^{64}Cu-DOTA 標識抗体の作製と生体内動態解析

榎本　秀一、劉　克約、西堀　正洋

目的：

前年度までに、抗 HMGB1 ラット単クローン抗体（#10-22）のビオチン標識化を試みたが、この標識体は抗原結合性を完全に消失することが、組換え体ヒト HMGB1 を固相化した Elisa で明らかとなった。そこで本年度は、抗体の標識法として新規に 1,4,7,10- tetraazacyclo dodecane- *N,N',N'',N'''*- tetraacetic acid（DOTA）を用いる。修飾抗体の抗原結合性について評価した後、^{64}Cu 放射性同位元素で ^{64}Cu-DOTA 標識を行い、生体内動態解析用 PET プローブとする。ラット MCAO モデルに標識抗体を尾静脈投与し、一定時間後に抗体の脳内移行を含む生体内動態についてのデータを PET 撮影により得る。さらに、抗体の脳内移行を、血液灌流脱血後の脳スライスを用いたオートラジオグラフィーで検討する。

結果と考察：

DOTA 修飾後抗体のＨＭＧＢ１結合性を Elisa 法で調べたところ、非修飾抗体の 60-70% の結合活性であることがわかった。前年度、抗体のビオチン標識により、抗原結合性が失われたのに対し、DOTA 修飾体ではかなりの程度抗原結合性が保持されることがわかった。表１に実験に用いた ^{64}Cu-DOTA 標識抗体のデータを示す。フリーの ^{64}Cu と ^{64}Cu-DOTA 標識抗体の分離は、ゲル濾過クロマトグラフィーによりおこなった（図１）。放射比活性として 1.2-1.6 MBq/μg protein の抗ＨＭＧＢ１あるいは対照（抗ＫＬＨ）抗体、各 60 μg を MCAO ラットに投与して実験をおこなった。図２に抗体投与１６時間後の PET 像を示す。抗ＨＭＧＢ１抗体、対照抗体ともに、脳内レベルは極めて低かったが、虚血側脳部位にだけ一定濃度分布していることがわかった。その程度は、抗ＨＭＧＢ１抗体の方が高かった（図２）。一方、灌流脱血後の脳スライスを用いたオートラジオグラフィーでは、対照抗体投与脳で、虚血部位への抗体移行が明瞭に認められたのに対し、抗ＨＭＧＢ１抗体の場合はその程度がかなり低かった（図３）。以上の結果から、抗ＨＭＧＢ１抗体は脳虚血部位への集積はおこっているが、脳内移行は限られていると推測できる。今回投与した用量は、以前の治療用量の約３０％量で、この用量によっても一定の治療効果が得られた可能性がある。その場合、ＢＢＢの構造破綻は最小限に抑えられた可能性があり、治療抗体の脳内移行が少ないという結果は、このことを示しているのかもしれない。

対照抗体（抗KLH抗体）

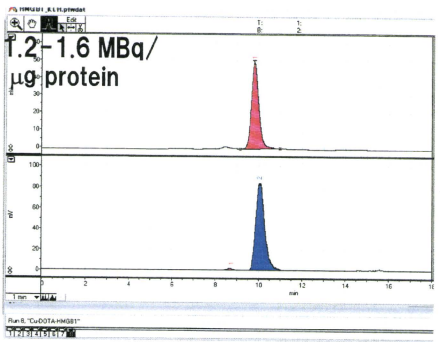
精製後抗体	容量=	110 ul		
	タンパク濃度=	1.321 ug/ul	タンパク量	145.31 ug
	RI=	208 MBq	←測定日時:	16:32:19
	添加したPBST量=	ul	放射能/容量	1.8900 MBq/ul
	HPLC用=	MBq	放射能/タンパク量	1.43 MBq/ug
	TLC用 (Pre)=	0.4 MBq	←測定日時:	17:40:31
	TLC用 (Post)=	2.15 MBq	←測定日時:	17:40:48

抗HMGB1抗体

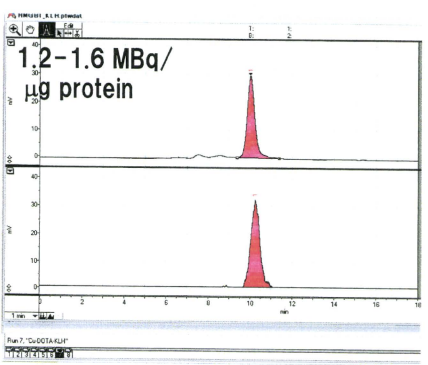
精製後抗体	容量=	105 ul		
	タンパク濃度=	1.586 ug/ul	タンパク量	166.53 ug
	RI=	261 MBq	←測定日時:	16:30
	添加したPBST量=	ul	放射能/容量	2.4900 MBq/ul
	HPLC用=	MBq	放射能/タンパク量	1.57 MBq/ug
	TLC用 (Pre)=	0.32 MBq	←測定日時:	17:39:36
	TLC用 (Post)=	2.55 MBq	←測定日時:	17:39:54

表1　^{64}Cu-DOTA 標識抗HMGB1抗体と対照抗体

対照抗体



抗HMGB1抗体



● 抗原結合性60-70%

図1 ^{64}Cu-DOTA 標識抗HMGB1抗体と対照抗体のゲルろ過クロマトグラフィー

	PET 1			PET 2		
個体番号	1	3		2	4	
ラット名	HMGB1-1	MGB1-2		KLH-1	KLH-2	
体重 (g:10/29)	258.2	256.4		265.9		
投与予定時刻						
投与前放射能 (MBq)	89.5	83.3		77.7	72.1	
投与前放射能 測定時刻	2010/11/12 18:12	2010/11/12 19:04		2010/11/12 18:40	2010/11/12 19:44	
投与時刻	18:19:00	19:19:00		18:44:00	19:54:00	
投与後放射能 (MBq)	1.7	0.75		1.53	0.52	
投与後放射能 測定時刻	18:19:00	19:19:00		18:45:00	19:52:00	
投与放射能 (MBq)	87.2311	81.4208		75.8864		

表2　MCAOラットに対する^{64}Cu-DOTA 標識抗HMGB1抗体と対照抗体の投与プロトコール

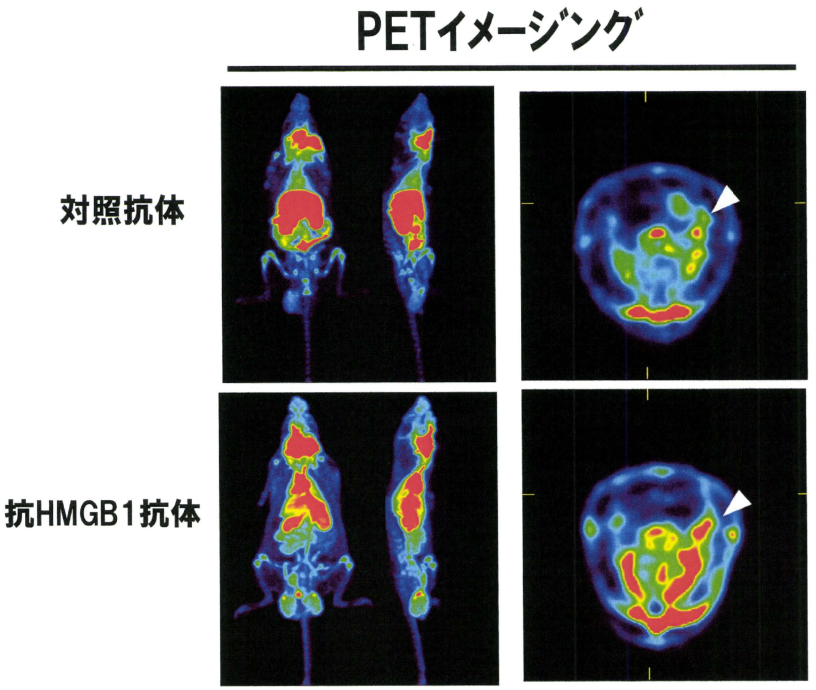


図2　^{64}Cu-DOTA 標識抗HMGB1抗体の生体内動態

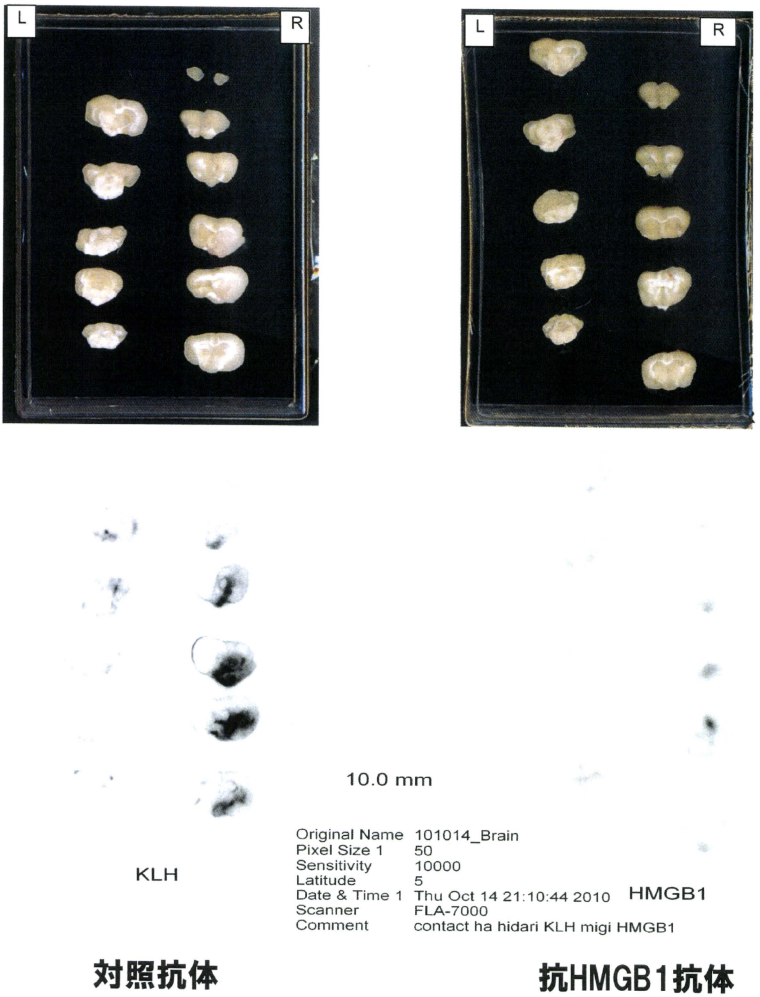


図3 ^{64}Cu-DOTA 標識抗HMGB1抗体と対照抗体の脳内移行

厚生労働科学研究費補助金（医療技術実用化総合研究事業）
分担研究報告書

抗 HMGB1 単クローン抗体の大量精製

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研究要旨
抗 HMGB1 ラット単クローン抗体（#10-22）を、脳梗塞治療用とアテローム動脈硬化症治療用抗体として継続して大量作製した。ヒト自己免疫疾患患者の血中抗 HMGB1 自己抗体の Elisa によるスクリーニングで、自己抗体陽性患者を同定することに成功した。

A. 研究目的

ラット脳梗塞ならびにマウスアテローム性動脈硬化症モデル実験に供するため、引き続き治療用抗体として抗 HMGB1 ラット単クローン抗体（#10-22）を大量産生・精製することが必要である。抗 HMGB1 完全ヒト抗体の作製に向けて、自己免疫疾患患者群から自己抗体産生患者を Elisa スクリーニングで同定する。

B. 研究方法

1. 前年度までに確立した精製法で、抗 HMGB1 ラット単クローン抗体（#10-22）を大量産生・精製する。
2. 病院倫理審査委員会で承認された内容をもとに、インフォームドコンセントの得られた自己免疫疾患患者群から自己抗体産生患者を Elisa スクリーニングで同定する。

C. 研究結果

現在の回転培養法で、50 ml の培地から約 50 mg の抗体が定常的に精製された。
自己免疫患者群５名から、３名の抗 HMGB1 自己抗体陽性患者を見出した。しかしながら、その力価は低かった。

D. 考察

現在の抗体供給体制で、概ね必要量を産生・精製することができるが、さらに効率化するためには新規に CO_2 インキュベータが必要となろう。自己免疫疾患患者グループから 60 %の頻度で抗 HMGB1 抗体陽性者を同定できたことは、今後患者数を増やすことによって、さらに多くの自己抗体陽性者を同定できる可能性を強く示唆する。

E. 結論

抗 HMGB1 ラット単クローン抗体(#10-22)の供給体制は整備されている。組換え体ヒト HMGB1 を固相化した Elisa プレートで、抗 HMGB1 自己抗体陽性患者を同定することができる。

F. 健康危険情報

該当なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1．特許取得

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2．実用新案登録

該当なし

3．その他

該当なし

抗体精製のプロトコールと抗 HMGB1 自己抗体の検出

友野　靖子、森　秀治

目的：

ラット脳梗塞ならびにマウスアテローム性動脈硬化症モデル実験に供するため、引き続き治療用抗体として抗 HMGB1 ラット単クローン抗体（#10-22）を大量産生・精製することが必要である。抗 HMGB1 完全ヒト抗体の作製に向けて、自己免疫疾患患者群から自己抗体産生患者を Elisa スクリーニングで同定する。

結果と考察

表１、表２に示すプロトコールで、50 ml の回転培養液から約 50 mg の単クローン抗体を得ることに成功している。個々のカラムクロマトグラフィーの溶出画分は、SDS-PAGE 後、クマシーブルー染色を施し、溶出分画の同定と純度検定をしている。

図１に示すように、Elisa プレートにヒト組換え体 HMGB1 を固相化し、抗 HMGB1 自己抗体スクリーニングを行なった。インフォームドコンセント後に得られた自己免疫疾患患者さんの希釈血清を用いて力価測定をおこなった（図１）。5 人の検査した患者さんの中では、3 名陽性と判定されたが、その力価は必ずしも高くないと評価された。さらに、スクリーニングを続ける必要がある。

抗体精製のプロトコール

MEP HyperCel (PALL Life Sciences)

MEPと0.1M PBS溶液を混ぜて、よく混合して、
遠心機により(2000rm/min、5min)遠心する。
PBS溶液で6回繰り返し、洗浄してから、
50mM Tris-HClで3回洗浄を繰り返す。

・抗体サンプルの結合

抗体試料(10ml/3mlゲル)を洗浄、平衡化完了のゲルに
加えて、緩やかに攪拌しながら、4℃ 2時間以上回転混合
する。
注意点: 4℃回転混合は2時間以上すること

・洗浄

遠心してから、上層液(素通り)を回収し、50mMTris-HCl
(pH7.5)で充分にゲルを洗う。 用量:50ml/回×4回

・タンパクの溶出

結合したゲルに50mMクエン酸ナトリウム(pH4)2mlを
加えて遠心し、回収用試験管にTris-HCl(pH8)を添加した。
(回収液の量:Tris-HCl液(pH8)の量=10:1)。50mM
クエン酸ナトリウム(pH4)を何回かに分けてゲルに加えて、
溶出、回収する。

・濃縮 (回収したタンパクが薄すぎ、容量が多すぎ時。
省略でも良い)

表1 MEP HyperCel による抗体の精製

抗体精製のプロトコール

陰イオン交換カラム

・Diethylaminoethyl Cellulose DE52
(Whatman international Ltd) カラムの製作。約150mlの
20mM phosphate buffer(pH7.0)でカラム平衡化。
サンプルをアプライする。

・カラム洗浄
20mM phosphate buffer(pH7.0)約30mlを流す。

・溶出
NaCl 0-0.3Mグラジエント溶出

ゲルろ過カラム

・上記溶出液を濃縮後、Sepharose 6B あるいは
Sephadex G-75にアプライし、抗体画分を回収する。

表2 陰イオン交換カラム とゲルろ過カラムによる抗体の精製

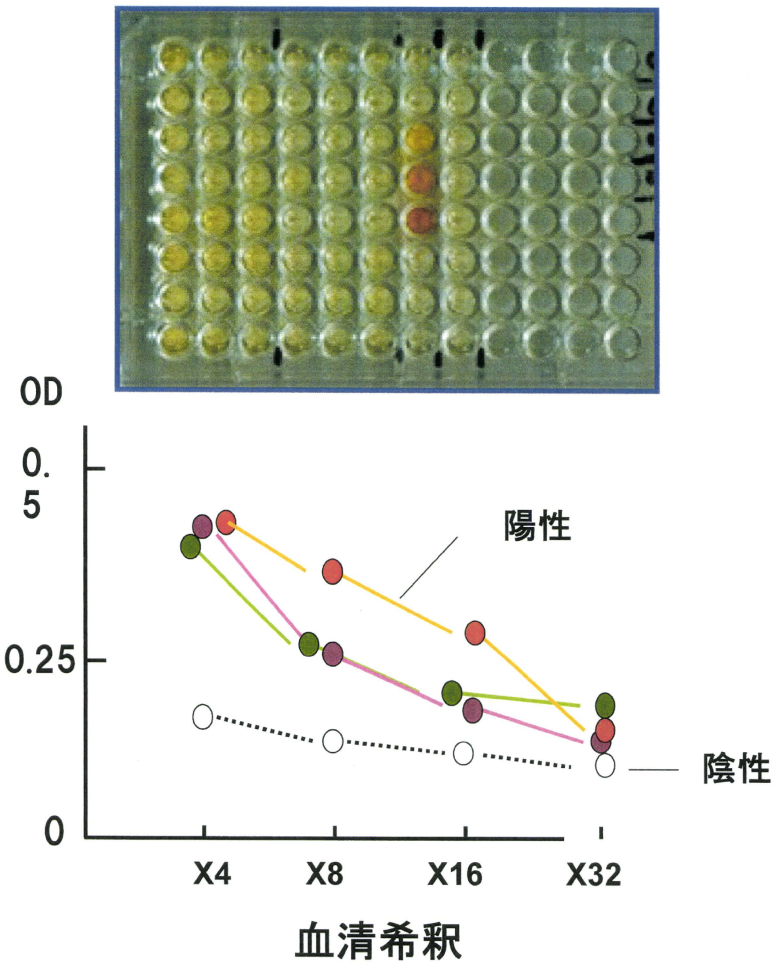

図1　ヒト血清中の抗HMGB1自己抗体の検出

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ohashi K. et al.	Advanced glycation end products enhance monocyte activation during human mixed lymphocyte reaction.	*Clin Immunol*	134	345-353	2010
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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Zhang J. et al.	Anti-high Mobility Group Box-1 Monoclonal Antibody Protects the Blood-Brain Barrier from Ischemia-Induced Disruption in Rats.	*Stroke*	42	*In press*	2011

研究成果の刊行物・別刷

European Journal of Pharmacology 627 (2010) 313–317

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

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

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ARTICLE INFO

Article history:
Received 25 May 2009
Received in revised form 25 September 2009
Accepted 14 October 2009
Available online 24 October 2009

Keywords:
Adhesion molecule
Advanced glycation end product
β_2-adrenoceptor
Monocyte

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.

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.

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doi:10.1016/j.ejphar.2009.10.034

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).

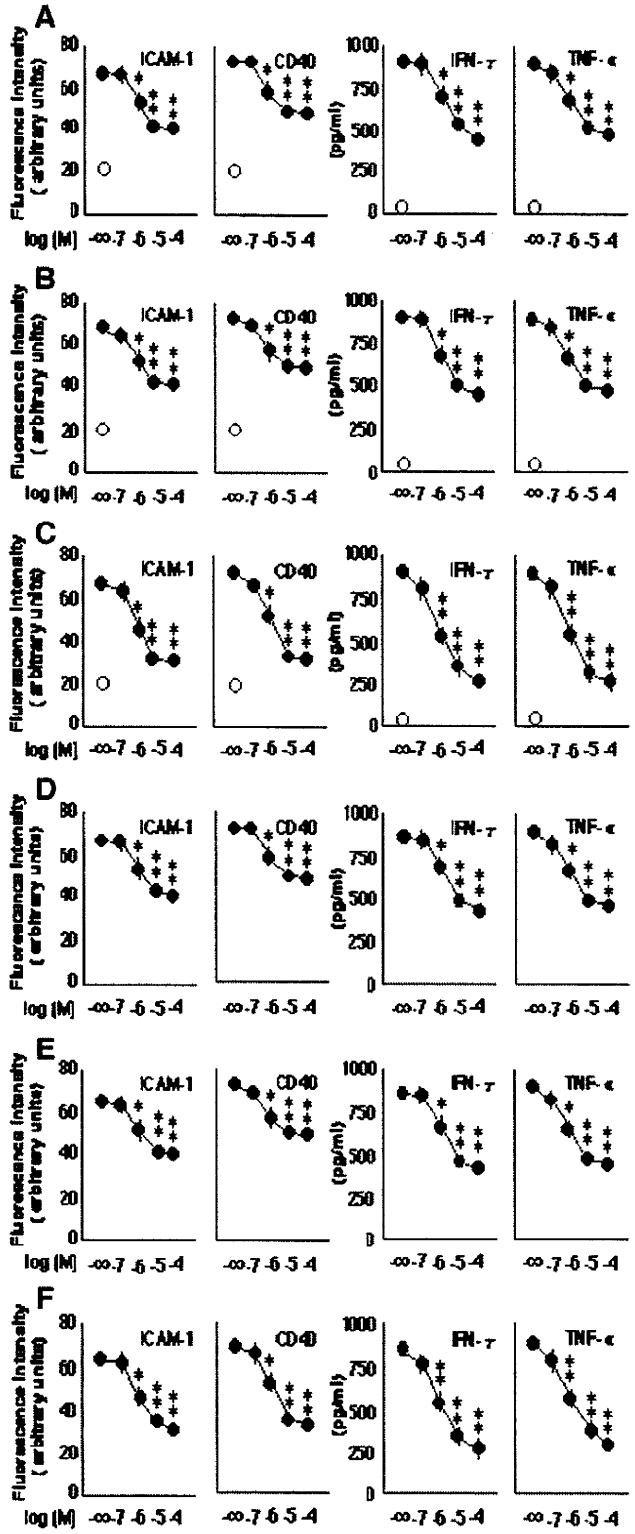


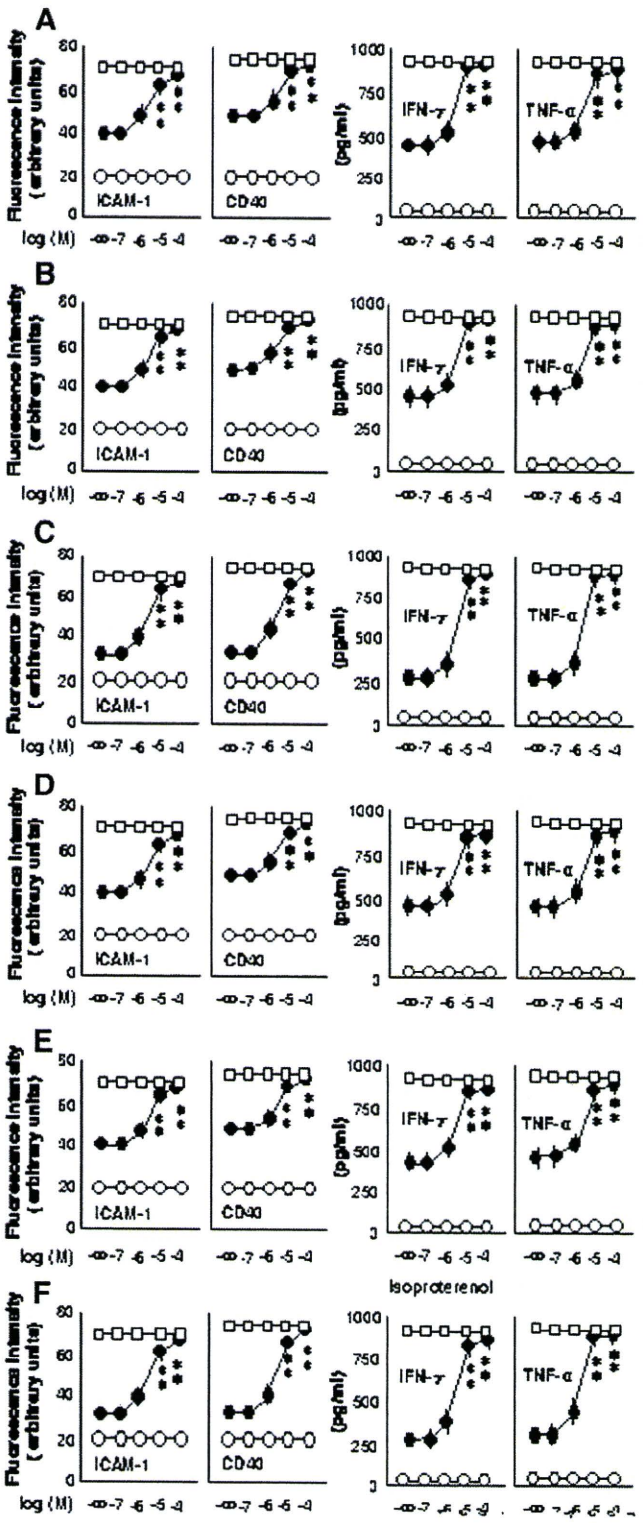
Fig. 1. The effects of epinephrine, norepinephrine and isoproterenol on AGE-2- and AGE-3-induced ICAM-1 and CD40 expressions on monocytes and the production of IFN-γ and TNF-α in peripheral blood mononuclear cells. Peripheral blood mononuclear cells at 1×10^6 cells/ml were incubated with AGE-2 and AGE-3 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



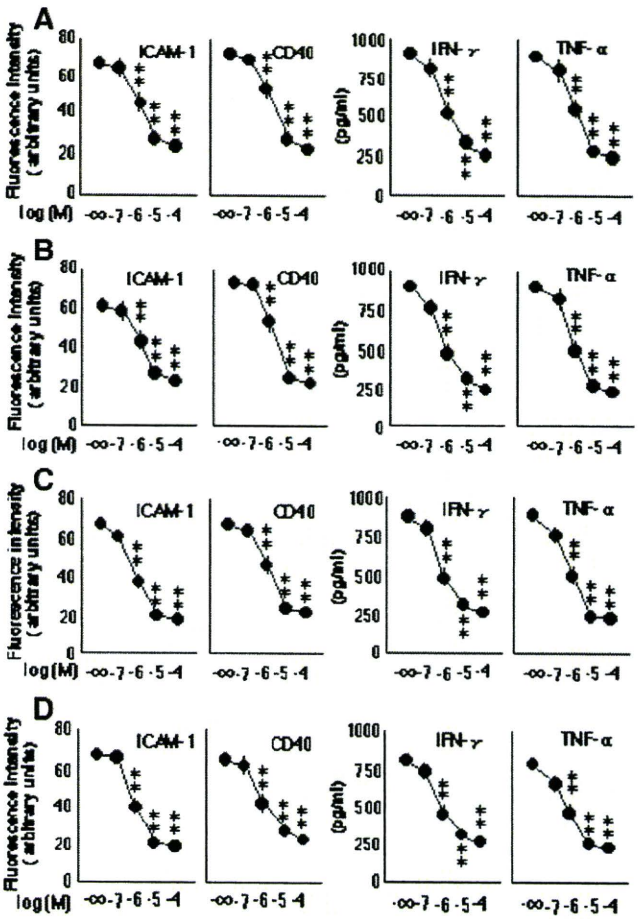


Fig. 3. The effects of selective β_2-adrenoceptor agonists on the action of AGE-2 and AGE-3. Peripheral blood mononuclear cells at 1×10^6cells/ml were incubated with β_2-adrenoceptor agonists, salbutamol and terbutaline, at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 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.

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 1×10^6cells/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.