抗 HMGB1 単クローン抗体の大量精製法の確立と抗体特性解析に関する研究

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目的：

ラットに完全フロイントアジュバントとともに HMGB1 を投与し、免疫成立後にリンパ節より採取した B リンパ球とマウスミエローマ細胞の融合細胞より、抗 HMGB1 抗体産生クローンを得る。これらの細胞を効率よく培養し、大量の分泌抗体を簡便に精製する方法を確立する。HMGB1 の全長にわたる１５アミノ酸長の人工合成ペプチドを用いて、エピトープを決定する。さらに、表面プラズモン共鳴法を用いて、抗体の平衡解離定数を測定する。

方法と結果：

モノクローナル抗体の作製は、ラットリンパ節法で行った 1、2)。抗原の免疫には Jcl: Wistar ラット（日本クレア）を用いた。抗原は仔牛胸腺よりトリクロロ酢酸ーアセトンで抽出し、CM－Sephadex カラムクロマトグラフィーで精製したＨＭＧＢ－１および２の混合物市販品（和光純薬）を用いた。抗原の PBS 溶液とフロイントコンプリートアジュバントとのエマルジョンを作製し、ラットの後ろ足皮下に 100μg/ラットで皮下注射した。抗原注射約 3 週間後に、ラットを麻酔下で開腹し、腫大した腸骨リンパ節を取り出し、同時に得られたラット血清中の抗体価を ELISA 法で測定し抗体価の上昇を確認した後、細胞融合に用いた。リンパ節由来のリンパ球とマウスミエローマ細胞（SP2/0) とを常法によりポリエチレングリコールを用いて細胞融合した。HAT により選択されたハイブリドーマを細胞融合後 7 日ー10 日後に、ELISA 法にて 1 次スクリーニングを行った。

ELISA 法はＨＭＧＢタンパク質を 96 穴プレート（ヌンク：日本インターメッド）に結合させ（0.3μg/well）、1%ウシ血清アルブミンでブロッキング後、細胞融合後の各ウェルの培養上清を 37℃で 1 時間反応させた。PBS で洗浄後、二次抗体として HRP 標識抗ラット免疫グロブリン(ダコ・ジャパン)を 1000 倍希釈して反応させた。PBS で洗浄後、OPD を発色基質として室温で発色させ、発色後 3M 硫酸で反応を停止させた。マイクロプレートリーダー（ＴＥＣＡＮ）で 490nm の吸光度を測定し、吸光度 0.13 以上を陽性ウェルとし、18 ラインを選んだ。

次に選んだ１８ラインを、市販品ＨＭＧＢ１，２混合物を用いたウェスタンブロットによる２次スクリーニングでアッセイし、陽性の 5 ラインを選択した。電気泳動は5－20%グラディエントゲル(アトー)を用い、定電流 20mA/ゲルで 60 分間通電した。セミドライ法で 100mA/ゲルで 60 分間ブロッティング後 PVDF 膜を洗浄し、5%スキムミルクで室温、60 分間ブロッキングした。洗浄後、１次抗体を 37℃、60 分間反応させ、洗浄後、2 次抗体として HRP 標識抗ラット免疫グロブリン (1000 倍希釈) (American Quolex 社) を室温で 60 分間反応させた。洗浄後、DAB を約 5～10 分間反応させ、蒸留水で洗浄して反応停止し風乾した。次に選択した５ラインをＥＬＩＳＡ法、ヒトＨＭＧＢ１組変えタンパク質を用いたウェスタンブロットによってクローニングし、#3-6, #4-1, #10-22, #11-19 の４クローンを確立した。また確立したクローンが分泌する免疫グロブリンのサブクラスを決定するために、二次抗体として HRP 標識抗ラット IgG1、IgG2a、IgG2b、IgG2c (Binding Site) を用いた ELISA 法を行った。高濃度モノクローナル抗体を得るために、クローニングにより得られたハイブリドーマ細胞株を回転培養装置 (グライナージャパン株) で２週間培養した。上清回収後、ELISA 法で抗体価を測定したところ、回転培養前の約 50 倍の抗体価の上昇を認めた。MepHypercel (日本ポール株)への３種類の単クローン抗体の結合条件を調べ、pH6.5~9.0 の条件において、よい結合が得られることを確認した。洗浄は 50 mM Tris-HCl, pH7.5 で、溶出は pH 3.5 のクエン酸ナトリウム緩衝液とした。当初、pH4.0 のクエン酸ナトリウム緩衝液を用いたが、クローン抗体によっては、この条件で溶出効率が低いことがわかった。エピトープ決定に用いた３種類のナイロン膜の内、Ultra Bind Membrane が特異的検出において、最も優れていた (Figure 1, Figure 2) 。HMGB1 のアミノ末端から、１５アミノ酸残基長で、５アミノ酸ずつオーバーラップするペプチドを４１本 (Figure 1) を人工合成し、これを Ultra Bind Membrane にブロットした。#10-22 は, HMGB1 の C 末端配列を認識した。一方、#11-19 と #4-1 クローンはともに、B-Box 内の LKEKYEKDIA 配列を認識した。ビアコアを用いた抗体親和性の測定では、抗原の直接カップリング法では、抗体の結合が検出されなかった。そこで、抗原としてヒスチジンタグ付き組み換え体 HMGB1 を作製し、抗ヒスタグ抗体による抗原キャプチャー法を用いることとした。この抗原固相化法により、#10-22、#11-19 と #4-1 各クローンの平衡解離定数はそれぞれ、1.5x 10^{-8}M, 3x10^{-7}M, 2.2x10^{-7}M と算出された (Figure 3) 。

考察と結論：

回転培養装置を用いて、ハイブリドーマ細胞の効率的な培養が可能であった。培養上清からの抗体精製法では、MepHypercel からの溶出に通常より、低いpH(3.5) の緩衝液を使用することで、回収率を上昇させることができた。#10-22 抗体の認識エピトープは HMGB1 のC末端配列であり、この配列はHMG ファミリーの中でもHMGB1に特異的な配列であった。一方、#11-19と#4-1 クローンの認識エピトープは、HMGB2 にも存在する配列であった。さらに、平衡解離定数の測定から、#10-22 クローン抗体の親和性が最も高いことがわかった。以上の結果から、脳梗塞その他の疾患治療抗体として、#10-22 クローン抗体が最も優れていると判断した。

結論として、ハイブリドーマ細胞の安定的で効率のよい培養法を確立し、高濃度の抗体を含む培養上清を得ることができた。これをスターティング材料として、疎水結合アフィニティカラムとゲル濾過カラムクロマトグラフィー法で、高純度の抗体を精製できるように条件を確立した。得られた単クローンの内、１種類（#10-22）が HMGB1 特異的抗体であり、かつ親和性が最も高いことが明らかにされた。以上の結果から、治療抗体として#10-22 が優れていると結論された。

Figure 1

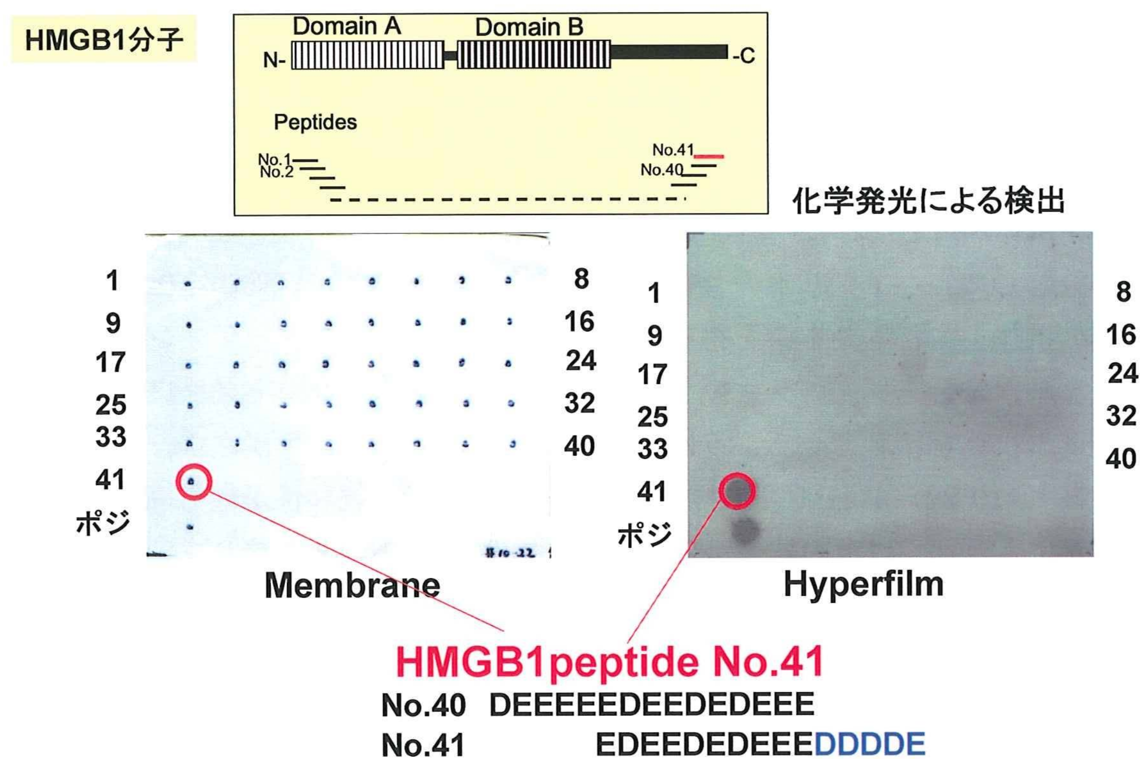
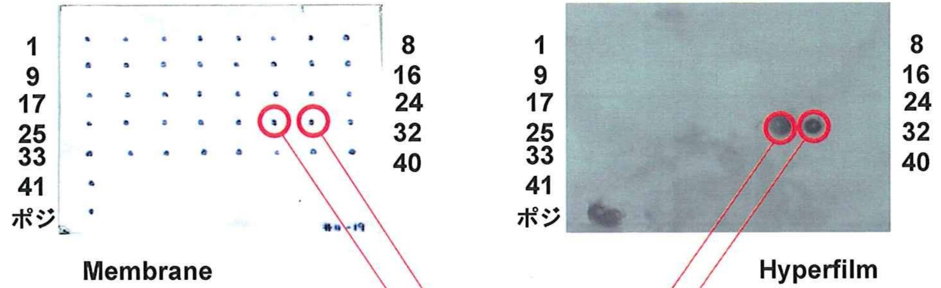


Figure 2

単クローン抗体(#11-19)のエピトープ



HMGB1peptide No.30,31

No. 30 KKAAKLKEKYEKDIA

No. 31 LKEKYEKDIAAYRAK

単クローン抗体(#4-1)のエピトープ

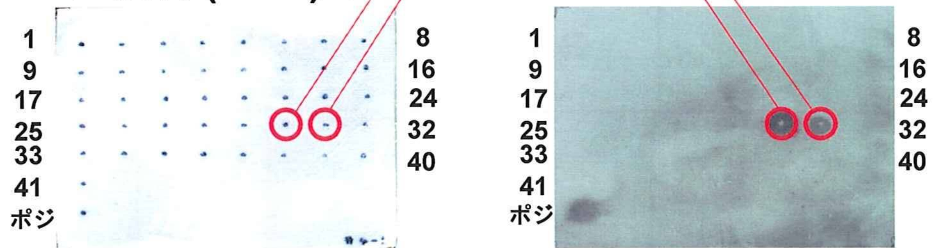
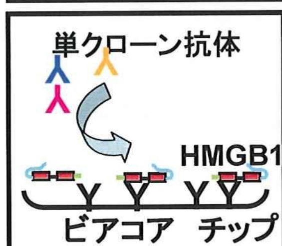
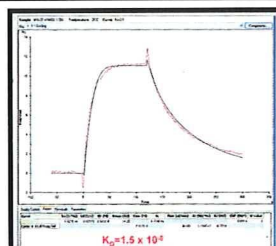


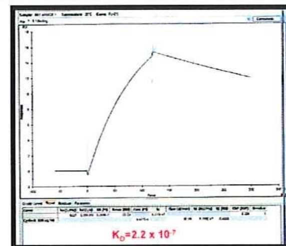
Figure 3

単クローン抗体の比較

クローン名	エピトープ	抗原 特異性	平衡解離定数 (Kd) 表面プラズモン 共鳴法
#10-22	C末端	HMGB1	1.5 x 10^{-8}M
#11-19	B-box	HMGB1/2	3.0 x 10^{-7}M
#4-1	B-box	HMGB1/2	2.2 x 10^{-7}M







研究成果の刊行に関する一覧表

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該当なし

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Advanced Glycation End Products Subspecies-Selectively Induce Adhesion Molecule Expression and Cytokine Production in Human Peripheral Blood Mononuclear Cells

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ABSTRACT

Advanced glycation end products (AGEs) are proteins or lipids that become glycated after exposure to diverse reducing sugars. Accumulation of AGEs induces diabetes complications. Microinflammation is a common major mechanism in the pathogenesis of diabetic vascular complications. Activation of monocytes/macrophages and T cells plays roles in the pathogenesis of atherosclerosis. The activation of T cells requires the enhanced expression of adhesion molecules on monocytes. AGEs activate monocytes by engaging the receptor for AGE (RAGE); however, little is known about the profile of agonist activity of diverse AGE moieties on monocytes. We investigated the effect of four distinct AGE subtypes (AGE-modified bovine serum albumin; AGE-2, AGE-3, AGE-4, and AGE-5) at concentrations ranging from 0.1 to 100 μg/ml on the expression of intercellular adhesion molecule-1, B7.1, B7.2, and CD40 on monocytes and its impact on the production of interferon-γ and tumor necrosis factor-α in human peripheral blood mononuclear cells. Among the AGEs examined, AGE-2 and AGE-3 selectively induced adhesion molecule expression and cytokine production. Antagonism experiments using antibodies against adhesion molecules demonstrated that cell-to-cell interaction between monocytes and T/natural killer cells was involved in AGE-2- and AGE-3-induced cytokine production. AGE-2 and AGE-3 up-regulated the expression of RAGE on monocytes. The effects of AGE-2 and AGE-3 were inhibited by nuclear factor-κB and p38 mitogen-activated protein kinase inhibitors. These results indicated that AGE-2 and AGE-3 activated monocytes via RAGE, leading to the up-regulation of adhesion molecule expression and cytokine production.

Advanced glycation end products (AGEs) are a heterogeneous class of compounds and modifications of proteins or lipids that become nonenzymatically glycated and oxidized after contact with aldose sugars (Schmidt et al., 1994). Accumulation of AGEs in disorders such as diabetes, renal failure, Alzheimer's disease, and natural aging has suggested their potential contribution to the pathogenesis of complications that typify these conditions (Brownlee, 1995; Takedo et al., 1996). Direct immunochemical evidence for the existence of six distinct AGE structures, including AGE-1, AGE-2, AGE-3, AGE-4, AGE-5, and AGE-6, was provided from the analysis of AGEs within modified proteins and peptides (Takeuchi and Yamagishi, 2004). Recently, it was demonstrated that toxic AGE structures, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3), have diverse biological activities on vascular endothelial cells, vascular smooth muscle cells, mesangial cells, Schwann cells, malignant melanoma cells, and cortical neurons (Oka-

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ABBREVIATIONS: AGE, advanced glycation end product; RAGE, Receptor for advanced glycation end product; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; sRAGE, soluble form of receptor for advanced glycation end product; ICAM, intercellular adhesion molecule; IFN, interferon; TNF, tumor necrosis factor; PBMC, peripheral blood mononuclear cells; Ab, antibody; SN50, H_2N-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Val-Gln-Arg-Lys-Arg-Gln-Lys-Leu-Met-Pro-OH; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; IL, interleukin.

moto et al., 2002). AGEs have also been suggested to have profound effects on inflammatory and immune cells (Figarola et al., 2007); however, it is still not clear which AGE subtypes play a role in the modulation of immune response.

Receptor for AGE (RAGE), a member of the immunoglobulin superfamily, was first described as a cell surface receptor for AGEs (Neeper et al., 1992). It is reported that AGEs ligate cell surface RAGE on the vascular endothelium, mononuclear phagocytes, vascular smooth muscle, and neurons to activate cell signaling pathways such as p44/p42 mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) (Yan et al., 1994), leading to redirect cellular function in a manner linked to the expression of inflammatory and prothrombotic genes important in the pathogenesis of chronic disorders such as diabetic microvascular disease and amyloidosis (Schmidt et al., 1994; Miyata et al., 1996; Park et al., 1998). Interestingly, AGEs up-regulate RAGE expression in various tissues, facilitating the AGEs-RAGE response by forming a positive feedback loop (Yamagishi and Imaizumi, 2005). Conversely, it is suggested that interruption of the interaction of AGEs with RAGE in vivo, by the administration of soluble form of RAGE (sRAGE), an extracellular ligand-binding domain of RAGE, reversed vascular hyperpermeability and suppressed accelerated atherosclerotic lesion development in diabetic rodents (Miyata et al., 1996; Park et al., 1998). Thus, ligation of AGEs with RAGE might play an important role in the development of various diabetic complications, including atherosclerosis.

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). Macrophages are recruited by abnormal endothelium over developing atherosclerotic plaques (Boyle, 2005). In addition, recruitment of monocytes/macrophages and T cells in diabetic glomeruli during the early stage of diabetes is considered to be involved in the progression of diabetic nephropathy (Sugimoto et al., 1997). Monocyte/macrophage activation, adhesion, and migration are key events in the pathogenesis of atherosclerosis (Figarola et al., 2007). Activated T cells induce the progression of inflammatory atherosclerotic plaques (Stoll and Bendszus, 2006). AGEs are implicated in the pathogenesis of atherosclerotic vascular disease of diabetic etiology (Stitt et al., 1997). The stimulation of RAGE is reported to induce plaque rupture in diabetic patients (Cuccurullo et al., 2006).

It is known that the enhanced expression of adhesion molecules, including intercellular adhesion molecule (ICAM)-1, B7.1, B7.2, and CD40, on monocytes results in the activation of T cells (Durie et al., 1994; Ranger et al., 1996; Camacho et al., 2001). We also found that cell-to-cell interactions mediated by the engagement between ICAM-1, B7.1, B7.2, and CD40, respectively, on monocytes and their ligands, lymphocyte function-associated antigen-1, CD28, and CD40 ligand, on T cells were involved in T-cell activation, leading to induce the production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α in human peripheral blood mononuclear cells (PBMC) (Takahashi et al., 2003). However, little is known about the effect of AGE subtypes on adhesion molecule expression as well as cytokine production. Therefore, we examined the effect of AGE-2, AGE-3, AGE-4, and AGE-5 on the expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes and the production of IFN-γ and TNF-α in PBMC to better understand the profile of each AGE in monocyte activation. Moreover, we analyzed changes in the expression of RAGE on monocytes induced by AGEs, the involvement of adhesion molecules in the cytokine-producing action of AGEs, and the relevant signal pathways triggered by AGEs.

Materials and Methods

Reagents and Drugs. Recombinant human IFN-γ, TNF-α, anti-IFN-γ Ab and anti-TNF-α Ab were purchased from BD Biosciences (San Jose, CA). SN50 and SB203580 were purchased from Calbiochem (San Diego, CA). AGE-modified bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) was prepared as described previously (Takeuchi et al., 2000). In brief, each protein was incubated under sterile conditions with glyceraldehyde-3-phosphate (AGE-2) (Sigma-Aldrich), glycolaldehyde (AGE-3) (Sigma-Aldrich), methylglyoxal (AGE-4) (Sigma-Aldrich), or glyoxal (AGE-5) (Tokyo Kasei, Tokyo, Japan) in 0.2 M phosphate buffer, pH 7.4, at 37°C for 7 days. BSA was incubated under the same conditions. AGE-BSA and BSA were dialyzed for 2 days at 4°C. The endotoxin concentration of AGEs at 100 μg/ml described above was measured at SRL (Okayama, Japan), and it was found to be 1.2 pg/ml. AGE-specific fluorescence was measured at 450 nm after excitation at 390 nm with a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). sRAGE was prepared as described previously (Goren et al., 2007). In brief, sRAGE was cloned into the *Escherichia coli* expression vector pASK-IBA32; some modifications to the pASK-IBA32 polylinker region were made for cloning purposes. sRAGE, from amino acids 23 to 340 (Neeper et al., 1992), was amplified by polymerase chain reaction using the following oligonucleotides (MWG Biotech, High Point, NC); the underlined bases designate restriction sites: 5′-CTGACCTATG CGGCCGCTGC TCAAAACATCACAGC-3′ and 5′-GACTGAATTC ATCAGTGATGATGGTGATGG TGAGTTCCCA GCCCTGATCC-3′. Anti-human RAGE rabbit polyclonal Ab was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Isolation of PBMC, Monocytes, and T Cells. Normal human PBMC were obtained from ten healthy volunteers after acquiring Institutional Review Board approval (Okayama University Institutional Review Board no. 106). Samples of 20 to 50 ml of peripheral blood were withdrawn from a forearm vein, after which the PBMC were prepared, and monocytes isolated from PBMC were separated by counterflow centrifugal elutriation as described previously (Takahashi et al., 2003). T cells were then enriched from PBMC by passing them through a nylon wool column to a purity of 85% T cells as determined by flow cytometry with FITC-conjugated anti-CD3 antibody. PBMC, monocytes, and T cells were then suspended at a final concentration of 1×10^6 cells/ml in the medium as described previously (Takahashi et al., 2003).

Measuring AGEs-RAGE Binding Using Microtiter Plate in Vitro. Ninety six-well flat-bottomed microtiter plates were coated with BSA-AGEs at increasing concentrations from 0.3125 to 20 μg/ml and were incubated at 4°C for 16 h with gentle shaking on an orbital microplate shaker. After three times washings with washing buffer (10 mM Tris-buffered saline containing 0.05% Tween 20, pH 7.5), plates were blocked with 10% BSA at 4°C for 16 h. The plates were then incubated with His-tagged sRAGE at 0.83 μg/ml in 10 mM Tris-buffered saline at 4°C for 16 h. After three times washings, nickel-nitrilotriacetic acid-horseradish peroxidase conjugate (QIAGEN, Osaka, Japan) was added to the well, and the incubation was performed at room temperature for 1 h. The reaction was developed by the addition of 0.15% H_2O_2 and 2.5 mM 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) (Tokyo Kasei Kogyo Co., Ltd., Tokyo Japan) in 0.2 M citrate buffer, pH 4.0.

Flow Cytometric Analysis for Adhesion Molecule Expression. For flow cytometric analysis, FITC-conjugated mouse IgG1 monoclonal (m) Ab against ICAM-1/CD54 and phycoerythrin-conju-

gated anti-CD14 mAb were purchased from Dako Denmark A/S (Glostrup, Denmark). FITC-conjugated mouse IgG1 mAb against B7.1 was purchased from Immunotech (Marseille, France), FITC-conjugated mouse IgG1 mAb against B7.2 and CD40 was from BD Biosciences Pharmingen (San Diego, CA), and FITC-conjugated IgG1 isotype-matched control was obtained from Sigma-Aldrich. Changes in the expression of human leukocyte antigens, ICAM-1, B7.1, B7.2, and CD40, on monocytes were examined by multicolor flow cytometry using a combination of anti-CD14 Ab with anti-ICAM-1, anti-B7.1, anti-B7.2, or anti-CD40 Ab. In addition, to analyze changes in the expression of human RAGE on monocytes by multicolor flow cytometry using a combination of phycoerythrin-conjugated anti-human CD14 mouse IgG (Dako Denmark A/S) and anti-human RAGE rabbit IgG (Santa Cruz Biotechnology, Inc.) followed by FITC-conjugated anti-rabbit IgG goat IgG (Dako Denmark A/S) was performed. PBMC at 1×10^6 cells/ml were incubated for 24 h. Cultured cells at 5×10^5 cells/ml were prepared for flow cytometric analysis as described previously (Takahashi et al., 2003). The cells were analyzed with a FACSCalibur (BD Biosciences). Data were processed using the Cell Quest program (BD Biosciences).

Cytokine Production. PBMC at 1×10^6 cells/ml were used for analyzing IFN-γ and TNF-α production. After culturing for 24 h at 37°C in a 5% CO_2, air mixture, the cell-free supernatant was assayed for IFN-γ and TNF-α protein by enzyme-linked immunosorbent assay (ELISA) using the multiple Abs sandwich principle (R&D Systems, Minneapolis, MN). The detection limit of ELISA for IFN-γ and TNF-α was 10 pg/ml.

Immunofluorescence Staining. For double immunofluorescence staining, PBMC at 1×10^6 cells/ml were incubated for 24 h in the presence or absence of AGE-2 AGE-3 and BSA at 100 μg/ml. The cultured cells were fixed in 1% paraformaldehyde and stained with anti-human CD14 mouse IgG (Dako Denmark A/S) followed by Alexa 555-conjugated anti-mouse IgG rabbit IgG (Millipore Bioscience Research Reagents, Temecula, CA) and anti-human RAGE rabbit IgG (Santa Cruz Biotechnology, Inc.) followed by Alexa Fluor 488-conjugated anti-rabbit IgG goat IgG (Millipore Bioscience Research Reagents), respectively. The stained cells were mounted for viewing by fluorescent confocal microscopy (Biozero BZ8000; Keyence, Osaka, Japan).

Western Blot Analysis. The effect of AGE-2 and AGE-3 on the expression of RAGE was determined by Western blot analysis. Anti-human RAGE rabbit IgG was obtained by immunization of rabbit with recombinant human sRAGE. The lysates of human monocytes or A549 cells, a human pulmonary epithelial cell line, were electrophoresed on SDS-polyacrylamide gel electrophoresis gel. The fractionated proteins were transferred to nitrocellulose membrane. The blotting of β-actin was used as a loading control.

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

Results

AGE Subtype Binding to sRAGE. To evaluate the binding of AGE subtypes to RAGE, we established the in vitro assay by using the immobilized AGE subspecies and the His-tagged sRAGE protein (Fig. 1). AGE-2 and AGE-3 showed relatively high-affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed a moderate affinity (8 times less potent than AGE-2 and AGE-3) for sRAGE. We confirmed that the immobilization efficiency of each AGE was the same when detected by anti-BSA Ab (data not shown).

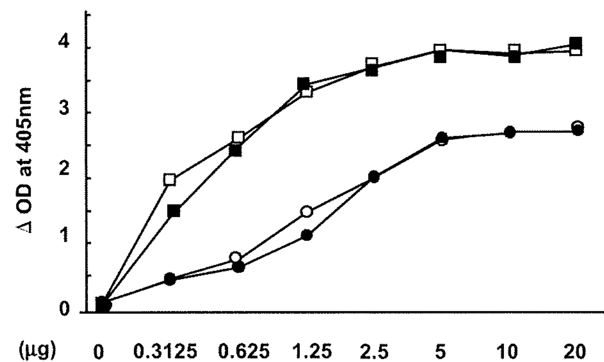

Fig. 1. AGE subtype binding to sRAGE. Ninety six-well flat-bottomed microtiter plates were coated with BSA-AGEs at increasing concentrations from 0.3125 to 20 μg/ml. After His-tagged sRAGE binding at 4°C for 16 h, the bound sRAGE was detected by nickel-nitrilotriacetic acid-horseradish peroxidase. Open squares (□) filled squares (■), open circles (○), or filled circles (●) represent the binding of AGE-2, AGE-3, AGE-4, and AGE-5 with sRAGE, respectively. The results are expressed as the means ± S.E.M. of triplicate determinations. When an error bar was within a symbol, the bar was omitted.

Time Course Effects of AGEs on the Expression of ICAM-1, B7.1, B7.2, and CD40 on Monocytes and the Production of IFN-γ and TNF-α in PBMC. To determine the proper incubation time, we examined the kinetics as shown in Figs. 2 and 3. In the absence of AGEs and BSA, the expression of ICAM-1, B7.1, B7.2, and CD40 moderately increased at 16 h and thereafter up to 24 and 48 h (Fig. 2A). AGE-2 and AGE-3 at 100 μg/ml significantly enhanced the expression of ICAM-1, B7.1, B7.2, and CD40 at 16 h and thereafter up to 24 and 48 h (Fig. 2, B, C, and G), whereas AGE-4, AGE-5, and BSA at 100 μg/ml had no effect at all (Fig. 2, D–F). In the absence of AGEs and BSA, the production of IFN-γ and TNF-α was under the detection limit during the incubation period (Fig. 3A). AGE-2 and AGE-3 at 100 μg/ml also time-dependently induced the production of IFN-γ and TNF-α in PBMC (Fig. 3, B and C). Neither AGE-4, AGE-5, nor BSA induced cytokine production (Fig. 3, D–F).

Dose-Response Relationship of AGEs on the Expression of ICAM-1, B7.1, B7.2, and CD40 on Monocytes, and the Production of IFN-γ and TNF-α in PBMC. The level of glyceraldehyde-derived AGE (AGE-2) is reported to be 17 μg/ml in the serum of patient with diabetes (Enomoto et al., 2006; Nakamura et al., 2007). It is reported that AGEs at the concentrations ranging from 50 to 200 μg/ml remarkably induce human monocyte adhesion to bovine retinal endothelial cells (Mamputu et al., 2004). AGEs at 200 μg/ml induce the expression of CD40, CD80, and CD86 and the production of IFN-γ in dendritic cells (Ge et al., 2005). Therefore, the effects of AGE-2, AGE-3, AGE-4, AGE-5, and BSA at concentrations ranging from 100 ng/ml to 100 μg/ml on the expression of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ and TNF-α were determined (Figs. 4 and 5). AGE-2 and AGE-3 concentration-dependently induced adhesion molecule expression with similar potency (Fig. 4, A and B), whereas AGE-4, AGE-5, and BSA showed no effect (Fig. 4, C–E). Consistent with the up-regulation of adhesion molecules, AGE-2 and AGE-3 concentration-dependently induced the production of IFN-γ and TNF-α (Fig. 5, A and B). The levels of IFN-γ and TNF-α production induced by AGE-2 at 100 μg/ml were 820 and 800 pg/ml, and those by AGE-3 at 100 μg/ml were 805 and 810 pg/ml, respectively. When we assumed that the effects of AGE-2 and AGE-3 were maximal at the concentration of 100 μg/ml, the ED_{50} values of AGE-2 for the induction of ICAM-1 expression and TNF-α produc-

Fig. 2. Time course effects of AGEs on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes. PBMC at 1×10^6 cells/ml were incubated with vehicle (A and G), AGE-2 (B and G), AGE-3 (C and G), AGE-4 (D), AGE-5 (E), or BSA (F and G) at 100 μg/ml for the indicated periods. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. FITC-conjugated IgG1 was used as an isotype-matched control Ab. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. *, $p < 0.05$ and **, $p < 0.01$ compared with the value for 0 h.

tion were calculated to be 3 and 5 μg/ml, and those of AGE-3 were 10 and 5 μg/ml, respectively. However, AGE-4, AGE-5, and BSA had no effect on cytokine production (Fig. 5, C–E).

Involvement of IFN-γ and TNF-α in the Effect of AGE-2 and AGE-3 on Adhesion Molecule Expression. We examined the involvement of IFN-γ and TNF-α in the

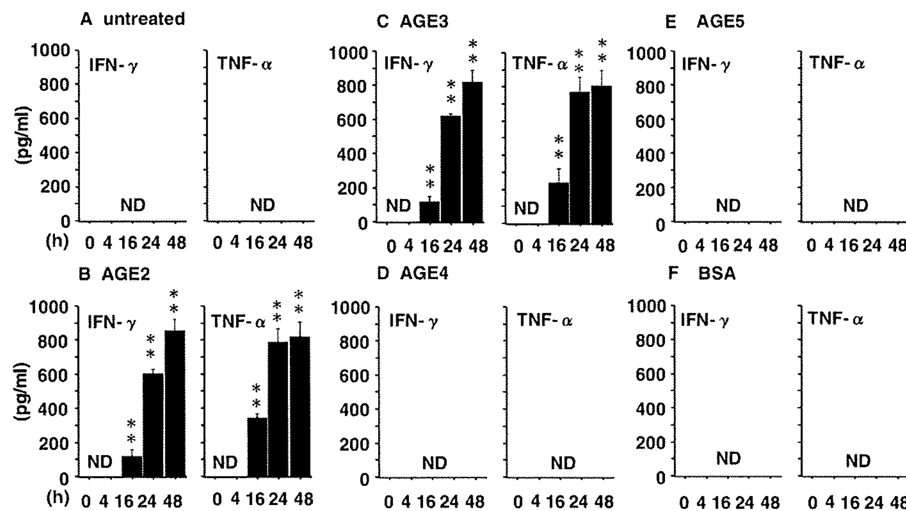

Fig. 3. The time-course effects of AGEs on the production of IFN-γ and TNF-α in PBMC. PBMC at 1×10^6 cells/ml were incubated with vehicle (A), AGE-2 (B), AGE-3 (C), AGE-4 (D), AGE-5 (E), or BSA (F) at 100 μg/ml for the indicated periods. The production of IFN-γ and TNF-α in PBMC was determined by ELISA. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the value for 0 h.

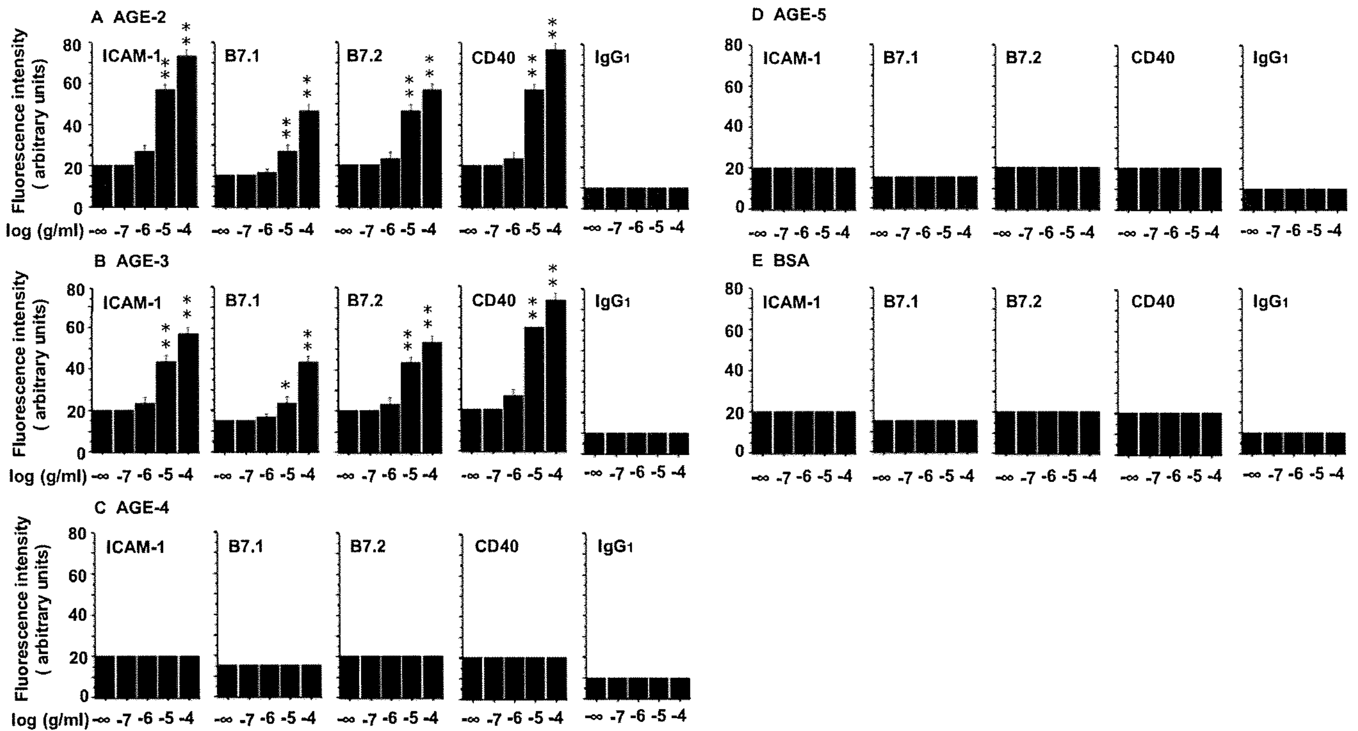

Fig. 4. Effect of AGEs on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes. PBMC at 1×10^6 cells/ml were incubated with AGE-2 (A), AGE-3 (B), AGE-4 (C), AGE-5 (D), or BSA (E) at increasing concentrations from 0.1 to 100 μg/ml for 24 h. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. FITC-conjugated IgG1 was used as an isotype-matched control Ab. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. *, $p < 0.05$ and **, $p < 0.01$ compared with the value for medium alone.

AGE-2 and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes (Fig. 6). IFN-γ and TNF-α at 10 ng/ml remarkably increased the expression of all adhesion molecules examined, and the expression reached a maximum level at 100 ng/ml. Moreover, the effects of cytokines and AGEs were additive. IFN-γ and TNF-α at 10 and 100 ng/ml enhanced the actions of AGE-2 and AGE-3, but those at 1 ng/ml had no effect (Fig. 6, A and B). Alternatively, anti-IFN-γ and anti-TNF-α Abs up to 100 ng/ml, which blocked the effect of exogenous IFN-γ and TNF-α at 100 ng/ml on adhesion molecule expression (data not shown), had no effect on the actions of AGE-2 and AGE-3 at 100 μg/ml (Fig. 6, C and D).

Involvement of Adhesion Molecule in the Effect of AGE-2 and AGE-3 on Cytokine Production. We examined the involvement of ICAM-1, B7.1, B7.2, and CD40 in the AGE-2- and AGE-3-induced production of IFN-γ and TNF-α in PBMC. As shown in Fig. 7, A and C, AGE-2 and AGE-3 concentration-dependently induced the production of IFN-γ and TNF-α in monocytes isolated from PBMC, exhibiting 20% of the amount obtained in PBMC, as shown in Fig. 5, B and C. AGE-2 and AGE-3 had no effect on the production of

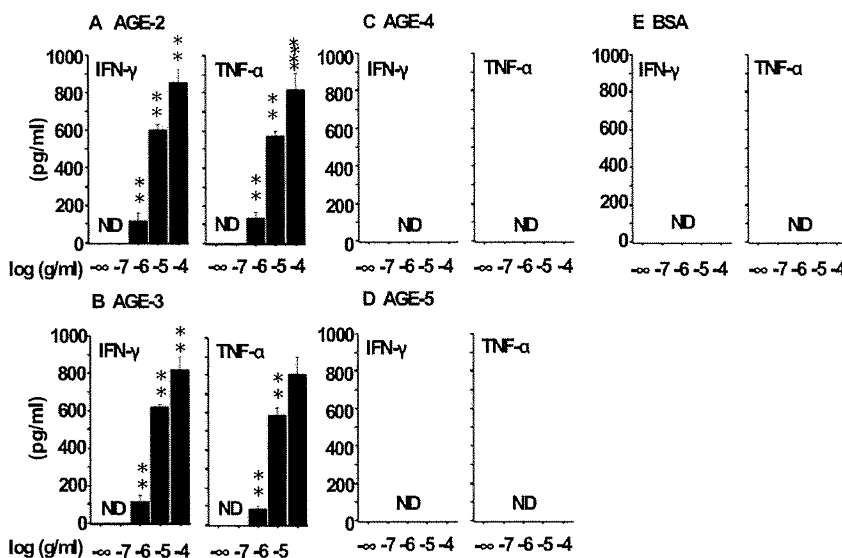

Fig. 5. Effect of AGEs on the production of IFN-γ and TNF-α in PBMC. PBMC at 1×10^6 cells/ml were incubated with AGE-2 (A), AGE-3 (B), AGE-4 (C), AGE-5 (D), or BSA (E) at increasing concentrations from 0.1 to 100 μg/ml for 24 h. The production of IFN-γ and TNF-α in PBMC was determined by ELISA. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the value for medium alone.

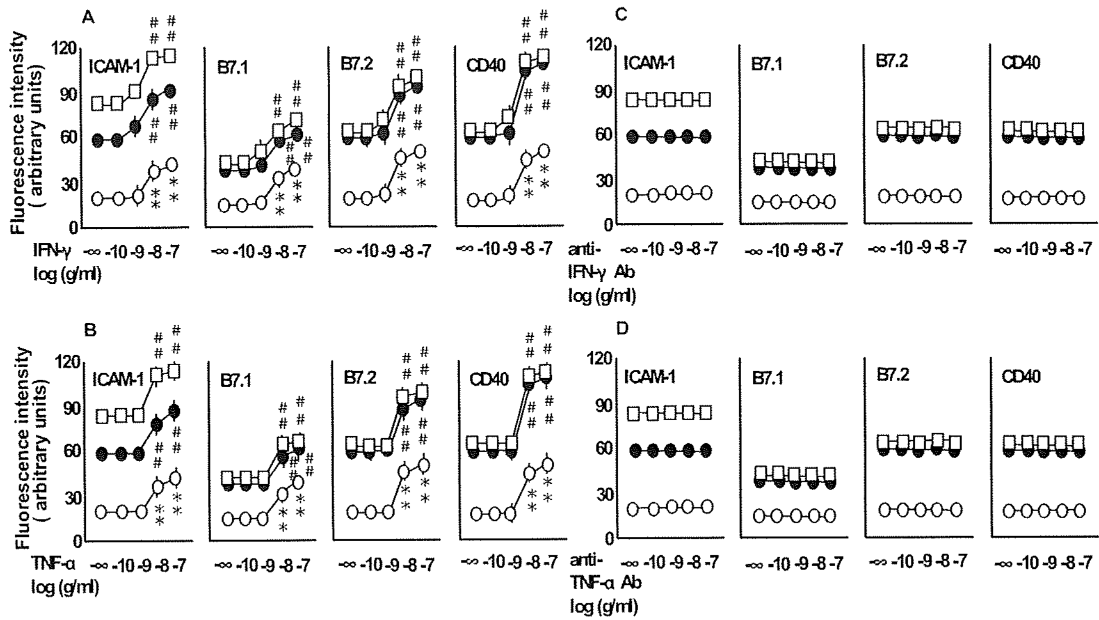

Fig. 6. Involvement of IFN-γ and TNF-α in the effect of AGE-2 and AGE-3 on adhesion molecule expression. PBMC at 1×10^6 cells/ml were incubated with IFN-γ (A), TNF-α (B), anti-IFN-γ (C), and anti-TNF-α (D) Abs at increasing concentrations from 0.1 to 100 ng/ml in the absence (○) or presence of AGE-2 (□) and AGE-3 (●) at 100 μg/ml for 24 h, and the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the value for medium alone. ##, $p < 0.01$ compared with the value for AGE alone. When an error bar was within a symbol, the bar was omitted.

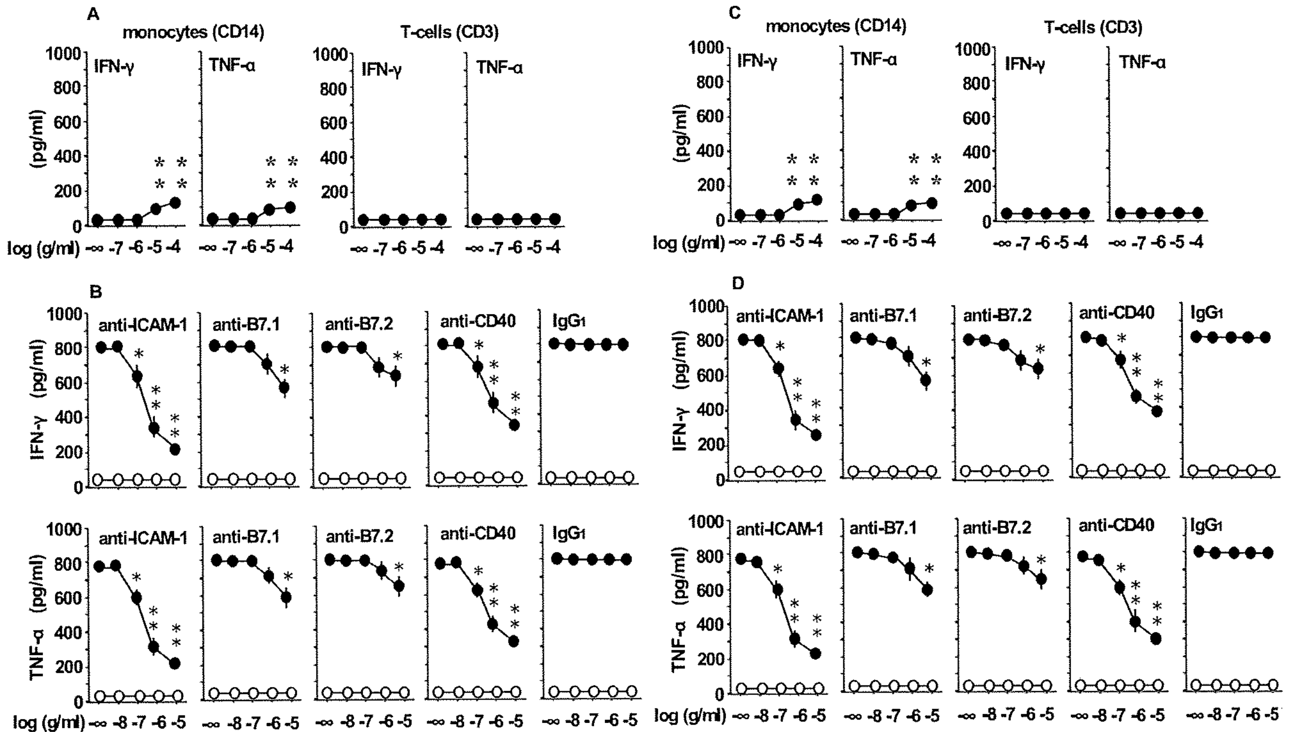

Fig. 7. Involvement of adhesion molecules in the effect of AGE-2 or AGE-3 on cytokine production. A and C, monocytes and T cells isolated from PBMC at 1×10^6 cells/ml were incubated with AGE-2 (A) or AGE-3 (C) at increasing concentrations from 0.1 to 100 μg/ml, and the production of IFN-γ and TNF-α was determined by ELISA. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the value for medium alone. B and D, PBMC at 1×10^6 cells/ml were incubated with anti-ICAM-1, anti-B7.1, anti-B7.2, and anti-CD40 Abs at increasing concentrations from 0.1 to 100 ng/ml in the absence (○) or presence (●) of AGE-2 (B) or AGE-3 (D) at 100 μg/ml, and the production of IFN-γ and TNF-α in PBMC was determined by ELISA. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. *, $p < 0.05$ and **, $p < 0.01$ compared with the value for AGE-2 or AGE-3 alone. If an error bar is within a symbol, the bar is omitted.

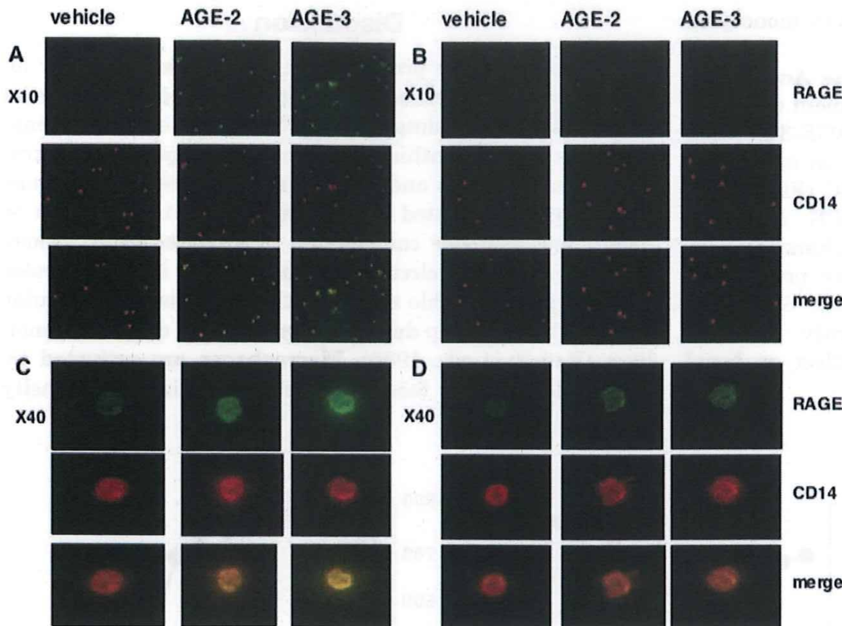

Fig. 8. Expression of RAGE on monocytes. The expression of RAGE on monocytes in the presence or absence of AGE1, AGE-2, AGE-3, AGE-4, AGE-5, and BSA at 100 μg/ml was analyzed by fluorescent microscopy. Staining with anti-RAGE rabbit IgG followed by Alexa 488-labeled goat anti-rabbit IgG as shown at 10× original magnification (A) and 40× original magnification (C). To block the engagement of anti-RAGE Ab with RAGE, anti-RAGE Ab was mixed with sRAGE for 24 h before use. The specificity of staining was confirmed by the absorption of primary Ab with sRAGE (B and D).

IFN-γ and TNF-α in T cells isolated from PBMC (Fig. 7, A and C). As shown in Fig. 7, B and D, anti-ICAM-1, anti-B7.1, anti-B7.2, and anti-CD40 Abs concentration-dependently inhibited the AGE-2- and AGE-3-induced production of IFN-γ and TNF-α in PBMC. The maximal inhibitory effect obtained by each Ab at 10 μg/ml was varied, and the rank order for the inhibition was as follows: ICAM-1 > CD40 > B7.1 > B7.2.

Expression of RAGE on Monocytes. The expression of RAGE on monocytes was determined by immunocytochemical staining (Fig. 8, A–D) and flow cytometry (Fig. 9A) using anti-RAGE Ab. AGE-2 and AGE-3 remarkably enhanced the expression of RAGE 24 h after stimulation, but AGE-4 and AGE-5 had no effect (data not shown). To confirm the binding specificity of anti-RAGE Ab to RAGE, we used a combination of anti-RAGE Ab with sRAGE for flow cytometry and immunocytochemical staining in the presence or absence of AGE-2 and AGE-3 (Figs. 8, B and D, and 9A). The detection of RAGE in the presence or absence of AGE-2 and AGE-3 was inhibited completely by the addition of sRAGE, indicating that anti-RAGE Ab recognized the extracellular domain of RAGE. As shown in Fig. 9B, Western blot analysis showed that RAGE is highly expressed on A549 cells, a human pulmonary epithelial cell line, as reported previously (Nakano et al., 2006). We confirmed that AGE-2 and AGE-3 at 100 μg/ml

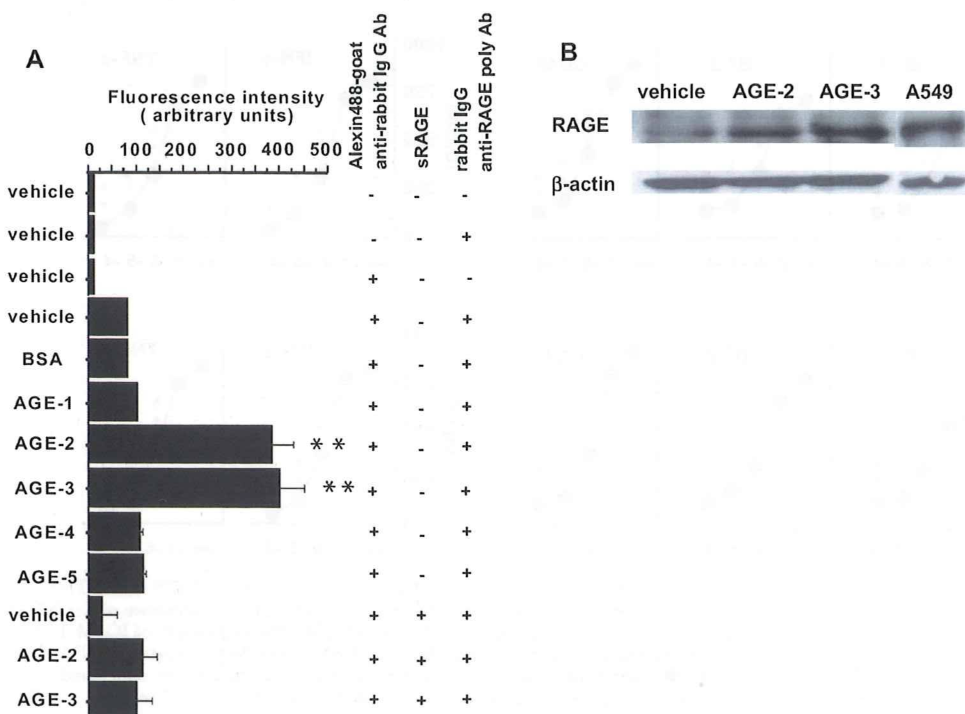

Fig. 9. Expression of RAGE on monocytes. A, expression of RAGE on monocytes in the presence or absence of AGE-1, AGE-2, AGE-3, AGE-4, AGE-5, and BSA at 100 μg/ml was examined by flow cytometry. The results of flow cytometry obtained under different conditions were quantified. To block the engagement of anti-RAGE Ab with RAGE, anti-RAGE Ab was mixed with sRAGE for 24 h before use. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $p < 0.01$ compared with the corresponding value for medium alone. B, protein levels in the presence or absence of AGE-2 or AGE-3 at 100 μg/ml were assessed by Western blot. Monocytes and A549 cells were cultured for 24 h at 37°C. RAGE levels were normalized to the level of β-actin.

induced the up-regulation of protein on human monocytes 24 h after the start of stimulation (Fig. 9B).

Involvement of NF-κB and MAPK in the Actions of AGE-2 and AGE-3. We examined the involvement of NF-κB and p38 MAPK activation in AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes and production of TNF-α and IFN-γ in PBMC (Fig. 10). A NF-κB inhibitor, SN50, and a p38 MAPK inhibitor, SB203580, reduced the AGE-2- and AGE-3-enhanced adhesion molecule expression as well as cytokine production. Even in the absence of AGE-2 and AGE-3, the SN50 and SB203580 inhibitors concentration-dependently inhibited adhesion expression, but neither had any effect on basal cytokine production.

Discussion

The diabetic state produces micro- and macrovascular lesions via various metabolic derangements, leading to crucial complications, including acquired blindness, end-stage renal failure, and neuropathies. In diabetic patients, the infiltration of macrophages and T cells into atherosclerotic plaques seems to be facilitated (Burke et al., 2004). Monocytes or macrophages, major components of atherosclerotic lesions and in contact with circulating plasma, play important roles in the hypercoagulable state and the progression of vascular injury, which develop during the progression of diabetes mellitus (Radoff et al., 1990). Macrophages are activated by atherosclerotic risk factors, including oxidized low-density

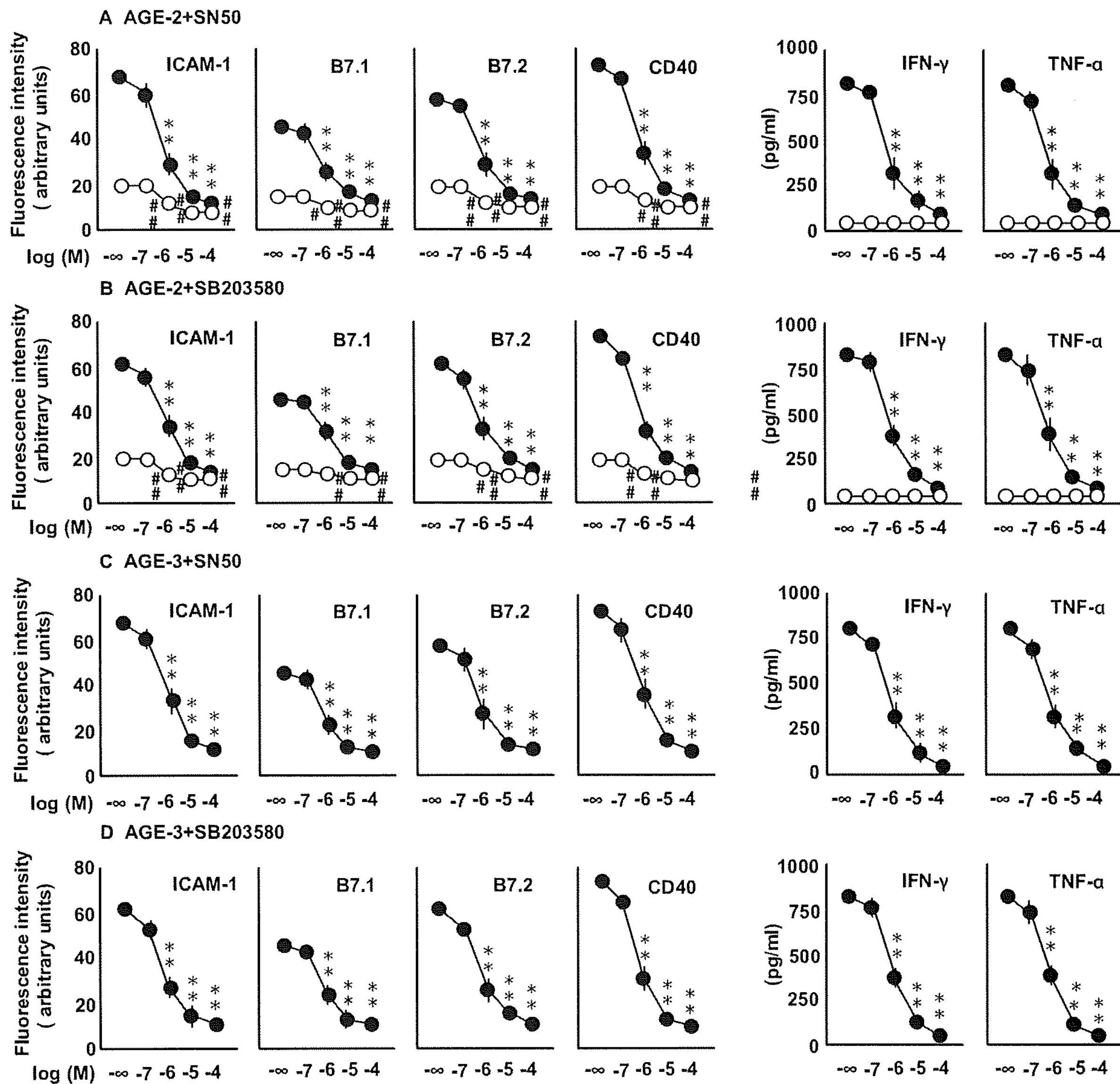

Fig. 10. Involvement of NF-κB and MAPK in the enhanced expression of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ and TNF-α by AGE-2 and AGE-3. PBMC at 1×10^6 cells/ml were incubated with AGE-2 (A and B) or AGE-3 (C and D) at 100 μg/ml in the presence of the NF-κB activation inhibitor SN50 and the p38 MAPK inhibitor SB203580 at increasing concentrations from 0.1 to 100 μM. The expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes was determined by flow cytometry, and the production of IFN-γ and TNF-α in PBMC was determined by ELISA. Open circles (○) represent the results in the absence of AGE, and filled circles (●) represent those in the presence of AGE. 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 alone. #, $p < 0.05$ and ##, $p < 0.01$ compared with the value for medium alone. If an error bar is within a symbol, the bar is omitted.

lipoprotein and AGEs, leading to death of cells and degradation of the extracellular matrix (Figarola et al., 2007). AGEs are involved in the cause of foam cell formation via the increased expression of oxidized low-density lipoprotein receptors in accelerated atherosclerotic lesions of patients with diabetes (Iwashima et al., 2000). NO by macrophages up-regulates the expression of Fas on vascular smooth muscle cells, priming them for apoptosis. The phenotypes of advanced diabetic nephropathy were prevented by administering an AGE inhibitor, (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide, in RAGE-transgenic mice crossbred with transgenic mice carrying human cDNA for inducible NO synthase under the control of the insulin promoter (Yamamoto et al., 2001). We therefore suggested that the activation of T cells by the enhancement of adhesion molecule expression on monocytes might result in the development of diabetic microangiopathy.

AGEs develop diabetic microangiopathy through the stimulation of RAGE (Stern et al., 2002). AGEs and RAGE are also detected in atherosclerotic plaque of diabetic patients (Cuccurullo et al., 2006). Takeuchi and Makita (2001) provided direct immunological evidence for the existence of four distinct AGE classes (AGE-2–5) among the AGE-modified proteins and peptides found in the serum of diabetic patients on hemodialysis. Antibodies, which cross-reacted with BSA modified by AGE-2, AGE-3, AGE-4, and AGE-5, recognized non–*N*-carboxymethyllysine AGE-2, AGE-3, AGE-4, and AGE-5 in serum samples obtained from type 2 diabetic patients, respectively. Among various subtypes of AGE, it has been shown that AGE-2 and AGE-3 are the main structures of AGEs that are detectable in the serum of diabetic patients (Takeuchi and Yamagishi, 2004). It is reported that AGE-2 induces diabetic microangiopathy (Takeuchi et al., 2000). Therefore, it has been suggested that blockade of the engagement of AGEs and RAGE is a target for the treatment of diabetic microangiopathy. However, little is known about the effect of AGE subtypes on the engagement of monocytes and T cells.

Several reports have shown a positive correlation between serum levels of TNF-α and circulating AGE levels. TNF-α promotes macrophage-induced apoptosis of human vascular smooth muscle cells (Boyle et al., 2003). Goren et al. (2007) reported that anti-TNF-α therapy, widely used in chronic inflammatory diseases in humans, improves healing of diabetes skin ulcers by targeting activated TNF-α-expressing macrophage subsets. Although it is reported that AGEs induce the production of TNF-α in monocytes (Boyle et al., 2001), little is known about the mechanism of enhancement of TNF-α production in monocytes. In the present study, we examined, for the first time, the affinity of AGE-2, AGE-3, AGE-4, and AGE-5 for RAGE by using in vitro assay system (Fig. 1). AGE-2 and AGE-3 have higher affinity for RAGE compared with AGE-4 and AGE-5. As shown in Figs. 2 to 5, we examined the effect of AGE subtypes on the expression of ICAM-1, B7.1, B7.2, and CD40 on monocytes, and the production of IFN-γ and TNF-α in PBMC. AGE-2 and AGE-3, but not AGE-4 and AGE-5, increased the expression of ICAM-1, B7.1, B7.2, and CD40 and the production of IFN-γ and TNF-α. Consistent with high-affinity binding of AGE-2 and AGE-3 to RAGE in vitro, our results concerning the adhesion molecule expression and cytokine production may support the idea that AGE-2 and AGE-3 function as toxic AGEs and play a central role in the pathophysiological processes associated with AGE formation. As shown in Fig. 6, A and B, the actions of AGE-2 and AGE-3 might be independent of the endogenous IFN-γ and TNF-α. IL-18 and IL-12, as well as IFN-γ and TNF-α, are associated with accelerated atherosclerosis (Gerdes et al., 2002; Wen et al., 2006). Recently, we reported that IL-18 induced the production of IFN-γ and TNF-α in PBMC (Takahashi et al., 2003). We found that the levels of IL-18 and IL-12, monocyte-derived cytokines, were under the detection limit (10 pg/ml) in the presence of AGE-2 and AGE-3 (data not shown). Anti-IL-18 and anti-IL-12 Abs had no effect on the AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2, and CD40 and production of IFN-γ and TNF-α (data not shown), indicating that the actions of AGE-2 and AGE-3 might be also independent of IL-18 and IL-12. As shown in Fig. 7, the enhanced production of IFN-γ and TNF-α by AGE-2 and AGE-3 may require the enhancement of cell-to-cell interaction between monocytes and T cells through the induction of plural adhesion molecule expression on monocytes.

As shown in Figs. 8 and 9A, we found, for the first time, the up-regulation of RAGE expression on monocytes by AGE-2 and AGE-3 using flow cytometry and immunocytochemistry. Moreover, we confirmed the up-regulation of RAGE protein induced by AGE-2 and AGE-3 using Western blot analysis (Fig. 9B). It is reported that the interaction of AGE-2 with RAGE alters intracellular signaling, gene expression, and the release of proinflammatory molecules in monocytes and macrophages (Sato et al., 2006). AGEs are reported to induce the activation of NF-κB in monocytes via RAGE (Hofmann et al., 1999). In endothelial cells, the activation of NF-κB induces the expression of cytokines, including IL-1 and transforming growth factor-β, and adhesion molecules, including ICAM-1 and vascular cell adhesion molecule-1 (Miyata et al., 1996). The interaction of AGEs and RAGE is reported to result in the up-regulation of RAGE through the activation of NF-κB in monocytes (Li and Schmidt, 1997). Consistent with this finding, pharmacological experiments using the inhibitors of NF-κB and p38 MAPK suggested that the activation of NF-κB and p38 MAPK might be in part involved in the effects of AGE-2 and AGE-3 on adhesion molecule expression and cytokine production (Fig. 10), although it is not excluded that other pathways may be involved in the actions of AGEs. Further studies on the signal pathway leading to the up-regulation of RAGE and adhesion molecule expression should be performed.

In conclusion, among AGEs, AGE-2 and AGE-3 selectively induced the expression of ICAM-1, B7.1, B7.2, and CD40, and the production of IFN-γ and TNF-α. Although adhesion molecule expression on monocytes was independent of endogenous cytokine production in the presence of AGE-2 and AGE-3, the induction of IFN-γ and TNF-α depended on the engagement of monocytes and T cells through the up-regulation of ICAM-1, B7.1, B7.2, and CD40. The actions of AGE-2 and AGE-3 might be through the stimulation and up-regulation of RAGE and the activation of NF-κB and p38 MAPK in monocytes. Together with these results, toxic AGE-dependent responses, including the enhancement of adhesion molecule expression on monocytes, may partially contribute to facilitation of the development of atherosclerotic plaques in diabetes.

Acknowledgments

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Short Communication

Specific Removal of Monocytes from Peripheral Blood of Septic Patients by Polymyxin B-immobilized Filter Column

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Lipopolysaccharide (LPS) is one of the major causes of septic shock. The polymyxin B-immobilized filter column (PMX) was developed for the adsorption of endotoxin by direct hemoperfusion and has been used for the treatment of LPS-induced septic shock. In this study, we demonstrated that PMX also specifically bound monocytes from the peripheral blood leukocytes of septic patients by mean of an analysis of bound cells using immunocytochemical and electron microscopic techniques. The specific removal of monocytes from septic patients may produce beneficial effects by reducing the interaction between monocytes and functionally associated cells including vascular endothelial cells.

Key words: septic shock, polymixin B-immobilized column, monocyte, adsorptive removal

Sepsis and septic shock, often associated with multiple organ failure, still remain important causes of morbidity and mortality in intensive care units. Many types of therapeutic trials for the treatment of septic shock have failed however, recent phase III studies using recombinant activated protein C demonstrated the effectiveness of this therapy [1, 2]. Lipopolysaccharide (LPS), one of the major causes of septic shock, together with LPS binding protein, binds to CD14 on the surfaces of monocytes/macrophages, leading to the activation of the signaling molecule complex of Toll-like receptor-4 (TLR-4) and MD2. Polymyxin B can bind LPS and neutralize its biological activity therefore, the polymyxin B-immobilized filter (PMX) column was developed for the adsorption of endotoxin by hemoperfusion [3]. There is now increasing evidence supporting the usefulness of this treatment, showing improvement of the survival rate in LPS-induced circulatory disorders and systemic inflammatory response syndrome. Moreover, the effectiveness of hemoperfusion with this column for treating septic shock beyond LPS endotoxemia [4] prompted us to investigate additional mechanisms. Since it is well known that different populations of leukocytes are activated during septic shock and change their adhesive phenotype, we hypothesized that some population of leukocytes may be adsorbed in the column and removed from the blood circulation after treatment. To examine this hypothesis, we investigated the cellular components in the PMX columns after direct hemofiltration in 4 septic patients.

The original diseases of the 4 patients were ileus of sigmoid colon (case 1; 69 ys male), embolism of superior mesenteric artery (case 2; 76 ys male), pye-

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