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The Novel Function of Advanced Glycation End Products in Regulation of MMP-9 Production

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Abstract

Background—Advanced glycation end products (AGEs), formed from proteins and peptides by nonenzymatic glycoxidation after contact with aldose sugars, have been implicated in the pathogenesis of age-related cardiac and vascular dysfunction. Our previous study demonstrated significantly elevated levels of AGE and the receptor for AGE (RAGE) in human abdominal aortic aneurysm (AAA) tissues. Inhibition of AGE signaling by targeted gene deletion of RAGE markedly reduced the development of aneurysm in a mouse model of AAA. We also showed that AGE may stimulate aneurysm formation by promoting metalloproteinase (MMP)-9 expression. In this study, we investigated the molecular mechanism underlying this novel function of AGE.

Methods—The murine macrophage cell line RAW 264.7 was pretreated with AGE, TGF- β , and MAPK inhibitors. The protein was collected for Western blot analysis. Culture supernatants were collected to determine MMP-9 activity by gelatin zymography.

Results—We found that AGE induced the production of MMP-9 in macrophages in a dose-dependent manner. This induction of MMP-9 was markedly diminished by pretreatment with TGF- β . To delineate the underlying molecular mechanism, we showed that AGE increased phosphorylation of p44/42 ERK, p38, JNK, and PI3K in macrophages. Moreover, AGE induced active p65 subunit of NF- κ B. Inhibition of ERK (UO126) or p38 (SB203580), but not PI3K (LY294002 or wortmannin), blocked AGE-induced MMP-9 expression. In contrast, inhibition of JNK (SP-600125) significantly enhanced the stimulatory effect of AGE on MMP-9. Furthermore, TGF- β suppressed AGE-induced expression of the active p65 subunit of NF- κ B.

Conclusions—Our data indicate that AGE induces MMP-9 through activation of ERK, p38 mitogen-activated protein and NF- κ B, a pathway that is antagonized by TGF- β . This finding in conjunction with previously reported AGE functions in inflammation suggests that anti-AGE therapies could be effective in the prevention of human AAA development and progression.

Keywords

MAP kinases; abdominal aortic aneurysm; signaling; TGF-beta

INTRODUCTION

Advanced glycation end products (AGEs) are a chemically heterogeneous group of compounds formed in the Maillard reaction when reducing sugars react nonenzymatically with amine residues on protein, lipids, and nucleic acids [1]. AGEs accumulate in the vessel

wall, where they may perturb vascular cell structure and function. AGEs have been identified as a significant pathological factor in the development of many age-related diseases such as atherosclerosis, myocardial infarction, diabetes mellitus, and Alzheimer's disease [1–3]. AGEs have been shown to trigger inflammatory responses and signaling pathways within cells and vascular tissue *via* interaction with the receptor for AGE (RAGE) [4].

RAGE, a member of the immunoglobulin superfamily of cell surface molecules, interacts with a broad range of ligands, including AGEs, amyloid- β peptide, amphoterin, and S100 protein [5, 6]. RAGE is located in the major histocompatibility complex locus on chromosome 6, which contains a multitude of overlapping and duplicated genes involved predominantly in inflammatory and immune responses [7]. It has been reported that the ligand-RAGE interaction evokes oxidative stress generation and elicits vascular inflammation in the blood vessel wall [8, 9].

We have previously shown that AGE and RAGE are highly expressed in the infiltrating macrophages of human aneurysm tissues compared with normal human aorta tissues. RAGE knockout mice are resistant to aneurysm formation, suggesting that AGE signaling may be integral to aneurysm formation [10]. Moreover, our *in vitro* study demonstrated a novel function of AGE, i.e., to induce MMP-9 expression in macrophages [10]. RAGE knockout mice showed deficient MMP-9 activity in an angiotensin II-mediated model of abdominal aortic aneurysm [10].

Interestingly, it has been reported that overexpression of transforming growth factor- β 1 (TGF- β 1) has the ability to stabilize preformed aortic aneurysms and reduce expression of MMP-9 [11]. TGF- β 1 has also been shown to suppress MMP-9 expression induced by tumor necrosis factor- α in monocytes [12]. However, in meningeal cells, TGF- β 1 stimulates the expression of MMP-9, a function that may in part be responsible for tumor metastasis [13]. However, the mechanism by which TGF- β 1 interacts with AGE signaling in the regulation of MMP-9 expression is unknown.

In this study, we explored the signaling mechanism through which AGE activates MMP-9 expression in macrophages. Moreover, we tested the interaction between TGF- β and AGE pathways in the regulation of MMP-9.

MATERIALS AND METHODS

Reagents

Human Glycated Albumin was purchased from Sigma Aldrich (St. Louis, MO). Chemical inhibitors for ERK 1 and 2 MAP kinase (UO126), p38 MAP kinase (SB203580), JNK MAP kinase (SP600125), PI3K (LY294002 and wortmannin) were obtained from Calbiochem (San Diego, CA). The NF- κ B chemical inhibitor phenylarsine oxide (PAO) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). All experiments were done *in vitro via* cell cultures and incubated at 37 °C with 5% CO₂-95% room air. RAW 264.7 macrophage cells were maintained in high glucose DMEM media with 10% FBS, and 100 units/mL of streptomycin and penicillin. All treatments with AGE and chemical inhibitors were carried out in high glucose DMEM media with 1% FBS.

Isolation of Peritoneal Macrophages and Bone Marrow

Mice were injected intraperitoneally with 2 mL of 4% (wt/vol) thioglycollate. Three days later, mice were sacrificed, and peritoneal cells were harvested by washing with ice-cold phosphate-buffered saline. After centrifugation, cells were resuspended in RPMI medium containing 5% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin and seeded into six-well plates. After 2 h, nonadherent cells were removed by washing with warm phosphate-buffered saline. The cells were then incubated in 2 mL of culture medium at 37 °C in a humidified 5% CO₂ incubator. Isolation of mice bone marrow macrophages was performed as previously described [14].

Gelatin Zymography

To analyze MMP-9 enzyme expression, treated cell supernatants were collected after 24 h and separated throughout 10% zymography gels. After electrophoresis, SDS was removed by washing the gels three times with buffer (50 mm Tris/HCl, pH 7.6, 150 mm NaCl, 5 mm CaCl₂, 2 μ M ZnCl₂, 0.1%, Triton X-100) for 30 min at room temperature with gentle agitation to renature enzymes. The gels were subsequently incubated in zymogen development buffer at 37 °C for 16 to 24 h. After briefly washing in water, gels were stained with Coomassie blue R-250 for 1 h. Gels were destained with 40% methanol and 5% acetic acid until clear white bands against a blue background were visible.

Western Blotting Analysis

Treated raw 264.7 cells were lysed and collected at specific time intervals. Samples consisting of 30 μ g of protein content and 25 μ L of a buffer mixture were run through a sodium dodecyl sulfate polyacrylamide (SDS-PAGE) based gel using electrophoresis as previously described [14]. Gels were then transferred to a nitrocellulose membrane *via* electroblotting. After gels were transferred, membranes were treated with 5% nonfat dry milk to prevent nonspecific antibody binding. The ERK MAP kinase, JNK MAP kinase, and p38 MAP kinase antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA). The NF- κ B subunit p65 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The membranes were treated with specific antibodies (diluted 1:1000 with 1X TBS-T buffer) and incubated overnight at 4 °C. After incubation, the membranes were washed three times for five min with 1X TBS-T buffer and then treated with the appropriate secondary antibody (diluted 1:10,000 with 1X TBS-T buffer) in order to detect the membrane bound first antibody. Protein signals were detected by treating membranes with enhanced chemiluminescence (ECL) Western blot reagents.

Statistical Analysis

Data are expressed as means $\pm$ SE. The data are presented as x-fold induction compared with control conditions. Statistical analysis was performed using a paired two-sided Student's t-test. Values of $P < 0.05$ were considered significant.

RESULTS

AGE Induces MMP-9 in Macrophages

We have recently shown that AGE induces MMP-9 expression in the RAW 264.7 macrophage cell line. To further confirm this novel finding, we tested AGE in mouse peritoneal macrophages and bone marrow macrophages. As shown in Figure. 1, AGE significantly increased MMP-9 production in macrophages isolated from these two different sources.

AGE Activates MAPK and PI3K Signaling Pathway

To understand the signaling mechanism underlying MMP-9 induction, we examined several signaling proteins that are known to be activated by AGE in other systems. Figure 2 demonstrates that all three MAP kinases, ERK, JNK, and p38, became rapidly activated upon AGE stimulation, evident by sustained phosphorylation that peaked between 30 min and 1 h. In contrast, the PI3K pathway became activated at much later time points, reflected by a phosphorylation of Akt that peaked between 4 to 8 h after treatment with AGE (Fig. 2). AGE did not alter protein levels of MAP kinases or Akt.

To delineate the signaling pathways that mediate AGE induced-MMP-9, we blocked each of these pathways with specific inhibitors: ERK1/2 by UO126 (10 μ M), JNK by SP600125 (10 μ M), p38 MAPK by SB203580 (10 μ M), and PI3K by LY294002 (10 μ M) or wortmannin (1 μ M). UO126 completely eliminated the ability of AGE to induce MMP-9 (Fig. 3A and B), which is mirrored by its profound effect on ERK phosphorylation/ activation (Fig. 3C). Inhibition of the p38 pathway also resulted in a significant reduction in MMP-9 expression, although to a lesser degree compared with ERK inhibition (Fig. 3A and B). In contrast, inhibition of PI3K pathway by LY294002 or wortmannin did not significantly change AGE-induced MMP-9 expression (Fig. 3A and B). Surprisingly, pretreatment of macrophages with the JNK inhibitor SP60125 significantly enhanced the MMP-9 induction (Fig. 2A and B). The inhibitory effect of SP60125 on JNK activation was confirmed by the inhibition of AGE-induced JNK phosphorylation (Fig. 3D). Taken together, these results indicate that AGE stimulates the expression of MMP-9 in an ERK1/2 and p38 MAPK-dependent mechanism.

AGE Induces Activation of NF- κ B

Next, we moved on to determine transcription factors that may be activated by AGE-RAGE-MAP kinase signaling. Since an NF- κ B binding site within the MMP-9 promoter is known to regulate MMP-9 gene expression, we hypothesized that AGE stimulates MMP-9 gene expression through activation of the NF- κ B pathway. We examined the activation of NF- κ B by evaluating its p65 subunit. Compared with control cells, macrophages that were treated with AGE for 4 h showed a markedly elevated level of p65 protein, an indication of NF- κ B activation (Fig. 4B). Next, we employed phenylarsine oxide (PAO), a protease inhibitor that blocks NF- κ B activation [15]. Treatment with PAO completely blocked AGE-induced MMP-9 activation (Fig. 4B).

TGF- β Suppresses AGE-Induced MMP-9

Next, we investigated whether TGF- β 1 could suppress the ability of AGE to induce MMP-9 expression in macrophage cells. Gelatin zymography revealed that TGF- β 1, at concentrations ranging from 0.1 to 5 ng/mL, profoundly diminished AGE-induced MMP-9 expression (Fig. 5A).

To understand how TGF- β 1 antagonizes AGE's regulation of MMP-9, we tested AGE-induced activation of NF- κ B in the presence of TGF- β 1. Addition of TGF- β 1 significantly suppressed AGE's ability to activate NF- κ B, as evidenced by diminished p65 level (Fig. 5B). In contrast, TGF- β 1 did not affect AGE's ability to activate ERK or JNK (Fig. 5C).

DISCUSSION

Increased expression of MMPs is a molecular characteristic of human aneurysmal tissues as well as advanced atherosclerotic plaques [16–19]. Animal studies demonstrated that upregulated MMPs contribute to pathogenesis of aneurysm or plaque vulnerability by causing degradation of extracellular matrix proteins [20, 21]. Our recent study has linked

MMP-9 expression to AGE and its receptor RAGE; both of which are elevated in aneurysm and atherosclerosis [10, 22]. Here, we demonstrated that AGE induces MMP-9 through an intracellular signaling pathway that is mediated by MAP kinases, but not PI3K.

The interaction of RAGE with AGE or other ligands triggers several signal transduction pathways, which in turn leads to the many pathological functions [23]. We found that AGE activates multiple signaling proteins in macrophages, including ERK, p38 MAPK, JNK, and PI3K/Akt. Activation kinetics differ amongst these mediators. Upon administration of AGE, all three MAP kinases became phosphorylated within 15 min. In contrast, the activation of PI3K was significantly delayed (by 4 h). ERK activation was not only most profound but also sustained for the longest time. The importance of ERK was demonstrated by the ability of ERK inhibitor to profoundly block MMP induction.

Our finding on ERK is consistent with the literature. Using the same macrophage cell line, other investigative groups have shown that ERK is a critical intracellular mediator of MMP-9 expression induced by matrix fragments [24–26]. More recently, Zhang *et al.* demonstrated that inhibition of ERK with the same inhibitor used in our study (UO126) or CI1040 significantly blocked aneurysm formation induced by angiotensin II (AngII) [27]. UO126 not only reversed AngII-stimulated tube formation by human umbilical vein endothelial cells (HUVECs), but also reversed the effect of AngII on MMP-2 secretion by HUVECs. Thus, ERK may contribute to aneurysm formation *via* stimulation of MMPs [27].

Although we found that AGE led to JNK activation, inhibition of this particular MAP kinase stimulated rather than inhibited MMP-9 expression. A similar observation has recently been reported by Lee and colleagues. These authors showed that SP600125 suppressed LPS-induced TNF- α and IL-6, but enhanced LPS induced MMP-9 expression [28]. Of note, the same JNK inhibitor (SP600125) was reported by Yoshimura *et al.* to inhibit aneurysm formation and to block TNF- α induced MMP-9 *in vitro* and *in vivo* [29, 30]. The precise reason for this discrepancy is not clear, however, Yoshimura *et al.* used human AAA walls in *ex vivo* culture with 50 μ M of SP600125 [31], while Lee *et al.* and our current study used RAW cells with 10 μ M of SP600125. The different results of SP600125 may be explained by the variation of experimental model and dosage of inhibitor.

The slow kinetics of PI3K activation suggests that this pathway is not essential for AGE's function in MMP-9. Indeed, neither wortmannin nor LY294002 affected AGE's ability to up-regulate MMP-9.

Our data suggest that TGF- β 1 is a potent inhibitor of MMP-9 expression in macrophages. This finding is consistent with other reports showing that TGF- β downregulates the stimulatory effects of TNF- α on MMP-9 gene expression and protein secretion [12]. However, the effect of TGF- β on MMP-9 expression may be cell- or tissue-dependent. In contrast to its inhibitory function in human lung fibroblasts and myometrical smooth muscle cells [32, 33] and macrophages (current study), TGF- β stimulates MMP-9 expression in human meningeal cells, skin fibroblasts, keratinocytes, and oral tumor cells [13, 34–36]. AAA wall infiltration has been found to be dominated by monocyte-macrophages [37], and macrophages are a major primary source of MMP-9 expression [10, 17]. Thus, the TGF- β -suppressing effect on MMP-9 in macrophages may play an important role in the prevention or stabilization of AAA formation [11].

Since AGEs are a group of compounds with heterogenous and homogenous structures, there is currently no universally established method to measure AGE concentrations in patients [38]. In a previous study, we demonstrated that the level of AGE is significantly elevated in aortic tissues of aneurysm patients using an antibody specific to carboxymethyl lysine (CML)-AGE [39]. However, we do not know the absolute concentration of AGE in these

aneurysmal samples. Consequently, it is difficult to know whether the concentration of AGE used in the current study is clinically relevant. Furthermore, the current study was performed in cultured cells. While the intracellular signaling pathways delineated from these *in vitro* studies are of importance to our understanding of AGE signaling as well as MMP-9 regulation, the deduced molecular interactions need to be further explored in animal experiments and clinical studies.

Abdominal aortic aneurysms are a common clinical problem that is in great need of pharmacologic treatments. MMP-9 has been implicated in the pathogenesis of AAAs [16], and interventions that block MMP-9 are particularly effective in suppressing aortic wall connective tissue degradation as well as improving the proteolytic balance in AAAs [40, 41]. Our data provides a new understanding of the important role of AGE and TGF- β in this process. The data also suggest AGE or ERK as potential new therapeutic targets in the prevention of AAA development and progression. However, it is necessary to emphasize the multifactorial nature of human aneurysm. It is possible that the ultimate pharmacologic treatment for aneurysm is a therapeutic strategy that targets multiple key pathways.

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FIG. 1.

Effects of AGE on MMP-9 activity in primary macrophages and bone marrow macrophages. (A) Mouse peritoneal macrophages were treated with control (300 $\mu\text{g/mL}$ albumin) or 100–300 $\mu\text{g/mL}$ of AGE for 24 h. (B) Mouse bone marrow was treated with control (300 $\mu\text{g/mL}$ of albumin) or 300 $\mu\text{g/mL}$ of AGE for 24 h. Supernatant was collected and assessed for MMP-9 and MMP-2 activity by gelatin zymography. (Color version of figure is available online.)

FIG. 2.

AGE activates MAPK and PI3K in macrophages. RAW 264.7 cells were treated with 300 μg/mL of AGE or with control for the indicated time periods. Cell lysates were prepared and analyzed by immunoblotting with phospho-specific and pan-antibodies against ERK (p44/42), p38, JNK, and Akt.

FIG. 3.

Effects of MAPK inhibitors or PI3K inhibitors on AGE-induced MMP-9. (A) Raw 264.7 cells were pre-incubated with ERK1/2 specific inhibitor UO126 (10 μ m), p38 MAPK inhibitor SB203580 (10 μ m), JNK inhibitor SP600125 (10 μ m), phosphatidylinositol-3 kinase inhibitor LY 294002 (10 μ m), or wortmannin (1 μ M) for 30 min; the cells were then treated with AGE (300 μ g/ mL) for 24 h. Supernatant was collected and assessed for MMP-9 and MMP-2 activity by gelatin zymography. (B) Relative MMP-9 levels were quantified by using the ImageJ program. Results represent mean $\pm$ SE of at least three separate experiments. * and # P < 0.05 compared with AGE treatment. (C) Raw 264.7 cells were pretreated with UO126 for 30 min, the cells were then treated with AGE for 1 h, and the cell lysates were analyzed by immunoblot using antibodies to ERK and phospho-ERK as

indicated. (D) Raw 264.7 cells were pretreated with SP600125 for 30 min, the cells were then treated with AGE for 1 h, and the cell lysates were analyzed by immunoblot using antibodies to JNK/SAP and phospho-JNK/SAP as indicated. (Color version of figure is available online.)

FIG. 4.

Effect of AGE on NF- κ B activation. (A) Raw 264.7 cells were treated with AGE for 2 or 4 h. Cell lysates were analyzed by immunoblot using anti-p65 antibody and β -actin antibody. (B) Raw 264.7 cells were preincubated with phenylarsine oxide (PAO) for 30 min, then the cells were treated with AGE for 24 h. Supernatant was collected and assessed for MMP-9 and MMP-2 activity by gelatin zymography. (Color version of figure is available online.)

FIG. 5. AGE induced MMP-9 activity is inhibited by TGF- β . (A) RAW 264.7 cells were pretreated with TGF- β 1 from 0.1 to 5 ng/mL for 20 min, followed by AGE (300 μ g/mL) for 24 h. Supernatant was collected and assessed for MMP-9 and MMP-2 activity by gelatin zymography. (B) Raw 264.7 cells were preincubated with TGF- β 1 (0.1–5 ng.mL) for 30 min, then the cells were treated with or without AGE (300 μ g/mL) for 24 h. Supernatant was collected and assessed for MMP-9 and MMP-2 activity by gelatin zymography. (C) Raw 264.7 cells were preincubated with TGF- β 1 for 30 min, then the cells were treated with AGE for 1 h, and the cell lysates were analyzed by immunoblot using anti-phospho-ERK or phospho-JNK antibodies. (Color version of figure is available online.)