Pharmacol Exp Ther 330: 826–833.

Yamagishi S, Imaizumi T (2005). Diabetic vascular complications: pathophysiology, biochemical basis and potential therapeutic strategy. Curr Pharm Des 11: 2279–2299.

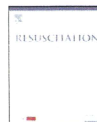
Resuscitation 82 (2011) 219–221



Contents lists available at ScienceDirect

Resuscitation

journal homepage: www.elsevier.com/locate/resuscitation



Experimental paper

Reduction of the infarct size by simultaneous administration of L-histidine and diphenhydramine in ischaemic rat brains☆

Naoto Adachi[a,*], Keyue Liu[b], Kanji Ninomiya[a], Eiko Matsuoka[a], Atsuko Motoki[c], Yumi Irisawa[d], Masahiro Nishibori[b]

[a] *Mabuchi Clinic, Kyoto-shi, Kyoto, Japan*
[b] *Department of Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama-shi, Okayama, Japan*
[c] *Department of Anesthesia, Japanese Red Cross Kyoto Daini Hospital, Kyoto-shi, Kyoto, Japan*
[d] *Department of Anesthesia, Noto General Hospital, Nanao-shi, Ishikawa, Japan*

ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form 20 August 2010
Accepted 20 October 2010

Keywords:
Brain ischaemia
Drug therapy
Inflammatory response
Neurones
Reperfusion
Stroke

ABSTRACT

Aims: While diphenhydramine is a histamine H_1 receptor antagonist, the agent has been shown to inhibit histamine-*N*-methyltransferase, a histamine inactivating enzyme in the brain. Since an increase in the brain concentration of histamine ameliorates reperfusion injury after cerebral ischaemia, effects of postischaemic administration of diphenhydramine were evaluated in rats treated with L-histidine, a precursor of histamine.
Methods: The right middle cerebral artery was occluded for 2 h, and the infarct size was determined 24 h after reperfusion of cerebral blood flow. Brain oedema was evaluated by comparing the area of the right hemisphere to that of the left hemisphere.
Results: Focal cerebral ischaemia provoked marked damage in saline-treated control rats, and infarct volumes in the striatum and cerebral cortex were 56 (49–63) mm^3 and 110 (72–148) mm^3, respectively (means and 95% confidence intervals, $n = 6$). Administration of L-histidine (1000 mg/kg, intraperitoneal) immediately after reperfusion did not affect the infarct size. Simultaneous administration of diphenhydramine (20 mg/kg, intraperitoneal) with L-histidine reduced the infarct size to 25% and 21% of that in the control group, respectively. The combination therapy completely reduced ischaemia-induced brain oedema.
Conclusion: Because histamine H_1 action does not influence ischaemic brain damage, elevation of the central histamine concentration by blockade of histamine-*N*-methyltransferase may be a likely mechanism responsible for the alleviation.

© 2010 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

We have shown that an increase in the histamine concentration in the brain alleviates reperfusion injury after brain ischaemia by reducing inflammatory responses through histamine H_2 receptors.[1–4] Although a single dose of L-histidine (1000 mg/kg), a precursor of brain histamine, failed to improve the outcome, simultaneous administration of metoprine, a competitive inhibitor of histamine-*N*-methyltransferase, an inactivating enzyme of brain histamine, reduced the size of brain infarction.[3]

There are several compounds that inhibit histamine-*N*-methyltransferase activity, such as amodiaquine, tacrine, chlorpromazine and vecuronium. However, adverse effects of these agents are problematic as well as those of metoprine. Diphenhydramine, a histamine H_1 receptor antagonist, is usually applied to various allergic diseases and has an inhibitory effect on histamine-*N*-methyltransferase.[5,6] Because the agent readily passes the blood–brain barrier, and no serious adverse effect has been reported, we investigated the effect of concomitant administration of diphenhydramine and L-histidine on ischaemic brain damage.

2. Methods

This study was approved by the Committee on Animal Experimentation at Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by Okayama Univer-

☆ A Spanish translated version of the abstract of this article appears as Appendix in the online version at doi:10.1016/j.resuscitation.2010.10.024.

* Corresponding author at: Mabuchi Clinic, Kakimoto-cho 590-4, Shimogyo-ku, Kyoto-shi, Kyoto 600-8357, Japan. Fax: +81 75 813 3810.
E-mail address: nadachi@m.ehime-u.ac.jp (N. Adachi).

0300-9572/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.resuscitation.2010.10.024

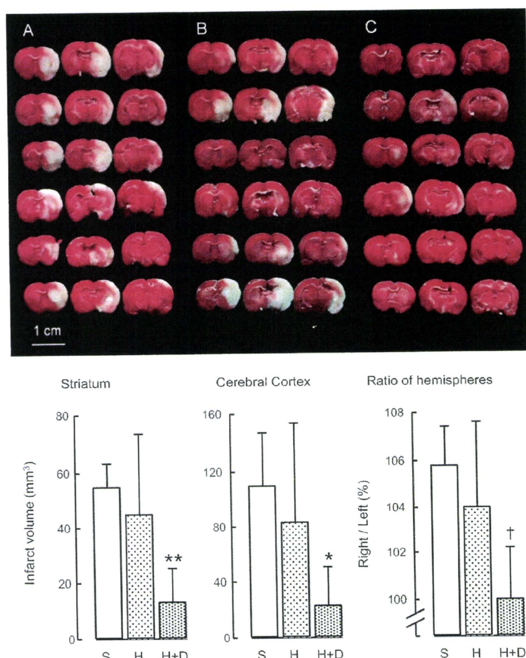


Fig. 1. Effects of postischaemic administration of L-histidine and diphenhydramine. The right middle cerebral artery was occluded for 2 h, and L-histidine (1000 mg/kg) and diphenhydramine (20 mg/kg) were intraperitoneally administered immediately after reperfusion of cerebral blood flow. The effect was evaluated by assessing the size of brain infarction 24 h after reperfusion with 2,3,5-triphenyltetrazolium chloride stain, which turns the viable tissue a deep red colour. All brain slices in the saline-injected control (A), L-histidine (B) and L-histidine plus diphenhydramine groups (C) are shown. The size of brain infarction was measured in the striatum and cerebral cortex. Using these brain slices, the percentage of the area of the right (ischaemic) hemisphere to that of the left (non-ischaemic) hemisphere was calculated as a measure of brain oedema. Values represent means and 95% confidence intervals ($n = 6$ each). $^{*}p = 0.02$, $^{**}p = 0.004$, $^{\dagger}p = 0.006$ as compared with the control group. S, saline; H, L-histidine; D, diphenhydramine.

sity Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. Male Wistar rats (Charles River, Yokohama, Japan) weighing 280–300 g were kept in groups in a room controlled at 23 ± 2 °C and maintained under an alternating 12-h light and 12-h dark cycle.

Eighteen rats were divided into three groups ($n = 6$ in each); the saline-injected control group, the L-histidine-injected group and the L-histidine plus diphenhydramine-injected group. The animal was anesthetised with 2% halothane in balanced 50% oxygen and 50% nitrous oxide, and kept under spontaneous ventilation. With the rat in the supine position, the right common carotid artery was exposed, and a thermocouple needle probe was inserted into the temporal muscle to maintain the brain temperature. After an intraperitoneal injection of heparin (100 units), the root of the right middle cerebral artery was occluded by insertion of a silicone-coated 4.0 nylon thread from the bifurcation of the internal and external carotid arteries. The tip of the thread was placed 18 mm distal from the bifurcation. After the surgical incision was sutured, the animal was allowed to recover from anesthesia. During surgery, the temperature of the temporal muscle was maintained at 37.0 ± 0.1 °C with a heating lamp. All rats showed paralysis of the contralateral limbs after recovery from anesthesia. The animal was anesthetised again 5 min before reperfusion of the blood flow, and the skin was reopened. Cerebral blood flow was resumed 2 h after middle cerebral artery occlusion by pulling the thread by 5 mm. Then, the animal was intraperitoneally injected with saline, L-histidine (1000 mg/kg) or L-histidine (1000 mg/kg) plus diphenhydramine (20 mg/kg). The surgical incision was sutured, and the animal was allowed to recover from anesthesia.

The animal that underwent 24 h of reperfusion was anesthetised with an intraperitoneal injection of sodium pentobarbital, and the brain was perfused with saline. Brain slices, 2-mm thick, between the coronal planes at the optic chiasma and caudal edge of the mammillary body were incubated for 30 min with 2% 2,3,5-triphenyltetrazolium chloride in 0.1 mol/L phosphate buffer (pH 7.4) at 37 °C. 2,3,5-Triphenyltetrazolium chloride is reduced by dehydrogenase enzymes, which exist in viable cells and result in a formazan precipitate, thereby turning the tissue a deep red colour. In contrast, nonviable cells in the infarcted area show a pale gray colour with the procedure. The infarct size in the striatum and cerebral cortex was then determined, using computer-aided planimetry by an investigator who was unaware of the particular treatment group. Using these brain slices, the ratio of the area of the right

hemisphere to that of the left hemisphere was obtained in each slice. Then, the average percentage of three slices was calculated in each animal.

The data were evaluated by Scheffé's tests, and p values less than 0.05 were considered statistically significant.

3. Results

Focal cerebral ischaemia for 2 h provoked marked damage in the striatum and surrounding cerebral cortex in saline-treated control animals (Fig. 1). The infarct volumes in the striatum and cerebral cortex were 56 (49–63) mm^3 and 110 (72–148) mm^3, respectively (means and 95% confidence intervals, $n = 6$). Postischaemic administration of L-histidine (1000 mg/kg) affected the infarct size in neither the striatum ($p = 0.64$) nor the cerebral cortex ($p = 0.65$). Simultaneous administration of diphenhydramine (20 mg/kg) with L-histidine reduced the infarct volume in both the striatum ($p = 0.004$) and cerebral cortex ($p = 0.02$), and the values were 25% and 21% of those in control animals, respectively.

The ratio of the area of the right hemisphere to that of the left hemisphere was 105.8% (104.0–107.6%) in the control group. Although a single dose of L-histidine did not affect the ratio ($p = 0.54$), simultaneous administration significantly reduced it ($p = 0.006$).

4. Discussion

In the present study, L-histidine administration did not ameliorate the size of brain infarction. The finding is consistent with our previous reports that L-histidine provides no benefits at any doses by a single dose after reperfusion.[3,7] Simultaneous administration of diphenhydramine reduced the size of brain infarction, and the magnitude of the decrease is similar to that obtained from combination therapy with L-histidine and metoprine.[3] The treatment with L-histidine and diphenhydramine markedly reduced ischaemia-induced brain oedema as well as the infarct size.

In our previous study, brain infarction produced by occlusion of the middle cerebral artery for 2 h was alleviated by postischaemic administration of L-histidine (1000 mg/kg × 2), immediately and 6 h after reperfusion.[2] The beneficial effect was completely abolished by topical administration of ranitidine, a histamine H_2 receptor antagonist, indicating that an increase in the histamine level contributes to the improvement through histamine H_2 receptors. Considering that diphenhydramine is a potent H_1 antagonist and has negligible affinity for histamine H_2 receptors, it is unlikely that the improvement of the outcome by diphenhydramine attributes to its blocking action on histamine H_1 receptors.

Methylation by the specific enzyme, histamine-*N*-methyltransferase, is the predominant pathway of histamine inactivation in the brain, and no high-affinity uptake for histamine has been reported in brain slices, homogenates and cultured neurones.[8–10] In a study on purified histamine-*N*-methyltransferase from the mouse brain, diphenhydramine showed a biphasic effect on histamine-*N*-methyltransferase activity.[5] At histamine concentrations below 10 μmol/L, diphenhydramine inhibited the enzyme activity. On the other hand, the agent markedly augmented it at histamine concentrations in excess of 10 μmol/L. Although the extracellular concentration of histamine increases in ischaemic brains, the concentration does not attain 10 μmol/L.[7,11] Therefore, diphenhydramine may show an inhibitory action on histamine-*N*-methyltransferase in cerebral ischaemia. Facilitation of the brain concentration of histamine by simultaneous administration may provide benefits by suppressing inflammatory responses during reperfusion through histamine H_2 receptors.

5. Conclusion

Since both L-histidine and diphenhydramine are readily transported to the brain across the blood–brain barrier, simultaneous administration of these compounds may be a new strategy for stroke.

Conflict of interest

N. Adachi, K. Liu, A. Motoki and M. Nishibori are concerned with patent applications/registrations related to reperfusion therapies with L-histidine. K. Ninomiya, E. Matsuoka and Y. Irisawa have no conflicts of interest.

References

1. Adachi N. Cerebral ischemia and brain histamine. Brain Res Rev 2005;50:275–86.
2. Adachi N, Liu K, Arai T. Prevention of brain infarction by postischemic administration of histidine in rats. Brain Res 2005;1039:220–3.
3. Motoki A, Adachi N, Semba K, Liu K, Arai T. Reduction in brain infarction by augmentation of central histaminergic activity in rats. Brain Res 2005;1066:172–8.
4. Hiraga N, Adachi N, Liu K, Nagaro T, Arai T. Suppression of inflammatory cell recruitment by histamine receptor stimulation in ischemic rat brains. Eur J Pharmacol 2007;557:236–44.
5. Taylor KM, Snyder SH. Histamine methyltransferase: inhibition and potentiation by antihistamines. Mol Pharmacol 1972;8:300–10.
6. Pollard S, Bischoff S, Schwartz JC. Modifications of brain HA metabolism induced by antihistamines. Agents Actions 1973;3:190–1.
7. Irisawa Y, Adachi N, Liu K, Arai T, Nagaro T. Alleviation of ischemia-induced brain edema by activation of the central histaminergic system in rats. J Pharmacol Sci 2008;108:112–23.
8. Hösli E, Hösli L. Autoradiographic localization of binding sites for [^{3}H]histamine and H_1- and H_2-antagonists on cultured neurons and glial cells. Neuroscience 1984;13:863–70.
9. Schayer RW, Reilly MA. Metabolism of ^{14}C-histamine in brain. J Pharmacol Exp Ther 1973;187:34–9.
10. Schwartz JC, Pollard H, Bischoff S, Rehault MC, Verdiere-Sahuque M. Catabolism of ^{3}H-histamine in the rat brain after intracisternal administration. Eur J Pharmacol 1971;16:326–35.
11. Adachi N, Itoh Y, Oishi R, Saeki K. Direct evidence for increased continuous histamine release in the striatum of conscious freely moving rats produced by middle cerebral artery occlusion. J Cereb Blood Flow Metab 1992;12:477–83.

High-Mobility Group Box Protein 1 Neutralization Reduces Development of Diet-Induced Atherosclerosis in Apolipoprotein E–Deficient Mice

Peter Kanellakis, Alex Agrotis, Tin Soe Kyaw, Christine Koulis, Ingo Ahrens, Shuji Mori, Hideo K. Takahashi, Keyue Liu, Karlheinz Peter, Masahiro Nishibori, Alex Bobik

Objective—High-mobility group box protein 1 (HMGB1) is a DNA-binding protein and cytokine highly expressed in atherosclerotic lesions, but its pathophysiological role in atherosclerosis is unknown. We investigated its role in the development of atherosclerosis in ApoE−/− mice.

Methods and Results—Apolipoprotein E–deficient (ApoE−/−) mice fed a high-fat diet were administered a monoclonal anti-HMGB1 neutralizing antibody, and the effects on lesion size, immune cell accumulation, and proinflammatory mediators were assessed using Oil Red O, immunohistochemistry, and real-time polymerase chain reaction. As with human atherosclerotic lesions, lesions in ApoE−/− mice expressed HMGB1. Treatment with the neutralizing antibody attenuated atherosclerosis by 55%. Macrophage accumulation was reduced by 43%, and vascular cell adhesion molecule-1 and MCP-1 expression was attenuated by 48% and 72%, respectively. CD11c+ dendritic cells were reduced by 65%, and the mature (CD83+) population was reduced by 60%. Treatment also reduced CD4+ cells by nearly 50%. mRNAs in lesions encoding tumor necrosis factor-α and interleukin-1β tended to be reduced. Mechanistically, HMGB1 stimulated macrophage migration in vitro and in vivo; in vivo, it markedly augmented the accumulation of F4/80+Gr-1(Ly-6C)+ macrophages and also increased F4/80+CD11b+ macrophage numbers.

Conclusion—HMGB1 exerts proatherogenic effects augmenting lesion development by stimulating macrophage migration, modulating proinflammatory mediators, and encouraging the accumulation of immune and smooth muscle cells. **(*Arterioscler Thromb Vasc Biol.* 2011;31:00-00.)**

Key Words: atherosclerosis ■ macrophages ■ HMGB1

Atherosclerosis is a chronic inflammatory disease characterized by intimal accumulation of atherogenic lipoproteins, extracellular matrix, smooth muscle cells, and inflammatory cells. Cytokines within atherosclerotic lesions play a key role in both the development and progression of atherosclerosis.[1,2] Recently, we and others have identified a novel cytokine in human atherosclerotic lesions, high-mobility group box 1 (HMGB1), that could be important for regulating development of atherosclerosis.[3–5] HMGB1 has 2 main functions. As a nuclear protein, it stabilizes nucleosomes and bending of DNA, which facilitates gene transcription. It can also be released from necrotic cells[6] or secreted by inflammatory cells, such as macrophages,[3] and natural killer cells,[7] triggering inflammation.[8] HMGB1-nucleosome complexes released by necrotic cells activate macrophages and dendritic cells to produce cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-10[9]; also, native secreted HMGB1 stimulates monocytes to secrete proinflammatory cytokines.[10] With respect to the vasculature, HMGB1 stimulates the migration and proliferation of vascular smooth muscle cells[4,11] and activates endothelial cells.[12] It induces the migration of macrophages, activates dendritic cells,[13,14] and is required for dendritic cells maturation.[14,15] Notably, dendritic cells control T-cell activation by secreting HMGB1.[16]

Structurally, HMGB1 has a tripartite domain organization. It contains 2 similar DNA-binding domains, HMG Box A and Box B, and a unique C-terminal domain consisting of an acidic tail of 30 amino acids all connected by short amino acid sequences.[17] The proinflammatory activity of HMGB1 has been localized to Box B, and antibodies raised against Box B prevent HMGB1 actions[17,18]; a RAGE-binding domain is localized within the C-terminal component of Box B and the segment connecting to the acidic tail.[19] In contrast, Box A attenuates HMGB1-induced release of proinflammatory cytokines.[20,21] The acidic tail is thought to be unstructured and interacts with specific basic residues in both boxes, possibly regulating their conformation.[22]

Received on: September 7, 2009; final version accepted on: November 2, 2010.

From the BakerIDI Heart and Diabetes Institute, Melbourne, Victoria, Australia (P.K., A.A., T.S.K., C.K., I.A., K.P., A.B.); Department of Pharmacology and Pathology, Okayama University Graduate School of Medicine, Okayama University, Okayama, Japan (S.M., H.K.T., K.L., M.N.).

Correspondence to Alex Bobik, BakerIDI Heart and Diabetes Institute, PO Box 6492 St Kilda Rd Central, Melbourne, Victoria 8008, Australia. E-mail alex.bobik@baker.edu.au

© 2010 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.110.218669

Downloaded from atvb.ahajournals.org by on November 20, 2010

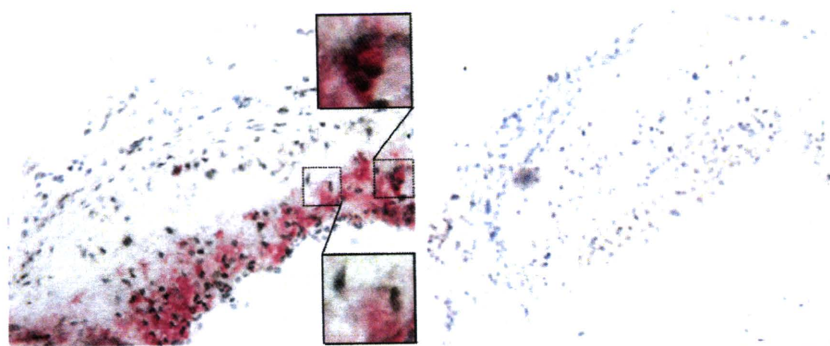


Figure 1. Immunohistochemical identification of HMGB1 expression in aortic sinus atherosclerotic lesions in ApoE−/− mice. Left, HMGB1 (red staining) was localized within intimal cells (top insert) and also diffusely distributed within lesions (bottom inset). Cells within the media did not express HMGB1. Right, Region similar to that shown in A incubated with nonspecific rabbit IgG instead of the primary rabbit HMGB1 IgG. Section was counterstained with hematoxylin. Scale bar represents 100 μm.

The proinflammatory effects of HMGB1 have been attributed to its interaction with a number of receptor types, which appears dependent on factors bound to HMGB1. RAGE, TLR2, and TLR4 appear to mediate proinflammatory effects in HMGB1-stimulated macrophages.[23,24] HMGB1 in DNA immune complexes and nucleosome complexes appears to exert proinflammatory effects via TLR9 and TLR2, respectively.[12,25] More recently, HMGB1 has also been shown to interact with CD24 and Siglec-10 to attenuate tissue damage induced immune responses[26]; a balance between these and the detrimental effects of HMGB1 has been suggested to determine the overall magnitude of its detrimental effects.[26]

HMGB1 has been implicated in a number of immune-driven diseases, including systemic lupus erythematosus, autoimmune diabetes, and arthritis.[9,27,28] In arthritis, it triggers inflammation by activating macrophages and inducing IL-1.[28] Because HMGB1 is overexpressed in atherosclerotic lesions[3–5] and activates immune processes that can augment atherosclerosis,[4,11,13–15] we examined its role in the development of atherosclerosis in apolipoprotein E–deficient mice (ApoE−/− mice). We used an anti-HMGB1neutralizing monoclonal antibody, which interacts specifically with the C-terminal sequence of HMGB1 within the acidic tail, to determine its role in atherosclerosis. This antibody reacts with HMGB1 and not HMGB2 and has been shown to inhibit HMGB1 responses in macrophages and ameliorates brain infarction induced by transient ischemia.[29]

Materials and Methods

Six-week-old ApoE−/− mice were fed a high-fat diet for 8 weeks and administered either an anti-HMGB1 monoclonal antibody or IgG2a control (400 μg IV twice weekly). At the end of the study, mice were killed with an overdose of pentobarbitone, and blood, aortic sinus and arch, spleen, and lymph nodes were collected for histological and molecular studies. Monocyte proliferation in vivo was assessed after administering bromodeoxyuridine (1 mg IP) for 3 days before the mice were killed. The chemotactic effects of HMGB1 on monocytes was assessed in mice 5 hours after administering HMGB1 (20 μg) into the peritoneal cavity.

A detailed Supplemental Methods section is available online at http://atvb.ahajournals.org.

Results

HMGB1 Expression in Atherosclerotic Lesions

HMGB1 is highly expressed in human atherosclerotic lesions, mostly by macrophages in which nearly 50% of the cells contained HMGB1 in their cytoplasm.[3] Therefore, we investigated whether atherosclerotic lesions in ApoE−/− mice also expressed HMGB1. HMGB1 was expressed within the aortic sinus of ApoE−/− mice and restricted to cells within the atherosclerotic intima (Figure 1). HMGB1 appeared to be cell associated, frequently within the cytoplasm and also diffusely distributed within lesions; the latter most probably reflected secreted HMGB1 (Figure 1).

Effect of HMGB1 Neutralization on Atherosclerotic Lesion Size

At 14 weeks of age (8 of weeks antibody treatment) total plasma cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were not different between mice treated with control or anti-HMGB1 neutralizing antibody (Supplemental Table I). However, analysis of Oil Red O–stained aortic sinus sections revealed significant differences in lipid deposition and atherosclerotic plaque size (Figure 2). Morphometric analysis showed that the anti-HMGB1 neutralizing antibody reduced lesion size compared with treatment with control antibody, 89 253±13 098 μm^2 versus 183 903±11 784 μm ($P<0.05$; Figure 2). Similarly, macrophage accumulation was reduced in lesions of mice treated with the neutralizing antibody. Cross-sectional area of the aortic sinus that stained with anti-CD68 antibody averaged 83 400±15 451 μm^2 in mice treated with the anti-HMGB1 neutralizing antibody compared with 141 364±12 608 μm^2 with control antibody (Figure 2). To determine whether the effects of the HMGB1 antibody were due to neutralizing circulating HMGB1, we measured plasma HMGB1 levels in control and anti-HMGB1 antibody treated mice using a specific HMGB1 ELISA. Plasma HMGB1 levels were undetectable (<1 ng/mL) in both instances, similar to earlier reports in nonatherosclerotic mice,[30] suggesting that effects of the neutralizing antibody were local at the site of developing lesions.

Effect of Neutralizing HMGB1 on Atherosclerotic Lesion Composition

Analysis of atherosclerotic lesion composition also revealed differences between the 2 groups in terms of cellular composition. Because HMGB1 can stimulate the migration of dendritic cells,[14,15] we investigated whether neutralizing HMGB1 activity attenuated their accumulation in developing lesions. Immunohistochemical studies and quantitative analysis indicated a marked reduction in dendritic cell accumulation, with CD11c staining averaging 8±1% of lesion area in

Downloaded from atvb.ahajournals.org by on November 20, 2010

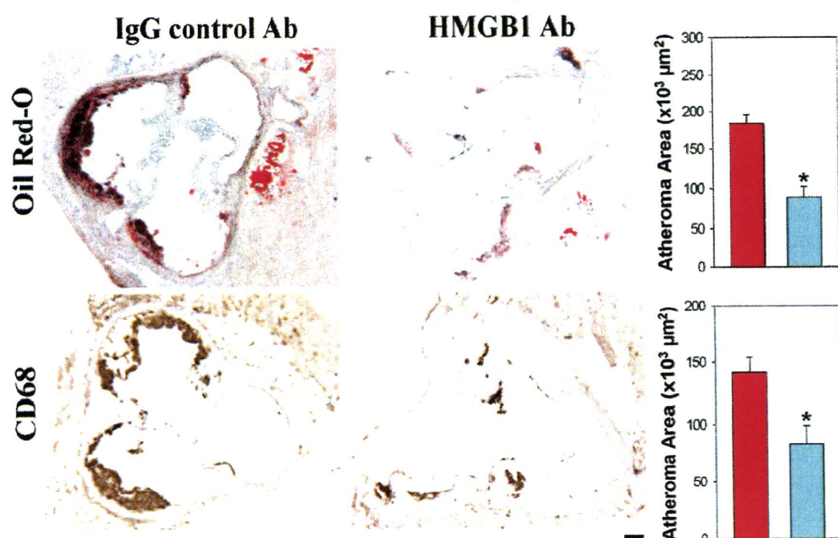


Figure 2. Photomicrographs of aortic sinus atherosclerotic lesions from ApoE−/− mice fed a high-fat diet and treated with control antibody (Ab; left) and anti-HMGB1 neutralizing antibody (right). Top sections show sections stained with Oil Red O and mean areas of staining (bar graph) in the 2 groups of mice. Bottom sections, Immunohistochemical staining using anti-CD68 (macrophage) antibody and mean areas of staining (bar graphs) in the 2 groups of mice. Red indicates control antibody; blue, anti-HMGB1 neutralizing antibody. *$P<0.05$ compared with control. Scale bar represents 100 μm.

aortic sinus of mice treated with anti-HMGB1 neutralizing antibody compared with 24±3% with control antibody ($P<0.05$; Figure 3). Because HMGB1 can also promote maturation of dendritic cells,[15] we investigated whether expression of CD83, a marker of dendritic cell maturation, was affected. CD83 expression in the lesions represented only a small fraction of the dendritic cell population, indicating that the dendritic cell population was largely immature (Figure 3). Treatment with the anti-HMGB1 antibody reduced the CD83 cell population by nearly 60% ($P<0.05$; Figure 3). Mature dendritic cells in atherosclerotic lesions are known to produce T-cell–attracting chemokines CCL19 and CCL21.[31] Consequently, we examined whether CD4+ T-cell accumulation was affected in mice treated with the anti-HMGB1 neutralizing antibody. Treatment with anti-HMGB1 neutralizing antibody reduced CD4+ T-cell accumulation in lesions by ≈50% ($P<0.05$; Figure 4). To determine the different T-cell subtypes that might be affected, we analyzed subset-specific mRNA expression. In lesions of anti-HMGB1 antibody–treated mice, we observed no change in expression of Tim-3, a known marker and negative regulator of Th1 lymphocytes ($P>0.05$; Figure 4)[32]; Tim-1, a marker of Th2 lymphocytes, was undetectable (Figure 4).[33]

Because HMGB1 stimulates the in vitro migration and proliferation of vascular smooth muscle cells,[4,11] and endothelial cells,[34] we also investigated whether it affected smooth muscle cell and endothelial cell numbers in developing lesions. Treatment with the anti-HMGB1 neutralizing antibody reduced smooth muscle cell accumulation within developing lesions by nearly 50% ($P<0.05$; Supplemental Figure I) but did not affect lesion-associated endothelial cells (Supplemental Figure I). Treatment with the neutralizing antibody was also associated with a 35% reduction in the number of proliferating cells within the lesions ($P<0.05$; Supplemental Figure I).

To further confirm that these cellular effects were largely restricted to developing atherosclerotic lesions, we also assessed lymphocyte populations in blood, spleen, and paraaortic lymph nodes. In these tissues anti-HMGB1 antibody treatment did not affect CD4+ T cells, B cells, natural killer

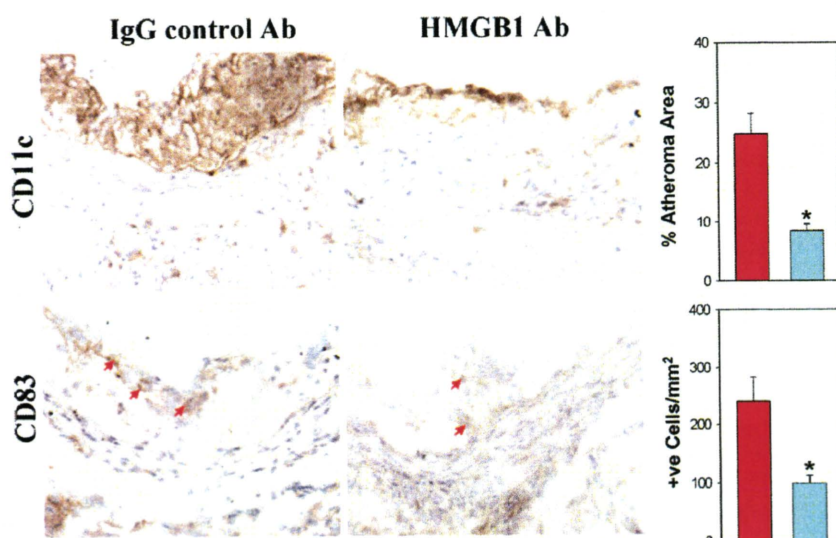


Figure 3. Immunohistochemistry of aortic sinus atherosclerotic lesions from control antibody (Ab; left) and anti-HMGB1 antibody (right)–treated ApoE−/− mice fed a high-fat diet. Cross-sections were stained with anti-CD11c antibody to detect dendritic cells (top) and anti-CD83, a marker of mature dendritic cells (bottom). Bar graphs represent the extent of immunostaining in the 2 groups. Red indicates control antibody; blue, anti-HMGB1 neutralizing antibody. *$P<0.05$ compared with control. Scale bar represents 100 μm.

Downloaded from atvb.ahajournals.org by on November 20, 2010

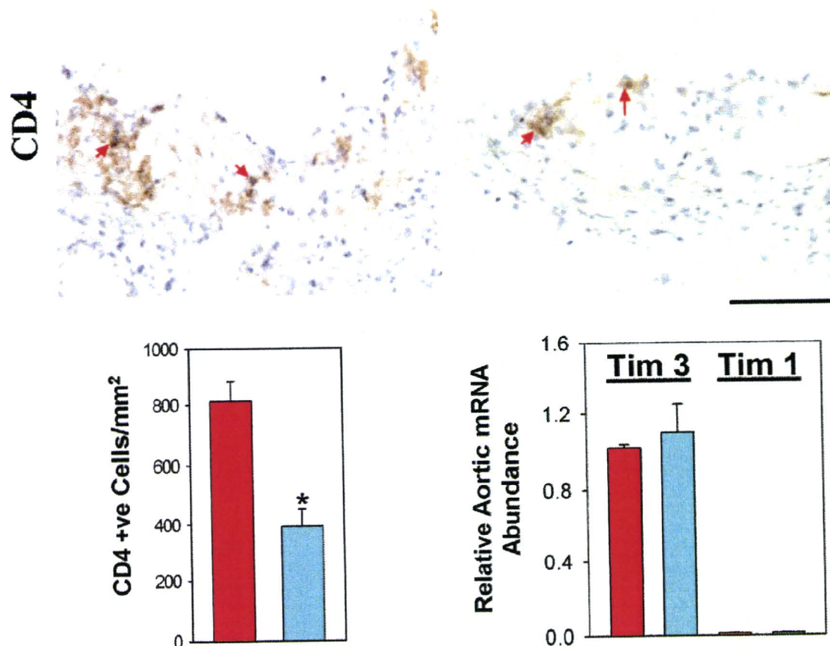


Figure 4. Distribution of CD4+ T cells in aortic sinus atherosclerotic lesions and their polarization following treatment of ApoE−/− mice with either control antibody or anti-HMGB1 neutralizing antibody. Top, cross-sections were stained with anti-CD4 antibodies to detect CD4+ lymphocytes in lesions of control (left) and anti-HMGB1 antibody (right)–treated mice. Bottom left, Bar graph indicating CD4+ lymphocyte density in the lesions. Bottom right, Bar graph indicating the relative abundance of mRNAs encoding Tim3 and Tim1 in lesions. Red indicates control; blue, anti-HMGB1 treatment. *$P<0.05$ compared with control. Scale bar represents 100 μm.

(NK) or NKT cell or CD4+Foxp3+ regulatory T-cell numbers (all $P>0.05$; Supplemental Figure II). In addition, we assessed monoblast/promonocyte proliferation, as well as monocyte and dendritic cell numbers in blood of control and anti-HMGB1–treated mice and dendritic cell numbers in lymph nodes. BrdU+CD11b^{hi}Ly-6C^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo} Ly-6G^{lo}CD22^{lo}, CD11b^{hi}Ly-6C^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, and CD11b^{hi}CD90^{lo}Cd49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo} monocytes in blood were unaffected by anti-HMGB1 antibody treatment ($P>0.05$; Supplemental Table II). Similarly, BrdU+ CD11c^{hi}CD11b^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, CD11c^{hi}CD11b^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, and CD11c^{hi}CD11b^{hi}I-A$^{b(hi)}$CD115^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo} monocyte-derived dendritic cells in blood were unaffected ($P>0.05$; Supplemental Table II). Also, CD11c^{hi}CD11b^{hi}I-A$^{b(hi)}$CD115^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo} monocyte-derived dendritic cells in inguinal and mediastinal lymph nodes were unaltered by anti-HMGB1 antibody treatment; CD11c+I-A^{b}+CD115+ are thought to emigrate from lesions to lymph nodes during regression of lesions.[35] Together, these results indicate that monocytes/dendritic cell numbers in blood do not account for the reduction in macrophages and dendritic cells in lesions of anti-HMGB1 treated mice. Similarly, emigration of dendritic cells from lesions does not appear to be affected by anti-HMGB1 antibody treatment.

HMGB1 Neutralization and Proinflammatory Mediators in Lesions

HMGB1 can increase the expression of a number of proinflammatory mediators including MCP-1, vascular cell adhesion molecule-1 (VCAM-1) and a variety of cytokines including IL-1β, IL-6, and tumor necrosis-α (TNF-α).[12,36,37] We investigated whether treatment with anti-HMGB1 neutralizing antibodies might affect expression of such proinflammatory mediators in developing lesions. Treatment with anti-HMGB1 neutralizing antibodies reduced expression of VCAM-1 in lesions by 50% and MCP-1 expression by nearly 70% (both $P<0.05$; Supplemental Figure III). There also tended to be reductions in the expression of TNF-α, IL-1β, and interferon-α, whereas IL-6 was unaffected (Supplemental Figure III).

HMGB1 and Cell Migration

Because anti-HMGB1 antibody treatment affected immune cell accumulation in lesions, in particular macrophages, to a much greater extent than proinflammatory cytokines, we examined the possibility that HMGB1 might be influencing macrophage migration to developing lesions. In vitro, HMGB1 stimulates macrophage migration.[13] To confirm the chemoattractant actions of HMGB1 on mouse macrophages, we first assessed its ability to stimulate mouse RAW264.7 macrophage migration using 24-well chemotaxis chambers. HMGB1 stimulated their migration, more than doubling the number of migrated macrophages ($P<0.05$; Figure 5). To determine whether HMGB1 also stimulated macrophage cell migration in vivo, we injected HMGB1 into the peritoneal cavity of mice and 5 hours later assessed its effects on peritoneal macrophage. HMGB1 markedly increased the number of F4/80+Gr1(Ly-6C)+ macrophages ($P<0.05$; Figure 5) and also increased F4/80+CD11b+ macrophages ($P<0.05$; Figure 5).

Discussion

We have previously reported that HMGB1 is expressed by macrophages in human aortic fatty streaks and fibrofatty lesions.[3] In advanced atherosclerotic plaques, HMGB1 is also expressed by vascular smooth muscle cells.[5] Also, serum HMGB1 levels have been associated with coronary artery disease.[38,39] In this study, we demonstrated that atheroscle-

Downloaded from atvb.ahajournals.org by on November 20, 2010

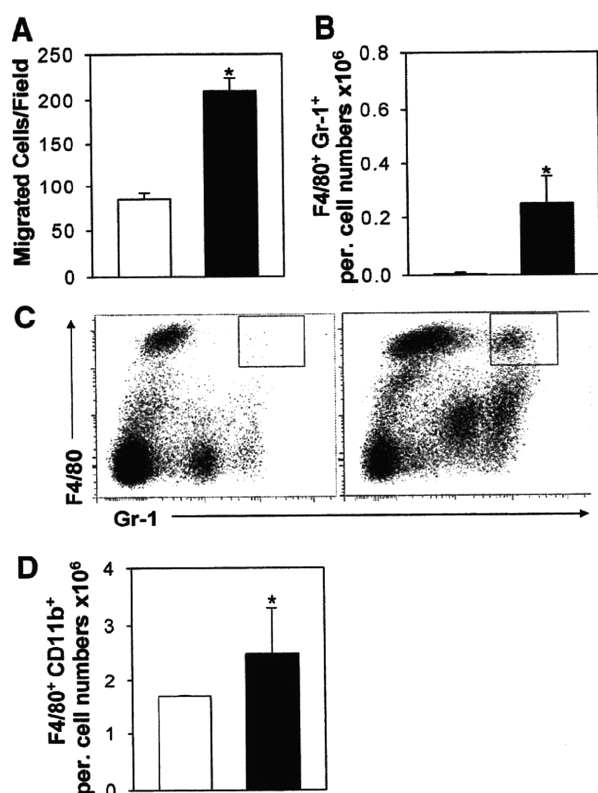


Figure 5. HMGB1-stimulated macrophage chemotaxis in vitro and intraperitoneal macrophage recruitment in vivo. A, Migration of mouse RAW264.7 macrophages in vitro in response to vehicle or HMGB1 (4 μg/mL). B, Number of F4/80+Gr-1(Ly-6C)+ macrophages recruited to the peritoneal (per.) cavity 5 hours after IP injection of vehicle or HMGB1 (20 μg/mL). C, Flow cytometric analysis of peritoneal lavage effluent demonstrating F4/80 and Gr-1 populations 5 hours after injection of vehicle or HMGB1. Boxed areas show the F4/80+Gr-1(Ly-6C)+ population. D, Number of F4/80+CD11b+ macrophages recruited to the peritoneal cavity 5 hours after IP injection of vehicle or HMGB1. *$P<0.05$ compared with vehicle control.

rotic lesions within the aortic sinus of ApoE−/− mice also express HMGB1, which contributes to lesion development. Neutralizing HMGB1 using a monoclonal antibody targeting amino acids within the acidic tail,[29] attenuated development of atherosclerosis. Our study suggests that HMGB1 in lesions rather than circulating HMGB1 contributes to atherosclerosis, stimulating macrophage migration and increasing the accumulation of other immune cell types, as well as proinflammatory mediators.

HMGB1 exerts multiple effects on monocyte/macrophages in vitro. It stimulates macrophage migration,[13] monocyte adherence and spreading, monocyte-matrix interactions,[40] and dendritic cell migration.[14] Our observations extend these findings and indicate that HMGB1 is also chemoattractive in vivo for macrophages; HMGB1 stimulated the migration of F4/80+Gr-1(Ly-6C)+ macrophages, a subtype known to accumulate in atherosclerotic lesions.[41] It does not appear to influence monoblast/promonocyte proliferation, monocyte numbers in blood, or efflux of dendritic cells from lesions.

Our studies suggest that CD4+ T cells in lesions are also affected by HMGB1; CD4+ T-cell numbers were reduced in lesions of mice treated with the HMGB1 neutralizing antibody but unaffected in blood, spleen, or lymph nodes. In vitro HMGB1 acts as a costimulatory factor together with T-cell receptor stimulation to augment proliferation.[42] Also, dendritic cells produce and respond to HMGB1 elevating IL-12 secretion, which in turn polarizes CD4+ T cells to the Th1 phenotype.[37] Our finding that Tim-3 expression is unaffected by anti-HMGB1 antibody treatment, despite reductions in CD4+ T cells, suggests that the function of these cells may be impaired; Tim-3 is a negative regulator of Th1 cell function.[32,43] Because HMGB1 appears to regulate immune cell numbers in lesions we also investigated whether it affected cytokine levels. In vitro, HMGB1 potently stimulates macrophages and endothelial cells to secrete proinflammatory cytokines, TNF-α, IL-1β, and IL-6.[12,37] Surprisingly, we found that neutralizing HMGB1 only had small effects on the expression of proinflammatory cytokines, suggesting that HMGB1 in lesions was affecting immune cell function rather than expression of proinflammatory cytokines in lesions. However, HMGB1 augmented the expression of proinflammatory mediators VCAM-1 and MCP-1.

In addition to affecting immune cells, HMGB1 can affect vascular smooth muscle cells, stimulating their migration[11] and proliferation.[4] Treatment with anti-HMGB1 neutralizing antibodies reduced smooth muscle cell accumulation and the number of proliferating cells within developing lesions. The intima is considered to be the "soil" in which atherosclerosis develops,[44] and attenuating its development/growth by neutralizing HMGB1 activity and reducing the intimal smooth muscle population could contribute to the reductions in lesion size. Although HMGB1 has also been shown to stimulate endothelial cell proliferation and angiogenesis, endothelial cells did not appear to be affected by treatment with the anti-HMGB1 neutralizing antibody.

Although our studies indicate that HMGB1 contributes to lesion development by stimulating macrophage migration and activating dendritic cells, the receptors through which these effects are mediated remain to be identified. It is interesting to note that the effects we observed on macrophages, dendritic cells, and T cells, as well as vascular smooth muscle cells, have been largely attributed to interactions with RAGE.[11,13,15,16] Inhibition of RAGE attenuates lesion development and is associated with reductions in macrophage and smooth muscle cell accumulation, as well as reductions in VCAM-1 expression,[45] effects that we observed following neutralization of HMGB1. However, we cannot exclude the possibility that HMGB1 also interacts with other receptors during development of atherosclerosis, including TLR2, TLR4, or the CD24/Siglec-10 system. Our findings provide an encouraging basis for the development of a novel therapeutic approach for atherosclerosis, possibly using newer formats of recombinant antibodies.

In conclusion, our data extend earlier findings on the expression of HMGB1 in human atherosclerotic lesions and demonstrate that locally, 1 expressed HMGB contributes to lesion development by stimulating macrophage migration and modulating proinflammatory mediators such as MCP-1 and VCAM-1. HMGB1 also indirectly contributes to accumula-

Downloaded from atvb.ahajournals.org by on November 20, 2010

tion of dendritic cells and CD4+ T cells. The findings define a new potential therapeutic target for atherosclerosis.

Sources of Funding

This study was supported by the National Health and Medical Research Council of Australia (to A.B.), the Australian National Heart Foundation (to A.B.), the Japanese Society for the Promotion of Science (to M.N.) and Japanese Ministry of Health, Labor and Welfare (to M.N.).

Disclosures

None.

References

1. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–871.
2. Tedqui A, Mallat Z. Cytokines in atherosclerosis. Pathogenic and regulatory pathways. *Physiol Rev*. 2006;86:515–581.
3. Kalinina N, Agrotis A, Antropova G, DiVitto G, Kanellakis P, Kostolias G, Ilyinskaya O, Tararak E, Bobik A. Increased expression of the DNA-binding cytokine HMGB1 in human atherosclerotic lesions: role of activated macrophages and cytokines. *Arterioscler Thromb Vasc Biol*. 2004; 24:2320–2325.
4. Porto A, Palumbo R, Pieroni M, Aprigliano G, Chiesa R, Sanvito F, Maseri A, Bianchi ME. Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein. *FASEB J*. 2006;20:E1955–E1963.
5. Inoue K, Kawahara K, Biswas KK, Ando K, Mitsudo K, Nobuyoshi M, Maruyama I. HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerotic lesions. *Cardiovasc Pathol*. 2007;16:136–143.
6. Scaffidi P, Mistell T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*. 2002;418:191–195.
7. NK/iDC interaction results in IL-18 secretion by DC at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1. *Blood*. 2005;106:609–616.
8. Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ. HMGB1 as a DNA-binding cytokine. *J Leukoc Biol*. 2002;72:1084–1091.
9. Urbonavicute V, Furnrohr BG, Meister S, Munoz L, Heyder P, De Marchis F, Bianchi ME, Kirschning C, Wagner H, Manfredi AA, Kalden JR, Schett G, Rovere-Querini P, Herrman M, Voll RE. Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. *J Exp Med*. 2008;205: 3007–3018.
10. Zimmermann K, Volkel D, Pable S, Lindner T, Kramberger F, Bahrami S, Scheiflinger F. Native versus recombinant high-mobility group B1 proteins: functional activity in vitro. *Inflammation*. 2004;28:221–229.
11. Degryse B, Bonaldi T, Scaffidi P, Muller S, Resnati M, Sanvito F, Arrigoni G, Bianchi ME. The high mobility group (HMG) boxes of the nuclear protein HMGB1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *J Cell Biol*. 2001;152:1197–1206.
12. Fiuza C, Bustin M, Talwar S, Tropea M, Gerstebberger E, Shelhamer JH, Suffredini AF. Inflammation-promoting activity of HMGB1 on microvascular endothelial cells. *Blood*. 2003;101:2652–2660.
13. Penzo M, Molteni R, Suda T, Samaniego S, Raucci A, Habiel DM, Miller F, Jiang H, Li J, Pardi R, Palumbo R, Olivotto E, Kew RR, Bianchi ME, Marcu KB. Inhibitor of NF-κB kinases α and β are both essential for high mobility group box 1-mediated chemotaxis. *J Immunol*. 2010;184: 4497–4509.
14. Yang D, Chen Q, Yang H, Tracey KJ, Bustin M, Oppenheim JJ. High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. *J Leuk Biol*. 2007;81: 59–66.
15. Dumitriu IE, Bianchi ME, Bacci M, Manfredi AA, Rovere-Querini P. The secretion of HMGB1 is required for the migration of maturing dendritic cells. *J Leuk Biol*. 2007;81:84–91.
16. Dumitriu IE, Baruah P, Valentinis B, Voll RE, Herrmann M, Nawroth PP, Arnold B, Bianchi ME, Manfredi AA, Rovere-Querini P. Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. *J Immunol*. 2005;174: 7506–7515.
17. Lotze M, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol*. 2005;5: 331–342.
18. Li J, Kokkola R, Tabibzadeh S, Yang R, Ochani M, Qiang X, Harris HE, Czura CJ, Wang H, Ulloa L, Wang H, Warren HS, Moldawer LL, Fink MP, Andersson U, Tracey KJ, Yang H. Structural basis for the proinflammatory cytokine activity of high mobility group box 1. *Mol Med*. 2003;9:37–45.
19. Treutiger CJ, Mullins GE, Johansson ASM, Rouhiainen A, Rauvala HME, Erlandsson-Harris H, Andersson U, Yang H, Tracey KJ, Andersson J, Palmblad. High mobility group 1 B-box mediates activation of human endothelium. *J Intern Med*. 2003;254:375–385.
20. Bianch ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. *Immunol Rev*. 2007;220:35–46.
21. Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, Susarla SM, Ulloa L, Wang H, DiRaimo R, Czura CJ, Wang H, Roth J, Warren HS, Fink MP, Fenton MJ, Andersson U, Tracey KJ. Reversing established sepsis with antagonist of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A*. 2004;101:296–301.
22. Wang Q, Zeng M, Wang W, Tang J. The HMGB1 acidic tail regulates HMGB1 DNA binding specificity by a unique mechanism. *Biochem Biophys Res Commun*. 2007;360:14–19.
23. Kokkola R, Andersson A, Ostberg T, Treutiger CJ, Nawroth P, Andersson U, Harris RA, Harris HE. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. *Scand J Immunol*. 2005;61:1–9.
24. Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaki A, Abraham E. Involvement of toll-like receptors 2 and 4 in cellular activation of high mobility group box 1 protein. *J Biol Chem*. 2004:279: 7370–7377.
25. Tian J, Avalos AM, Chen B, Senthil K, Wu H, Parroche P, Drabic S, Golenbock D, Sirois C, Hua J, An LL, Audoly L, Rosa GL, Bierhaus A, Naworth P, Marshak-Rothstein A, Crow MK, Fitzgerald KA, Latz E, Kiener PA, Coyle AJ. Toll-like receptor 9-dependent activation by DNA containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol*. 2007;8:487–496.
26. Chen GY, Tang J, Zheng P, Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. *Science*. 2009;323: 1722–1725.
27. Han J, Zhong J, Wei W, Wang Y, Huang Y, Yang P, Purohit S, Dong Z, Wang MH, She JX, Gong F, Stern DM, Wang CY. Extracellular high-mobility group box 1 acts as an innate immune mediator to enhance autoimmune progression and diabetes onset in NOD mice. *Diabetes*. 2008;57:2118–2127.
28. Kokkola R, Li J, Sundberg E, Aveberger AC, Palmblad K, Yang H, Tracey KJ, Andersson U, Harris EH. Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis Rheum*. 2003;48: 2052–2058.
29. Liu K, Mori S, Takahashi HK, Tomono Y, Wake H, Kanke T, Sato Y, Hiraga N, Adachi N, Yoshino T, Nishibori M. Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB J*. 2007;21:3904–3916.
30. Ueno H, Matsuda T, Hashimoto S, Amaya F, Kitamura Y, Tanaka M, Kobayashi A, Maruyama I, Yamada S, Hasegawa N, Soejima J, Koh H, Ishizaka A. Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am J Resp Crit Care Med*. 2004; 170:1310–1316.
31. Erbel C, Sato K, Meyer FB, Kopecky SL, Frye RL, Goronzy JJ, Weyand CM. Functional profile of activated dendritic cells in unstable atherosclerotic plaques. *Basic Res Cardiol*. 2007;102:123–132.
32. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA, Freeman GJ, Kuchroo VK. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature*. 2002;415: 536–541.
33. Khademi M, Illes Z, Gielen AW, Marta M, Takazawa N, Baecher-Allan C, Brundin L, Hannerz J, Martin C, Harris RA, Hafler DA, Kuchroo VK, Olsson T, Piehl F, Wallstrom E. T cell Ig-and mucin-domain-containing molecule-3 (TIM-3) and TIM-1 molecules are differentially expressed on human Th1 and Th2 cells and in cerebrospinal fluid-derived mononuclear cells in multiple sclerosis. *J Immunol*. 2004;172:7169–7176.
34. Mitola S, Belleri M, Urbinati C, Coltrini D, Sparatore B, Pedrazzi M, Melloni E, Presta M. Extracellular high mobility group box-1 protein is a proangiogenic cytokine. *J Immunol*. 2006;176:12–15.
35. Llodra J, Angeli V, Liu J, Trogan E, Fisher EA, Randolph GJ. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes

Downloaded from atvb.ahajournals.org by on November 20, 2010

regressive but not progressive plaques. *Proc Natl Acad Sci U S A.* 2004; 101:11779–11784.

36. Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlabndsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, Tracey KJ. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med.* 2000;192:565–570.
37. Messmer D, Yang H, Telusma G, Knoll F, Li J, Messmer B, Tracey KJ, Chiorazzi N. High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization. *J Immunol.* 2004;173: 307–313.
38. Yan XX, Lu L, Peng WH, Wang LJ, Zhang Q, Zhang RY, Chen QJ, Shen WF. Increased serum HMGB1 level is associated with coronary artery disease in nondiabetic and type 2 diabetic patients. *Atherosclerosis.* 2009; 205:544–548.
39. Hu X, Jiang H, Zhou X, Xu C, Lu Z, Cui B, Wen H. Increased serum HMGB1 is related to severity of coronary artery stenosis. *Clin Chim Acta.* 2009;406:139–142.
40. Rouhianen A, Kuja-Panula J, Wilkmamn E, Pakkanen J, Stenfors J, Tuominen RK, Lepantalo M, Carpen O, Parkkinen J, Rauvala H. Regulation of monocyte migration by amphoterin (HMGB1). *Blood.* 2004;104: 1174–1182.
41. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6C^{hi} monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheroma. *J Clin Invest.* 2007;117:195–205.
42. Rossini A, Zacheo A, Mocini D, Totta P, Facchiano A, Castoldi R, Sordini P, Pompilio G, Abeni D, Capogrossi MC, Germani A. HMGB1-stimulated human primary fibroblasts exert a paracrine action on human and murine cardiac stem cells. *J Mol Cell Cardiol.* 2008;44:683–693.
43. Golden-Mason L, Palmer BE, Kassan N, Townshend-Bulson L, Livingston S, McMahon BJ, Castelblanco N, Kuchroo V, Gretch DR, Rosen HR. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol.* 2009;83:9122–9130.
44. Schwartz SM, de Blois D, O'Brien ER. The intima: soil for atherosclerosis and restenosis. *Circ Res.* 1995;77:445–465.
45. Bucciarelli LG, Wendt T, Qu W, Lalla E, Rong LL, Goova MT, Moser B, Kislinger T, Lee DC, Kashyap Y, Stern DM, Schmidt AM. RAGE blockade stabilizes established atherosclerosis in diabetic apolipoprotein E-null mice. *Circulation.* 2002;106:2827–2835.

Downloaded from atvb.ahajournals.org by on November 20, 2010

Supplemental Material

Peter Kanellakis et al. HMGB1 Neutralization Reduces Development of Diet-Induced Atherosclerosis in Apolipoprotein E-Deficient Mice

Supplemental Materials and Methods

Animal Experiments

Twenty four male ApoE-/- mice on the C57BL/6 background were obtained from the Precinct Animal Facility, AMREP, Melbourne, Australia and fed a high-fat diet containing 0.15% cholesterol and 21% fat (Speciality Feeds) from 6 weeks of age for 8 weeks. Mice (12 per group) also received either a control IgG2a against *Keyhole Limpet* hemocyanin (400μg iv twice weekly) or anti-HMGB1 monoclonal antibody (400μg iv twice weekly) for the duration of the dietary feeding. This dose regime was based on our earlier findings that 400μg anti-HMGB1 i.v. is highly effective in reducing cerebral infarct volumes,[1] and the long plasma half-life of IgG2a, approximately 5 days.[2,3] In vitro, 1μg of the anti-HMGB1 neutralizing antibody attenuates the stimulatory effect of 10μg HMGB1 on macrophages, assessed as an increase in ICAM-1 expression, by 67% whilst 100μg of antibody attenuates this response by 93%.[1] At the end of the study mice were killed with an overdose of pentobarbitone (120mg/kg i.p.), blood collected by cardiac puncture and aortic sinus and arch collected for histology and molecular studies. To assess the effects of the anti-HMGB1 antibody treatment on monocytes and dendritic cells and proliferation of their precursors an additional 4 mice in each group were treated with bromodeoxyuridine (1mg, i.p. daily) for three consecutive days prior to culling. All experiments were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee.

Monoclonal Antibodies

Monoclonal antibodies (anti-HMGB1 and control) were purified from culture media of growing hybridomas producing the anti-HMGB1 and anti-*Keyhole Limpet* hemocyanin

Downloaded from atvb.ahajournals.org by on November 20, 2010