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# ADVANCED GLYCATION END PRODUCTS STIMULATE PLASMINOGEN ACTIVATOR ACTIVITY VIA GM-CSF IN RAW 264.7 CELLS

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The effects of advanced glycation end products (AGE) on the plasminogen activator (PA) activity were investigated with murine macrophage cell line RAW 264.7 cells. AGE-bovine serum albumin (BSA) showed a dose-dependent induction for the urokinase-type PA (uPA) activity. The uPA induction by AGE-BSA was effectively suppressed by the antibody against granulocyte-macrophage colony-stimulating factor (GM-CSF). The uPA activity of these cells was also induced by ligands for the macrophage scavenger receptor (MSR). These data provide evidence that AGE-BSA stimulates the uPA activity via GM-CSF through MSR in RAW cells. These findings, taken together with a recent demonstration of endocytic uptake of AGE-proteins by MSR *in vitro* and the presence of AGE-proteins in atherosclerotic lesions, strongly suggest that the uPA induction by AGE-proteins via MSR plays an important role in human atherogenesis. 

In the Maillard reaction, proteins react with glucose to form a Schiff base and Amadori products. Further incubation of these early products leads to advanced glycation end products (AGE), which are characterized by fluorescence, a brown color and intra- or intermolecular cross-linking (1, 2). Recent immunological demonstration of AGE-proteins in several human and animal tissues has emphasized a potential role of AGE in normal aging and disease processes such as diabetic complications and atherosclerosis (3-6). The possible link of AGE to these disease processes has been pursued from the cell surface receptor, or binding protein for AGE (the AGE receptor). AGE-proteins are known to be endocytosed by macrophages or macrophage-derived cells via the AGE-receptor. At the same time, their interaction with

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## Abbreviations

AGE, advanced glycation end products; PA, plasminogen activator; GM-CSF, granulocyte-macrophage colony-stimulating factor; BSA, bovine serum albumin; MSR, macrophage scavenger receptor; LDL, low-density lipoprotein; RT-PCR, reverse transcriptase-polymerase chain reaction; IL-1, interleukin-1; TNF, tumor necrosis factor.

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macrophages is known to induce several biological responses *in vitro* such as secretion of TNF and IL-1β and the cell growth of mouse peritoneal macrophages (7, 8).

It is generally accepted that the activation of a protease system(s) is essential for the cellular growth and migration (9). Plasminogen activator (PA), a protease catalyzing enzymatic conversion of plasminogen to plasmin which directly mediates degradation of a broad range of matrix proteins, or indirectly activates procollagenase into active collagenase, is known as a main enzyme catalyzing degradation of extracellular matrix proteins (10). In this study we examined whether AGE-proteins could induce PA in RAW 264.7 cells, the cell line established from murine macrophages.

## MATERIALS AND METHODS

**Chemicals**. Bovine serum albumin (BSA), fetal calf serum (FCS), polyinosinic acid, polycytidylic acid, and fucoidin were purchased from Sigma Chemical Co. RPMI 1640, macrophage-serum free medium (M-SFM), murine recombinant GM-CSF and anti-murine GM-CSF antibody were purchased from Gibco laboratories. Other chemicals were of the best grade available from commercial source.

**Ligand preparation**. AGE-BSA were prepared as described previously (11). Endotoxin was removed from AGE-BSA with endotoxin-adsorbent (Pyro Sep C: Daicel Chemical Industries, Ltd., Tokyo, Japan) by the method described previously (12) and the level of endotoxin in each sample was measured by Endospacy (Seikagaku Corporation). Endotoxin levels of the AGE-BSA and other ligands used for the present experiments were less than 100 pg/ml which had no effect on the PA activity of RAW cells. Acetylated LDL and oxidized LDL were prepared as described previously (13). Maleylated BSA was prepared as described previously (14). Protein concentrations were determined by bicinchoninic acid protein assay reagent (Pierce) using BSA as a standard .

**Cell culture and preparation of cells**. Unless otherwise specified, all cellular experiments were performed at 37°C in a humidified atmosphere of 5% $CO_2$ in air. RAW 264.7 cells were cultured in RPMI 1640 containing 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 10% FCS. SV40 MES 13 cells were cultured in 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing and 5% FCS.

**PA activity assay**. RAW cells (1 x $10^6$) were seeded in a 12-well culture plate (22.1 mm in diameter, Corning) in 1.0 ml of RPMI 1640 containing 10% FCS. The cells were replaced with 1.0 ml of prewarmed M-SFM containing various concentrations of AGE-BSA or other reagents, and incubated for 20 h. Cells were then lysed with 1.0 ml of 0.5% Triton X-100 for 30 min at room temperature and sonicated in ultrasonic cleaner for 10 min. The plasminogen activator activity of cell lysates was then determined as described previously (15). Briefly, 50 μl of each cell lysate was added to 50 μl of a substrate solution which contained 20 μg of D-Val-Leu-Lys-pNA, a substrate for plasmin and 0.25 μg of plasminogen, and the mixture was incubated for 3 h, followed by measurement of the absorbance at 405 nm in spectrophotometer (Titertek Multiskan, Labosystems). Human urokinase (U8627: Gibco) was used as a standard for PA.

**RT-PCR**. Total RNA was isolated from the precipitated cells by TRI zol Reagent (Gibco BRL). The total RNA (1.0 μg) was reverse transcribed using oligo dT (Gibco BRL) with RNase H-free reverse transcriptase (Superscript II; Gibco BRL). Thirty cycles of amplification were carried out using a thermal cycle program (94°C for 30 sec, at 55°C for 1 min, at 80°C for 1.5 min for uPA, 94°C for 30 sec, at 57°C for 1 min, and at 80°C for 2 min for tPA, 94°C for 30 sec, at 56°C for 1 min, and at 81°C for 1.5 min for PAI-1, and 94°C for 1 min, at 57°C for 2 min and at 74°C for 3 min for ß-actin) in a Gene Amp PCR System 9600 (Perkin Elmer), and the products were analyzed by agarose gel electrophoresis.

**Design of primers**. Sequences utilized for the preparation of forward (FP), and reverse (RP) PCR primers were as follows:

| | | | |
|---|---|---|---|
| uPA | (16) | FP; 5'-CAT CAA AAA CCT GCT ATC AT-3' | RP; 5'-ACC TCA AAC TTC ATC TCT CC-3'. |
| tPA | (17) | FP; 5'-CAG TGT TCT GTC GCT GAA GC-3' | RP; 5'-AGC AGG AAC TGA TCA GCA CC-3' |
| PAI-1 | (18) | FP; 5'-ATG ACC ACA GCG GGG AAA AC-3' | RP; 5'-AAC TTA GGC AGG ATG AGG AG-3' |

The size of RT-PCR products were expected to be 574, 596, and 692 base pairs for mRNA encoding uPA, tPA, and PAI-1 respectively. The primers for mouse ß-actin were purchased from Clontech (Palo Alto, CA).

## RESULTS

### AGE-BSA stimulates the uPA activity in RAW cells

Fig. 1 shows the effects of AGE-BSA on PA activity of RAW cells. The total PA activity of cell lysate was increased from 750 to 1,100 μunits/mg cell protein by 10 μg/ml of AGE-BSA, and further increased up to 1,300 μunits/mg cell protein by 40 μg/ml of AGE-BSA in a dose-dependent manner. However, a further increase in the concentration to 80 μg/ml resulted in a significant suppression of the activity to 1,100 μunits/mg cell protein. In a sharp contrast, parallel incubations with BSA used as a control had no effect on the PA activity of these cells. These data strongly suggest that the AGE-BSA has a capacity to induce the PA activity in RAW cells. Two types are known for PA, urokinase-type (uPA) and tissue-type (tPA) (10). RT-PCR analysis showed that AGE-BSA could induce the expression of uPA, but not tPA (Fig 2, lane 2). Since SV40-MES13 cells used as a positive control exhibited a significant expression of both uPA and tPA under the identical conditions (Fig. 2, lane 4), it is likely that AGE-BSA stimulates the uPA activity in RAW cells.

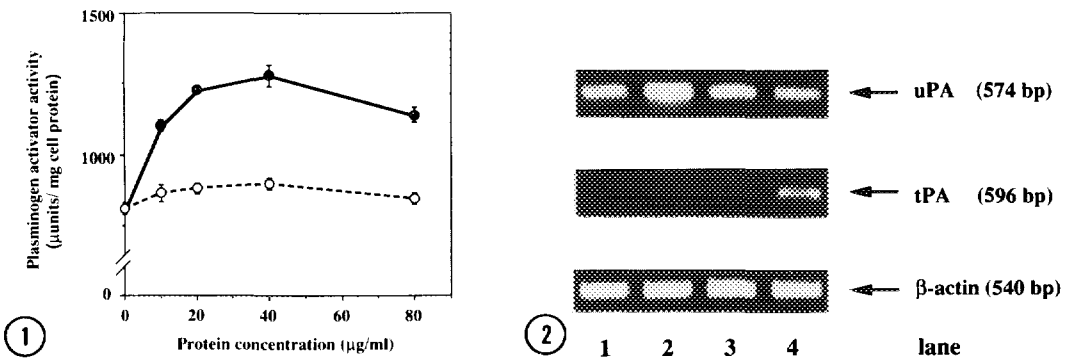


**Fig. 1. Effect of AGE-BSA on plasminogen activator activity of RAW cells.**
RAW cells (1 x $10^6$) were incubated for 20 h at 37°C with indicated concentrations of AGE-BSA (●) or BSA (○) . The cells were washed, lysed with 0.5% Triton X-100 and sonicated, followed by determination of the PA activity as described under "Materials and Methods." Data represent the mean of four separate experiments. Error bars represent SD.

**Fig. 2. Effect of AGE-BSA on uPA and tPA mRNA in RAW cells.**
RAW cells (2.5 x $10^6$) were incubated without (lane 1) or with 50 μg/ml AGE-BSA (lane 2) or BSA (lane 3). Similarly, SV40-MES13 cells were incubated without ligands (lane 4). After incubation for 6 h at 37°C, the total RNA was extracted and uPA (upper), tPA (middle), and β-actin (lower) cDNAs were amplified by PCR for 30 cycles and analyzed on a 1.5% agarose gel.

**AGE-BSA stimulates the PA activity via GM-CSF through MSR in RAW cells**

Since our previous study has demonstrated that AGE-proteins could stimulate the expression of GM-CSF (8), we examined whether GM-CSF was involved in AGE-BSA-induced uPA expression in RAW cells. As shown in Fig. 3, the PA activity was stimulated from 750 to 1,300 μuints/mg cell protein with 40 μg/ml of AGE-BSA. This increase was completely suppressed to a basal level by the presence of an anti-GM-CSF antibody, whereas a nonimmune IgG had no effect. Under these conditions, murine recombinant GM-CSF alone (100 ng/ml) could induce the PA activity of these cells up to 1,270 μunits/mg cell protein, a level similar to the case with 40 μg/ml of AGE-BSA and this increase was suppressed to a basal level by the same antibody, while the nonimmune IgG had no effect. These finding strongly suggest that AGE-BSA stimulates the uPA activity by inducing GM-CSF in RAW cells. Therefore, a likely mechanism is that AGE-BSA interacts with these macrophages to induce GM-CSF, which then stimulates the uPA activity of these cells in an autocrine or paracrine fashion. We have recently shown that MSR plays a major role in endocytic uptake of AGE-proteins by macrophages or macrophage-derived cells (19). We therefore examined the role of MSR in the AGE-BSA-

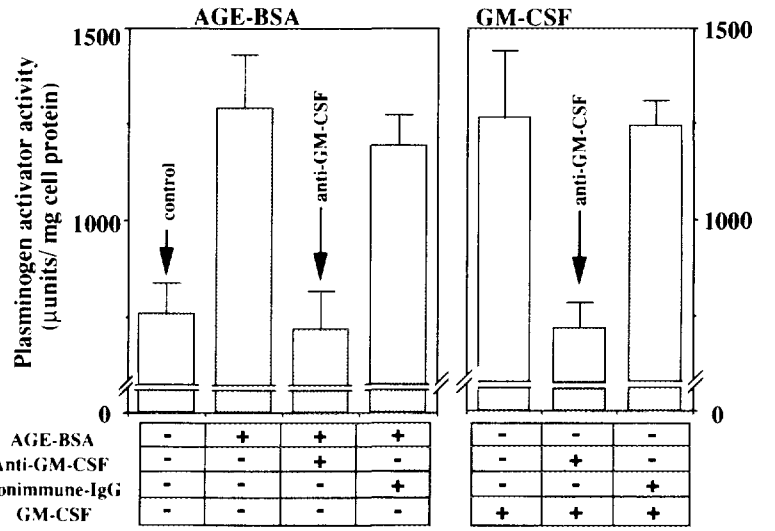


**Fig. 3. Effect of anti-GM-CSF antibody on AGE-induced PA activity.**
RAW cells (1 x $10^6$) were incubated with 40 μg/ml of AGE-BSA in the absence or presence of 50 μg/ml of anti-GM-CSF antibody. The control experiments were done with 50 μg/ml of nonimmune mouse IgG. The cells were also incubated with 100 ng/ml of recombinant GM-CSF in the absence or presence of 50 μg/ml of anti-GM-CSF antibody or nonimmune IgG. After incubation for 20 h at 37°C, PA activity was determined as described under "Materials and Methods." Data represent the mean of three separate experiments. Error bars represent SD.

induced stimulation of the PA activity in RAW cells. Ligands for MSR such as maleylated BSA, oxidized LDL and acetylated LDL could induce the PA activity (Fig. 4). Similar stimulating effects were also observed for polyanions such as fucoidin and polyinosinic acid, well-known inhibitors for MSR, whereas polycytidylic acid which had no affinity for MSR (20) failed to induce the PA activity. These findings strongly suggest that MSR of RAW cells plays an important role in the induction of the PA activity by AGE-BSA.

**High concentration of AGE-BSA induced PAI-1 mRNA**

The PA activity in situ is regulated by both PA and plasminogen activator inhibitor (PAI) (10). We tested the involvement of PAI-1 in the suppression of the uPA activity by a high concentration of AGE-BSA (80 μg/ml) (Fig. 1). RT-PCR analyses showed that the expression of mRNA of PAI-1 was negligible with 10 to 50 μg/ml of AGE-BSA, whereas it became significant by incubation with 100 μg/ml of AGE-BSA. It is likely therefore that a significant suppression of the PA activity by a high concentration of AGE-BSA might be accounted for by concomitant induction of PAI-1 (Fig. 5).

## DISCUSSION

Interaction of AGE-proteins with macrophages is known to induce several cytokines (7, 8, 12, 21). Yui et al. (8) have recently demonstrated that AGE-proteins are able to induce cell

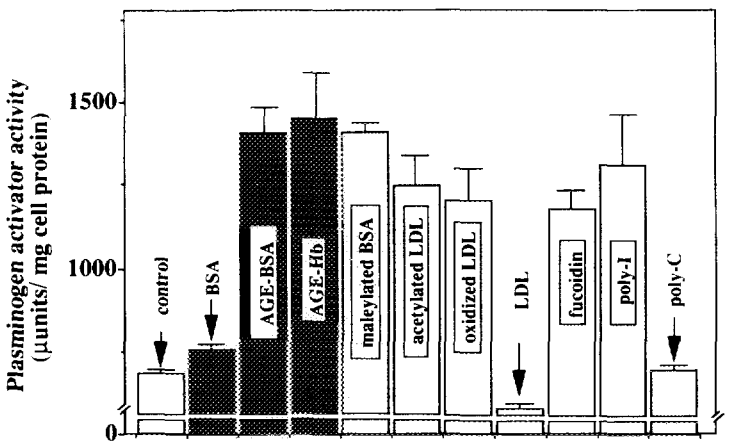


**Fig. 4. Effects of several ligands on PA activity in RAW cells.**
RAW cells (1 x $10^6$) were incubated for 20 h at 37°C with 40 μg/ml of BSA, AGE-BSA, and AGE-hemoglobin (Hb), or 80 μg/ml of maleylated BSA, acetylated LDL, oxidized LDL, LDL, fucoidin and polycytidylic (poly-C), or 10 μg/ml of polyionosinic acid (poly-I). PA activity was measured as described under "Materials and Methods." Data represent the mean of three separate experiments. Error bar represent SD.

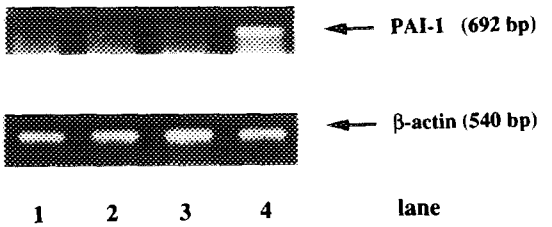


**Fig. 5. Effect of AGE-BSA on PAI-1 mRNA in RAW cells.**
RAW cells (2.5 x $10^6$) were incubated for 9 h at 37°C without (lane 1) or with 10 (lane 2), 50 (lane 3),or 100 (lane 4) μg/ml of AGE-BSA. PAI-1 (upper) and β-actin (lower) cDNAs were amplified by PCR for 30 cycles and analyzed on a 1.5% agarose gel.

growth of mouse peritoneal macrophages with a concomitant expression of GM-CSF mRNA, suggesting the macrophage growth induced by AGE-proteins is mediated by GM-CSF. Since GM-CSF is known to induce the uPA activity in human monocytes (22), two pathways are possible for AGE-proteins to induce the uPA in RAW cells; one is the induction of GM-CSF by AGE-proteins with a subsequent induction of uPA by GM-CSF, and the other is a direct pathway without GM-CSF induction. In the present study, the AGE-induced uPA production was completely inhibited by the anti-GM-CSF antibody and exogenously added GM-CSF could in fact induce uPA in these RAW cells (Fig. 3). Therefore, it is likely that AGE-proteins induce uPA via GM-CSF.

It is interesting to ask what kind of the AGE-receptor is involved in AGE-BSA-induced uPA production in RAW cells. Our recent study using Chinese hamster ovary cells overexpressing the type II MSR has clearly shown that the capacity of these cells to take up and degrade acetylated LDL (a ligand for MSR) increases significantly concurrent with their capacity to take up and degrade AGE-BSA, suggesting MSR plays a major role in the endocytic uptake of AGE-proteins by macrophages (19). This notion is also supported by the present result that under the identical conditions, the uPA induction was also stimulated by ligands for MSR such as acetylated LDL, oxidized LDL, maleylated BSA, fucoidin and polyinosinic acid, whereas LDL or polycytidylic acid, inert ligands for MSR, did not have such an effect. Thus, these findings strongly indicate that AGE-proteins induce the uPA activity through MSR.

Although the physiological significance of the AGE-stimulated uPA induction in macrophages is not clear, three lines of evidence support a potential link of this phenomenon to atherosclerotic processes. First, MSR was preferentially expressed by macrophage-derived foam

cells in the early atherosclerotic lesions (22). Second, AGE-proteins were demonstrated to occur not only in these foam cells but also in an extracellular space of atherosclerotic lesions (5). Finally, the uPA activity of macrophages of these lesions was much higher than circulating monocytes (23). Therefore, it seems reasonable to expect that the uPA induced in the atherosclerotic lesions might play some role in remodeling of vascular walls by proteolytic degradation of extracellular matrix proteins.

In summary, to our knowledge, this is the first report suggesting that AGE-proteins might regulate the uPA activity in macrophages via MSR. Further elucidation of this mechanism might shed a light in a pathological role of AGE in disease states *in vivo*.

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