Effects of Advanced Glycation End-products on the Proliferation and Fibronectin Production of Smooth Muscle Cells

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The aim of this study was to evaluate the effects of advanced glycation end-products (AGEs) on the proliferative activity and fibronectin production of smooth muscle cells (SMCs). AGE-bovine serum albumin (AGE-BSA) was prepared by incubation with D-glucose at 37°C for 60 days. Cultured SMCs were obtained from explants isolated from porcine abdominal aorta and used between passages 3 and 10. The proliferative activity of SMCs was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay and by incorporation of 3H-thymidine into DNA. Fibronectin production was assessed by competitive ELISA assay for both fibronectin secreted into the culture medium (M-FN) and cell-associated fibronectin (C-FN), i.e., both intra- and peri-cellular fibronectin. The MTT assay revealed that AGE-BSA did not produce any change in optical density (A570) of SMCs at concentrations of up to 20 µg/ml, but decreased that of SMCs at a concentration of 40 µg/ml. The addition of PDGF (5 ng/ml) induced an increase in 3H-thymidine incorporation into DNA of quiescent SMCs, while the addition of AGE-BSA (20 µg/ml) had no effect. In contrast, AGE-BSA significantly increased C-FN of SMCs (30.8±8.58 ng/µg TP), compared to unmodified BSA (16.5±4.19 ng/µg TP). However, no difference in M-FN levels was observed between cells treated with AGE-BSA and unmodified BSA. The addition of anti-transforming growth factor (TGF) -β antibody restored the levels of C-FN in SMCs cultured in 20 µg/ml of AGE-BSA, suggesting that TGF-β might act as an intermediate factor in AGE-induced fibronectin production by SMCs. Our results suggest that interaction of AGE-modified proteins with SMCs may play a role in the development of atherosclerosis in diabetic and non-diabetic patients.

Key words: Advanced glycation end products (AGEs), Fibronectin, Proliferation, Smooth muscle cells (SMCs)

Introduction

Advanced glycation end-products (AGEs), which result from non-enzymatic glycation of proteins, are thought to contribute to the development of diabetic complications, including macro- and micro-angiopathies (1, 2). However, the interaction of glucose with proteins in vivo is not restricted to diabetic patients (3-5). We have shown that AGE-modified collagen increased in the aorta of non-diabetic subjects with age (6). A recent study in individuals suffering from hyperlipidemia showed that glycated LDL was susceptible to oxidation and increased complex formation with matrix proteins (7). Thus, non-enzymatic glycation of proteins, including collagen and LDL, may play a role in the development of human atherosclerotic lesions. However, the detailed mechanism by which AGEs accelerate atherosclerosis has not been elucidated.

Recent studies have focused on the ligand activity of
AGEs in various cell types, such as macrophages, mesangial cells, endothelial cells and fibroblasts (8, 9). Vlassara (10) indicated that specific binding of AGE-modified proteins to smooth muscle cells (SMCs) might be associated with increased cellular proliferation, and mediated by growth factors, including platelet-derived growth factor (PDGF). Conversely, AGE-modified protein has been shown to fail to induce the growth of rabbit smooth muscle cells (11). In this study, we investigated whether AGE-modified bovine serum albumin (AGE-BSA) promoted proliferation of and fibronectin (FN) production by cultured SMCs, and whether transforming growth factor-β (TGF-β) contributed to the interaction between AGE-modified protein and SMCs.

Materials and Methods

Cell culture

Cultured SMCs were obtained from explants isolated from porcine abdominal aorta and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS, Sanko Junyaku Co., LTD, Funabashi, Japan), as previously described (12). The following experiments were performed using SMCs between passages 3 and 10.

Preparation of AGE-BSA

AGE-BSA was prepared according to the method of Horiuchi et al. (13). Briefly, 1.6 g bovine serum albumin (BSA) (Wako Junyaku Co., Osaka, Japan) was dissolved with 3.0 g D-glucose (Wako Junyaku Co.) in 10 ml 0.3 M sodium phosphate buffer (pH 7.4). This solution was sterilized by Millipore filtration (pore size: 0.45 μm) and then incubated at 37°C for 60 days. Unmodified BSA (unmod-BSA) was prepared by incubation in 0.3 M sodium phosphate buffer without any D-glucose. After incubation, each sample was dialyzed against 10 mM sodium phosphate-buffered saline (PBS) to remove any excess free glucose. To verify the formation of AGEs, the AGE content of each solution was determined by competitive enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody (6D12) against N-(carboxymethyl)lysine (CML), one of the major AGE chemical structures (13). Monoclonal anti-CML antibody was a generous gift from Prof. S. Horiuchi (Department of Biochemistry, Kumamoto University Medical School). The Maillard reaction-related fluorescence of each solution was measured with a spectrophotofluorometer (Shimazu Co., Kyoto, Japan) at an excitation wavelength of 370 nm and an emission wavelength of 440 nm (14). PBS was used as background fluorescence. Protein content was measured using a BCA protein assay kit (Pierce, Rockford, IL).

MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay

To examine the proliferative and cytotoxic effects of AGEs on SMCs, an MTT assay was performed using a commercially available colorimetric assay kit (Chemicon International, Inc., Temecula, CA). Briefly, cells were seeded on 12-well multiplates (Falcon, Franklin Lakes, NY) at 2 x 10^4 cells per well in medium containing 10% FCS, and cultured for four days. The medium was changed every other day. The cells were washed three times with Dulbecco’s PBS (Nissui, Tokyo) and then maintained in fresh medium containing 2% plasma-derived serum (PDS) for 48 hours to arrest cell growth. After washing twice with PBS, the cells were incubated in experimental medium supplemented with 2% PDS and various concentrations of AGE-BSA or unmod-BSA. After two or four days, SMCs were harvested by trypsinization and suspended in medium supplemented with 10% FCS. A 0.1 ml aliquot of the cell suspension was added to each well of a 96-well flat-bottomed tray (Falcon), MTT solution (0.01 ml, 5 mg/ml) was added to each well, and the tray was then incubated at 37°C for three hours. An isopropanol/HCl solution (0.01 ml) was added and the absorbance at 570 nm was read on a micro-ELISA plate reader (Titertek Multiskan MCC, Flow Laboratories, Inc., USA). Results were expressed as mean optical density (A_570) in duplicate.

Assessment of DNA synthesis

To examine the effect of AGEs on DNA synthesis in SMCs, SMCs were exposed to porcine PDGF (R & D Systems, Inc., Minneapolis, MN), AGE-BSA and unmod-BSA for 48 hours. Cells were seeded at a density of 2 x 10^4 cells per well and then cultured in medium with 10% FCS. After three days, the cells were washed three times with PBS and incubated in DMEM containing 2% PDS for 48 hours to arrest cell growth. To reinitiate DNA synthesis, quiescent cells were stimulated with fresh medium containing 2% PDS, 5 ng/ml PDGF, 20 μg/ml AGE-BSA or 20 μg/ml unmod-BSA. To examine changes in the incorporation rate of ^3H-thymidine, the cells were pulse-labeled with 2 μCi/ml of ^3H-thymidine (New England Nuclear, Boston, MA) for two hours at 0, 8, 24, 32 and 48 hours after reinitiation. The medium was discarded, and the cells were washed three times with cold PBS and then three times with cold 5% trichloroacetic acid (TCA), after which they were incubated with 5% TCA at 4°C for 15 min. The cells were washed twice more with cold 5% TCA, and the resulting TCA-insoluble material was dissolved with 1 ml of 0.2 N NaOH at room temperature for 30 min. Cell lysates were added to 6 ml of a scintillator (ACS II, Amersham, Canada) and the radioactivity was determined with a liquid scintillation counter (TRI-CARB 300C(D)/460C(D) Packard). Other aliquots of the cell lysates were subjected to protein determination by a protein assay kit (Pierce). To further examine changes in the amount of ^3H-thymidine incorporation after reinitiation, quiescent SMCs were incubated with 2% PDS, 5 ng/ml PDGF, 20 μg/ml AGE-BSA or 20 μg/ml unmod-BSA, together with...
Fibronectin production by SMCs

SMCs were plated in 6-well multiplates at a density of 5 × 10⁵ cells per well. After 48 hours, the cells were washed three times with PBS and incubated in fresh medium containing 0.5% FCS for two days to arrest cell growth. After three washes with PBS, cells were incubated with medium containing various concentrations of AGE-BSA or unmod-BSA for three days. The medium was removed from the wells and immediately centrifuged for 20 min at 10,000 × g to remove cellular debris. The supernatants were stored at −80 °C until assay for C-FN. The FN concentration in all samples was determined in duplicate with a competitive ELISA plate reader. The amount of M-FN was calculated using a protein assay kit.

Statistical analysis

Data were expressed as mean ± SD. One-way ANOVA and Bonferroni’s multiple comparison test were used to determine the statistical significance of differences among more than three groups. Differences between two groups were analyzed with the unpaired t-test. A p value of less than 0.05 was considered statistically significant.

Results

Characterization of AGE-BSA

The intensity of the Maillard reaction-related fluorescence (units/mg) of samples was expressed as their fluorescence subtracted from background fluorescence. AGE-BSA solution (1.351.6 units/mg) exhibited a significantly higher fluorescence than unmod-BSA solution (1.71 units/mg) or freshly prepared BSA solution (0.69 units/mg). Figure 1 shows the inhibition curves of AGE-BSA, unmod-BSA, and freshly prepared BSA by a competitive ELISA assay using monoclonal anti-CML antibody. The monoclonal anti-CML antibody reacted with AGE-BSA, but not with freshly prepared BSA. Unmod-BSA reacted weakly with monoclonal anti-CML antibody. However, the mean CML content of AGE-BSA solution was 66-fold greater than that of unmod-BSA solution.

MTT assay

Figure 2 shows the mean values of optical density (A570) of SMCs at two and four days after incubation with various concentrations of AGE-BSA or unmod-BSA. No difference in the optical density of SMCs was observed between AGE-BSA and unmod-BSA at two days. At four days, the optical density of SMCs in AGE-BSA was similar to that in unmod-BSA at concentrations of up to 20 μg/ml. However, 40 μg/ml of AGE-BSA induced a significant decrease in the mean optical density of SMCs compared with 40 μg/ml of unmod-BSA or less than 20 μg/ml AGE-BSA, indicating that incubation with AGE-
BSA at a concentration 40 μg/ml for four days was apparently cytotoxic to SMCs. Thus, the following experiment was performed using a concentration of 20 μg/ml of AGE-BSA and unmod-BSA.

Incorporation of ³H-thymidine into SMCs

Figure 3A shows the serial changes in the incorporation rate of ³H-thymidine after the addition of AGE-BSA (20 μg/ml), unmod-BSA (20 μg/ml) or PDGF (5 ng/ml). SMCs incubated with 2% PDS for 48 hours incorporated little ³H-thymidine, suggesting that the cells were growth-arrested (Fig. 3, 0 hr). The addition of PDGF resulted in a marked increase in the incorporation rate of ³H-thymidine of SMCs, but subsequently (after 48 hours) returned to the resting state. In contrast, AGE-BSA and unmod-BSA had no effect on the incorporation rate of ³H-thymidine of SMCs. The time course of ³H-thymidine incorporation into SMCs is shown in Fig. 3B. PDGF significantly increased ³H-thymidine incorporation into SMCs compared with AGE-BSA and unmod-BSA. However, no difference in ³H-thymidine incorporation into SMCs was observed between AGE-BSA, unmod-BSA and PDS.

Effects of AGE-BSA on fibronectin production by SMCs

Levels of M-FN and C-FN were determined in SMCs cultured in 5, 10, 20 or 40 μg/ml of AGE-BSA or unmod-BSA for three days (Fig. 4). Cells cultured in more than 20 μg/ml AGE-BSA exhibited significantly greater C-FN levels than those cultured in AGE-BSA of less than 10 μg/ml. The mean values of C-FN were 10.0 ± 2.47,
AGEs and SMC Proliferation and FN Production

A

B

Fig. 3. Serial changes in the rate (A) and amount (B) of ^3H-thymidine incorporation into SMCs incubated with 2% plasma-derived serum, 5 ng/ml platelet-derived growth factor, 20 μg/ml AGE-BSA or unmod-BSA. A: SMCs were pulse-labeled with 2 μCi/ml ^3H-thymidine for two hours at the indicated time intervals after reinitiating DNA synthesis. B: The amount of ^3H-thymidine incorporated into SMCs was determined at the indicated time intervals after reinitiating DNA synthesis. Each symbol represents the mean±SD (n=6). DPM: decay per minute, *p < 0.01; versus PDS, AGE-BSA and unmod-BSA by Bonferroni’s multiple comparison test.

Fig. 4. Effects of various concentrations of AGE-BSA (open circles) and unmodified BSA (solid circles) on fibronectin production by SMCs. The levels of cell-associated fibronectin (C-FN) and tissue culture-supernatant fibronectin (M-FN) were determined by a competitive ELISA system. The values show ng fibronectin per μg total protein in the cell lysate or culture medium (ng/μg TP). Each symbol represents the mean±SD (n=6). a: p < 0.01; versus AGE-BSA of less than 10 μg/ml by Bonferroni’s multiple comparison test, versus 20 μg/ml of unmod-BSA by unpaired t-test. b: p < 0.01; versus AGE-BSA of less than 10 μg/ml by Bonferroni’s multiple comparison test. c: p < 0.01; versus AGE-BSA of less than 20 μg/ml by Bonferroni’s multiple comparison test. d: p < 0.05; 0 μg/ml of unmod-BSA by Bonferroni’s multiple comparison test.

12.0±3.63, 30.8±8.58 and 26.2±8.17 ng/μg TP at 5, 10, 20 and 40 μg/ml of AGE-BSA, respectively. Cells incubated with up to 20 μg/ml of unmod-BSA exhibited no change in their C-FN levels, the mean values of which were 14.4±3.55, 16.3±3.34, 16.5±4.19 and 26.0±9.50 ng/μg TP at 5, 10, 20 and 40 μg/ml, respectively. Analysis of data showed a significant difference in the C-FN level between AGE-BSA and unmod-BSA at a concentration of 20 μg/ml. However, no difference in C-FN levels was found between AGE-BSA and unmod-BSA at a concentration of 40 μg/ml. M-FN levels did not change in cells cultured in AGE-BSA or unmod-BSA at concentrations of up to 20 μg/ml. Although it was increased in both AGE-BSA and unmod-BSA at 40 μg/ml, no significant difference was observed between them. Mean M-FN levels were 3.13±0.45, 4.53±2.00, 5.47±1.37 and 10.7±2.63 ng/μg TP at 5, 10, 20 and 40 μg/ml of AGE-BSA, respectively. Mean M-FN levels were 5.41±1.41, 4.59±1.18, 4.68±1.67 and 7.32±2.16 ng/μg TP at 5, 10, 20 and 40 μg/ml of unmod-BSA, respectively.
Effect of anti-TGF-β antibody on fibronectin production by SMCs

To determine whether TGF-β contributes to the increase in C-FN production by SMCs cultured in AGE-BSA, the effect of anti-TGF-β antibody on C-FN production by SMCs was examined. As shown in Fig. 5, the addition of antibody blunted the increase in C-FN levels of SMCs cultured in AGE-BSA for three days. The mean values of C-FN were 35.8±12.5 and 15.5±8.54 ng/µg TP in AGE-BSA and AGE-BSA plus anti-TGF-β antibody, respectively.

Discussion

Recent in vivo studies (6, 15, 16) have reported the presence of AGE-protein adducts in atherosclerotic lesions and diabetic glomerulosclerosis. AGEs possess ligand activity for several specific surface receptors on various cells, which modulate various cellular functions, including migration and proliferation (8, 9). Yui et al. (9) showed that the growth of murine macrophages was induced by AGE-modified proteins. Conversely, AGE-modified matrix has been shown to inhibit the proliferative activity of cultured mesangial cells (17, 18). The effect of AGE-modified proteins on the proliferative activity of SMCs remains controversial (10, 11). Since atherosclerotic lesions are caused by proliferation of vascular SMCs, we were interested in the effect of AGE-modified proteins on the proliferative activity of SMCs. In this study, MTT analysis showed that the optical density (A570) of SMCs was similar between AGE-BSA and unmod-BSA at concentrations of up to 20 µg/ml. SMCs incubated with 40 µg/ml of AGE-BSA exhibited significantly lower optical density than those incubated with unmod-BSA. The addition of PDGF (5 µg/ml) induced an increased ³H-thymidine incorporation into DNA of quiescent SMCs, but 20 µg/ml of AGE-BSA did not. These results indicate that AGEs may not stimulate the proliferative activity of SMCs. However, diabetes is well known to accelerate atherosclerotic lesions. One possible explanation that could account for this discrepancy between the in vitro and in vivo findings is that the AGE-modified proteins do not interact directly with SMCs to mediate SMC proliferation. AGE-modified proteins may stimulate SMC proliferation through various cytokines and growth factors, including cachectin/TNF, IL-1β, and PDGF released from macrophages (19-21).

Several in vivo studies (22, 23) have shown that fibronectin accumulates in the connective tissue of the aorta and glomeruli in diabetes mellitus. AGEs have been shown to enhance synthesis of extracellular matrix components of mesangial cells, including fibronectin, laminin, and type IV collagen (18, 24). In this study, cell-associated fibronectin of SMCs was significantly greater in AGE-BSA than in unmod-BSA, in concordance with results of those previous studies (18). The aorta from diabetic patients has been shown to contain an increased fibronectin content, with both saline-soluble and saline-insoluble components (22). The former is thought to be derived from plasma fibronectin, and the latter from locally-synthesized fibronectin. Thus, AGE-modified proteins may contribute to increased accumulation of fibronectin in the artery through upregulation of fibronectin synthesis by SMCs. In this study, the amount of cell-associated fibronectin of SMCs was significantly different between AGE-BSA and unmod-BSA at a concentration of 20 µg/ml, but not at a concentration of 40 µg/ml. The MTT assay revealed a significantly decreased optical density of SMCs incubated with AGE-BSA at a concentration of 40 µg/ml compared with that of SMCs incubated with unmod-BSA. AGEs may have a cytotoxic effect on SMCs, which may result in decreased cell-associated fibronectin of SMCs.

The mechanism by which AGEs stimulate fibronectin synthesis by cells is not fully understood. Several reports have described overexpression of TGF-β in glomeruli and arteries of diabetes (26, 27). A recent study (28) demonstrated that AGE stimulation caused overexpression of TGF-β mRNA and increased secretion of TGF-β by mesangial cells. Thus, TGF-β is a likely candidate for mediating increased production of fibronectin by SMCs in this in vitro model. The present study revealed that PDGF but not AGE-BSA stimulated SMC proliferation.
AGE-BSA significantly increased the level of cell-associated fibronectin in SMCs compared to unmod-BSA. Finally, the addition of anti-TGF-β antibody restored the level of cell-associated fibronectin in SMCs incubated with AGE-BSA. Thus, TGF-β may mediate increased SMC fibronectin synthesis induced by AGE-BSA, which would be consistent with the previous report (28). Interestingly, in this study, addition of anti-TGF-β antibody was inhibitory on increased SMC fibronectin production in AGE-BSA after 72 hours, but not after 48 hours (data not shown). Various cells have been shown to synthesize and release TGF-β as a biologically inactive molecule (29). Activation of latent TGF-β occurs in vitro by extremes of pH, heat and plasmin (29, 30). This study could not identify mediators that activate AGE-BSA-induced TGF-β. The mechanism by which AGEs enhance fibronectin production via activation of TGF-β has yet to be elucidated.

The biological role of increased SMC fibronectin production due to AGE-modified proteins in atherogenesis cannot be elucidated from this study. Fibronectin is known to be a pericellular matrix glycoprotein, which interacts with cells and other matrix components. There have been reports (31, 32) that fibronectin accumulates in early lesions induced by mechanical injuries or atherogenic diets. Recently, SMCs obtained from hypercholesterolemic animals have been shown to have elevated expression of fibronectin mRNA and fibronectin synthesis (33, 34). We previously showed that AGE-adducts increased in the collagen matrix of lesion-free intima in non-diabetic subjects with age (6). Thus, AGE-adducts may play a role in atherogenesis by enhancing fibronectin production by SMCs.

In the present study, we demonstrated that, 1) vascular SMCs cultured in AGE-BSA exhibited no change in proliferative activity, but did show increased fibronectin production, and 2) TGF-β may mediate stimulation of SMC fibronectin production by AGE-BSA. These data suggest that AGEs may play a role in the development of atherosclerosis in both diabetic and non-diabetic patients through increased fibronectin production.

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