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Roles of Advanced Glycation Endproducts（AGE）and Receptor for AGE on Vascular Smooth Muscle Cell Growth

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Abstract

Proliferation of vascular smooth muscle cells（VSMC）represents an essential event in the developement of diabetic atherosclerosis. Previous studies suggest that several cytokines and growth factors mediate the proliferation capability in VSMC from diabetic animals. In addition, advanced glycation end products（AGE）and receptor for AGE（RAGE）are important for pathologic features of diabetic complications. In the present study, we attempted to clarify the roles of AGE and RAGE in the proliferation of VSMC using streptozotocin（STZ）-treated rat sera and aortic SMC prepared from non-diabetic rats. AGE levels increased in the diabetic sera, which enhanced the growth of VSMC in proportion to their diabetic periods. AGE-bovine serum albumin（BSA）prepared *in vitro* also exhibited a stimulatory effect on VSMC growth. The endocytic uptake of AGE and enhanced RAGE expression in VSMC after culture with diabetic sera were observed. In addition, anti-AGE and anti-RAGE antibodies inhibited these stimulatory effects on VSMC growth. These findings suggest that AGE in diabetic rat sera may cause an enhanced effect on VSMC proliferation. However, the concentrations of AGE in diabetic sera were much lower than that of AGE-BSA which demonstrated a significant stimulatory effect on VSMC growth. The magnitude of the VSMC growth-enhancement by the diabetic sera was markedly greater than that by the AGE-BSA solution. In conclusion, the AGE-RAGE interaction in VSMC, in addition to growth factors induced by AGE, contributes to the stimulatory effect of diabetic sera on VSMC proliferation which can accelerate atherosclerosis.
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Key words: streptozotocin, smooth muscle cell, advanced glycation end products（AGE）, receptor for AGE（RAGE）

Introduction

Since diabetic macroangiopathy is rapidly accelerated atherosclerosis with clinical and pathological features of atherosclerosis commonly seen in non-diabetic patients[1,2], proliferation of vascular smooth muscle cells（VSMC）represents an essential event in the developement of diabetic atherosclerosis in addition to various forms of insult to the endothelium[3,4].

Prior to proliferation, migration of VSMC from the media to the intima of the aorta and transition from the contractile to synthetic phenotype occur during the development of atherosclerosis[5,6]. Furthermore, it has been reported that these processes can be simulated in vitro[7]. The growth rates of cultured VSMC

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obtained from diabetic patients[8] or diabetic animals[9–11] are higher than non-diabetic controls. Several studies have suggested that the increase in proliferation capability in VSMC from diabetic creatures is related to several cytokines and growth factors including interleukin-1 β[12], platelet derived growth factor (PDGF)[10], basic fibroblast growth factor (bFGF)[13] and heparin binding epidermal growth factor like-growth factor (HB-EGF)[14]. However, the process in which VSMC gains the enhanced ability of proliferation is still not completely clarified.

Recent studies have suggested that the central pathologic features of diabetic complications are correlated with the formation of advanced glycation end products (AGE), the ultimate result of non-enzymatic glycation and oxidation of protein and lipids. Although AGE binds to several cell-surface sites including receptor for AGE (RAGE)[15], galectin-3/p 60/p 90[16] and macrophage scavenger receptor (MSR)[17], the RAGE appears to play a major role to alter cellular functions[15,18]. RAGE has been reported to be expressed by SMC as well as endothelial cells, monocytes/macrophages, mesangial cells and nerve cells[19]. However, the relationship between AGE and VSMC is still poorly understood. In the present study, we evaluated the proliferation capability in VSMC in relation to AGE formation and binding to RAGE, using STZ-treated rat sera and aortic SMC prepared from non-diabetic rats.

Materials and Methods

Preparation of AGE-bovine serum albumin (BSA)

AGE-BSA was prepared by incubating 20% BSA (Sigma, St. Louis, USA) and 1.67 M glucose in 0.5 M phosphate buffer (pH 7.4) for 12 weeks at 37℃. AGE formation was confirmed by measurement of fluorescence at 440 nm of wavelenght. Control non-glycated BSA was obtained by incubation of the same solution in the same manner omitting glucose. Endotoxin levels were checked using a kit for endotoxin test (Limulus J Single Test®, Wako). The AGE-BSA solutions at the concentrations used in this study were confirmed to be endotoxin-free (<2.5 U/m*l* of endotoxin).

Preparation of diabetic sera

In each experimental group, four male Wistar rats at 12, 16, 20 and 24 weeks old were injected with streptozotocin (STZ, Sigma, St. Louis, USA) intraperitoneally (60 mg/kg) to induce hyperglycemia. At 12, 16, 20 and 24 weeks after STZ administration, the animals aged 36 weeks were sacrificed, and blood sera were prepared. Non-diabetic control rats were age matched with the diabetic rats at the time of sacrifice. All rats had free access to standard rat chow and water. This experiment was approved by the Animal Research Ethics Board of Nippon Medical School, Japan.

Assessment of AGE in diabetic sera

AGE levels of rat sera were determined by a non-competitive enzyme-linked immunosorbent assay (ELISA) according to the method of Horiuchi et al[20]. The standard curve was constructed with AGE-BSA (0.01 μg/m*l*～50 μg/m*l*). Each well of a 96-well microtiter plate was coated with 100 μ*l* of samples diluted at 1: 10,000 or 1: 20,000 with 0.05 M carbonate buffer (pH 9.6) overnight at 4℃ and washed three times with 0.01 M Phosphate buffered saline (PBS) containing 0.05% Tween 20 (Buffer A, pH 7.4). The following steps were performed at room temperature. Each well was then blocked non-specific reaction with 200 μ*l* of 2.0% skim milk, 0.5% BSA and 0.5% gelatin in 0.05 M carbonate buffer (pH 9.6) for 1 hour and washed three times with Buffer A. Each well was incubated for 1 hour with 50μ*l* of an anti-AGE monoclonal antibody (dilution = 1: 1,000; 6D12, Wako, Osaka, Japan) diluted with Buffer A containing 0.3% BSA. The wells were then washed three times with Buffer A and incubated with 100 μ*l* of horseradish peroxidase (HRP)-labeled gout anti-mouse IgG antibody (dilution = 1: 1,000; MBL, Nagoya, Japan) for 1 hour. After washing three times with Buffer A, the wells were reacted with 100 μ*l* of 20 μg/m*l* *o*-phenylenediamine (OPD) as a colorimetric substrate (Sumitomo Bakelite, Tokyo, Japan) for 1 hour. The reaction was terminated by addition of 100 μ*l* 1 M sulfuric acid, and then the absorbance at 490 nm was read on a micro-ELISA plate reader (Immuno Mini, Nage Nunc International, Tokyo, Japan).

Culture of VSMC

Vascular smooth muscle cells (VSMC) were isolated by collagenase digestion[6,7,9] from the thoracic aorta of non-diabetic Wistar rats (body weight 250 g) and cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco BRL Eggenstein, Germany) with 10 % fetal calf serum (FCS), penicillin (100 units/m*l*) and amphotericin B (2.5 μg/m*l*) in a humidified atmosphere of 5% CO_2 at 37℃[21]. The cells were passaged every 4～6 days and were used within passages at the fourth generation.

Assay of VSMC growth

Subcultured rat VSMC (first to fourth generation) were removed by 15 min incubation in 0.05% trypsin with 5.3 mM EDTA. VSMC plated on a 24-well plate at a density of 5×10^3 cell per well were cultured for 24 hours in DMEM supplimented with 10% FCS. These cells were maintained for 48 hours in DMEM supplimented with 0.5% FCS to arrest their growth. After 24 hours, the medium was changed and cells were incubated for further 48 hours with fresh DMEM with 0.5% FCS. Thenceforth, the medium was replaced with each test condition medium, a variety of concentrations of rat sera or AGE-BSA in DMEM as described later. The cell growth was evaluated every 48 hours until the 8 th day by 3(4, 5-dimethyl-thiazole-2 yl) 2, 5-diphenyl-tetrazolium bromide (MTT) assay [22]. That is, 200 μ*l* of MTT solution (5 mg/m*l* in PBS) was added to each well, and the cells were incubated for 4 hours at 37℃. Then, the medium was discarded and 100 μ*l* of isopropanol with 0.04 N HCl was added to each well to dissolve the formazan that had been formed. The solutions were transferred to 96-well plates to measure absorbance at 590 nm.

The growth of VSMC measured by MTT assay was preliminarily compared with cell counting. The results of the MTT assay under a variety of rat serum concentrations closely correlated with a hemocytometer (data not shown).

Inhibitory effect of anti-RAGE polyclonal antibody or anti-AGE antibody (6D-12) on enhanced VSMC growth by STZ treated serum or AGE-BSA

VSMC were cultured in 24-well plates in DMEM supplemented with FCS at a plating density of 5×10^3 cells per well similar to other culture experiments. After 24 hours, the medium was changed and cells were incubated for further 48 hours with 0.5% FCS in DMEM. Then, the media were replaced with the indicated concentrations of anti RAGE rabbit serum[23] or non-immunized rabbit serum (Sigma, St. Louis, USA). After culture for 24 hours, the media were changed to 10% diabetic rat serum 24 weeks after STZ treatment to assay cell growth after 72 hours.

VSMC were prepared by the same method as described above. Then, the media were replaced with 1% of an anti-AGE monoclonal antibody (6D12) or 1% of anti-RAGE rabbit serum[23]. After culture for 24 hours, the media was changed to 5 μg/m*l* of AGE-BSA in DMEM to assay cell growth after 96 hours.

Western blotting analysis of AGE and RAGE in cultured VSMC

VSMC were plated in 24-well plates in DMEM supplemented with 10% FCS (final volume: 1 m*l*/well). These cells were then cultured at a plating density of 6×10^5 cells per well. After 24 hours, the medium was changed and cells were incubated for further 48 hours with fresh DMEM with 0.5% FCS. The medium was replaced with 7% and 10% sera after 16 and 24 weeks of the diabetic condition, respectively. After 72 hours, the cells were lysed in lysis buffer for 15 min at 4℃. Proteins (10 μg/m*l* for each sample) extracted from the media and cells were electrophoresed and transferred onto a polyvinylidene difluoride membrane (MILLIPORE, Bedford, MA) for Western blot analysis [24,25]. Similar anti-AGE and anti-RAGE antibodies as used in other experiments were used as the primary antibodies, followed by treatment with alkaline phosphatase conjugated to anti-rabbit IgG or goat anti-mouse IgG (Kirkegaard & Perry Lab, Gaithersdurg, Maryland, USA) as the secondary antibody. Staining intensity was developed with nitro blue tetrazolium-bromochloro-indolyl phosphate (NBT-BCIP) mixture.

Immunocytochemistry of AGE and RAGE in cultured VSMC

For immunocytochemistry of AGE, VSMC were likewise cultured in a 75 m*l* flask with DMEM containing 10% STZ treated rat serum or control rat serum for 4 days, and were then detached with 0.05%

trypsin to prepare paraffin embedded cell blocks. For immunocytochemistry of RAGE, VSMC cultured on chamber slides were fixed by 4% buffered paraformaldehyde solution after incubation for 2 days in DMEM containing 10% STZ treated rat serum or control rat serum. Immunostaining was carried out by the labeled streptavidin-biotin complex (LsABC) method. Reaction with the primary antibodies was performed overnight at 4℃ in a humidifying chamber, using a 1: 500 dilution of an anti-AGE monoclonal antibody (6D12) or 1: 200 dilution of the anti-RAGE polyclonal antibody. Controls were treated with non-immune serum instead of primary antibody as well as the omission of the primary antibody. After washing in PBS three times for 5 minutes each time, biotinylated anti-mouse IgG rabbit serum (Zymed Laboratories, South San Francisco, CA, USA) or anti-rabbit IgG goat serum (Nichirei) was used as the secondary antibody for AGE or RAGE immunostaining for 30 minutes, respectively. Bound antibodies were detected with a streptavidin-peroxidase complex (Nichirei) using daiminobenzidine tetrahydrochloride (Sigma) as the substrate and counterstaining was performed with Mayerr's hematoxylin.

Statistical analysis

All values are reported as means ± SE unless stated otherwise. Unpaired t-test, one way factorial analysis of variance (ANOVA), Fisher's PSLD test and Bonferroni correction/Dunnett's test were used for statistical comparison. A value of $P < 0.05$ was considered statistically significant.

Results

Diabetic condition of the animals

At two days after the injection, glucosuria appeared and blood glucose rose to 416 ± 29 mg/dl (mean ± SE). In addition, blood sugar rose to 583 ± 41 mg/dl (mean ± SE) at one week after STZ administration. All rats manifested continuous weight loss. In some animals cataracts were observed at 12 weeks after STZ administration. The AGE concentration in diabetic sera, determined by ELISA, ranged approximately from 10 to 18 ng/ml while those in the control sera were around 2.5 ng/ml (**Fig. 1**). Significant differences between the diabetic and control sera were found at 16 and 20 weeks after the onset of diabetes.

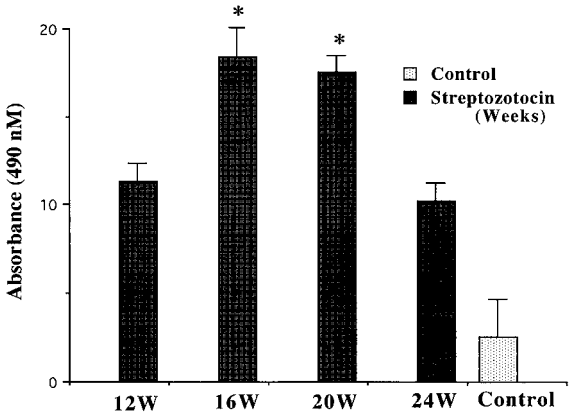

Fig. 1 AGE concentration in diabetic sera
The sera were obtained from diabetic rats 12, 16, 20 and 24 weeks after STZ administration and age-matched control rats. Values are the means ± SE of 3 independent experiments.
*P<0.05 vs. the control sera.

Effect of diabetic sera on VSMC growth

A variety of concentrations (1, 3, 5, 7, 10 and 15%) of rat sera in DMEM were prepared from the serum after 24 weeks of the diabetic condition. Firstly, effects of these diabetic sera on VSMC growth were assayed. VSMC cultured in 7, 10 and 15% sera for six days exhibited much higher absorption in MTT assay than those in 1, 3 and 5% sera (**Fig. 2**). Then effects of 10% sera after 12, 16, 20 and 24 weeks of the diabetic condition on VSMC growth for six days were evaluated.

VSMC were cultured in 96-well plates for 96 h in EDTA containing 10% STZ treatment rat sera. These VSMC were plated at 5×10^3 cell per well and their growth rate were dertermined by MTT assay. Diabetic rat sera enhanced the growth of VSMC prepared from non-diabetic rats. The serum at 12 weeks after STZ treatment did not influence VSMC growth. The sera at 16, 20 and 24 weeks after STZ treatment significantly enhanced the cell growth 2.3, 4.4 and 5.9-fold greater than the control serum, respectively (**Fig. 3**).

Effects of AGE-BSA on VSMC growth

Fig. 4 shows the effects of AGE-BSA at the indicated concentrations on VSMC growth. The VSMC proliferation was significantly enhanced under the

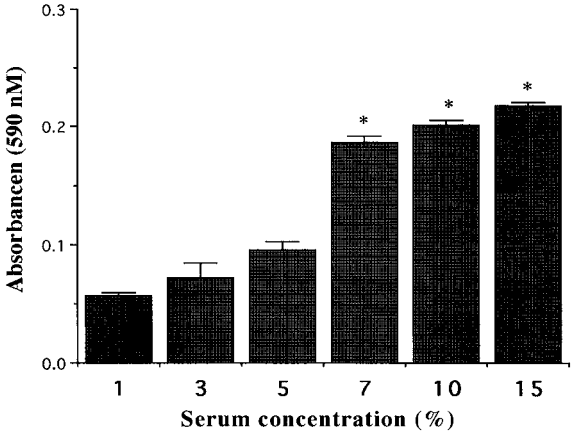

Fig. 2 Effect of diabetic sera 24 weeks after STZ treatment on VSMC growth
MTT assay of VSMC growth after incubation for 96 hours in 1, 3, 5, 7, 10 and 15% diabetic rat sera 24 weeks after STZ treatment was performed. Values are the means ± SE of 3 independent experiments. *$p < 0.05$ vs. 5% serum.

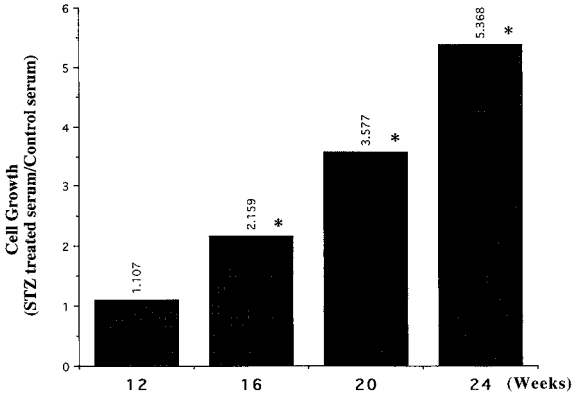

Fig. 3 Effect of diabetic sera after various ill duration on VSMC growth
MTT assay of VSMC growth after incubation for 96 hours in 10% diabetic rat sera 12, 16, 20 and 24 weeks after STZ treatment was performed. Results are expressed as ratio to control i.e., STZ treatment serum/control serum. Values are the means ± SE of 3 independent experiments. *$p<0.05$ vs. the control sera.

presence of AGE-BSA at a concentration of 5 to 10 μg/m*l* in comparison with non-glycosylated BSA. On the other hand, the cell growth under non-glycosylated BSA even at a concentration of 10 μg/m*l* was not significantly different from the control DMEM (data not shown).

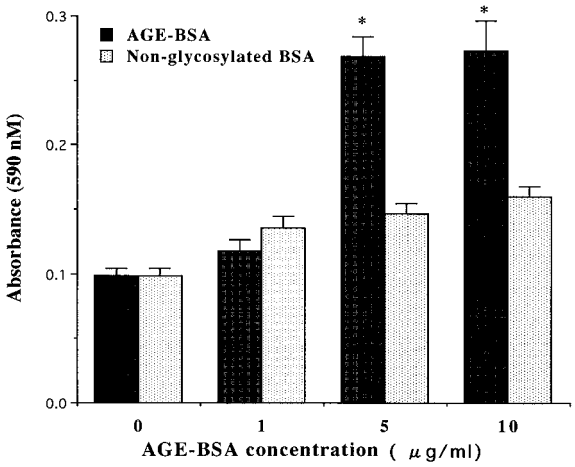

Fig. 4 Effects of AGE-BSA on VSMC growth
The effects of various concentrations of AGE-BSA on VSMC growth were evaluated by MTT assay. VSMC (1×10^4 cells/well) were plated on 24-well plates in DMEM plus 0.5% FCS with AGE-BSA for 96 hours. The VSMC proliferation was significantly enhanced under the presence of AGE-BSA at 5 to 10 μg/m*l* in comparison with non-glycosylated BSA. Values are the means ± SE of 3 separate experiments. *$p<0.05$ vs. non-glycated BSA.

Inhibitory effect of antibodies against AGE and RAGE on enhanced VSMC growth

To confirm that AGE-RAGE interaction participates in growth enhancement of VSMC (1×10^4 cells/well on 24-well plates) by diabetic rat serum or AGE-BSA, we evaluated the effects of anti-RAGE and anti-AGE (6D-12) antibodies against enhanced VSMC growth by STZ treated serum or AGE-BSA. The enhanced proliferation of VSMC by 10% diabetic rat serum 20 weeks after STZ treatment was significantly inhibited by anti-RAGE rabbit serum at 5 and 10 μ*l*/m*l* of concentrations in comparison with the control non-immunized rabbit serum (**Fig. 5 A**). However, the growth level with 10 μ*l*/m*l* of anti-RAGE antibody was around 60% of the stimulated growth, while the growth level with the non-diabetic serum was around 27% of the stimulated growth as shown in figure 3. Then the inhibitory effects of anti-RAGE antibody were considerably low. On the other hand, the enhanced effect of VSMC growth by 5 μg/m*l* of AGE-BSA was almost completely inhibited by anti-AGE antibody as well as anti-RAGE antibody (**Fig. 5 B**).

Western blotting analysis of AGE and RAGE in cultured VSMC

Western blot analysis confirmed that AGE was significantly more accumulated in VSMC cultured hours with diabetic rat serum than those cultured with the control non-diabetic serum (**Fig. 6A**). Although RAGE expression was not so different between the diabetic rat serum 16 weeks after STZ treatment and the control serum, STZ treated rat serum with 24 weeks diabetic duration induced RAGE expression in VSMC (**Fig. 6 B**).

Immunocytochemistry of AGE and RAGE in cultured VSMC

Immunoreactive AGE signals of were strongly positive in the cytoplasm of VSMC cultured with STZ treated rat serum while those were almost negative in VSMC cultured with the non-diabetic rat serum (**Fig. 7 A, B**). Although RAGE was detected in VSMC cultured with the non-diabetic rat serum as well as STZ treated rat serum, the intensity of immunostaining in VSMC cultured with the non-diabetic rat serum was much weaker (**Fig. 7 C, D**).

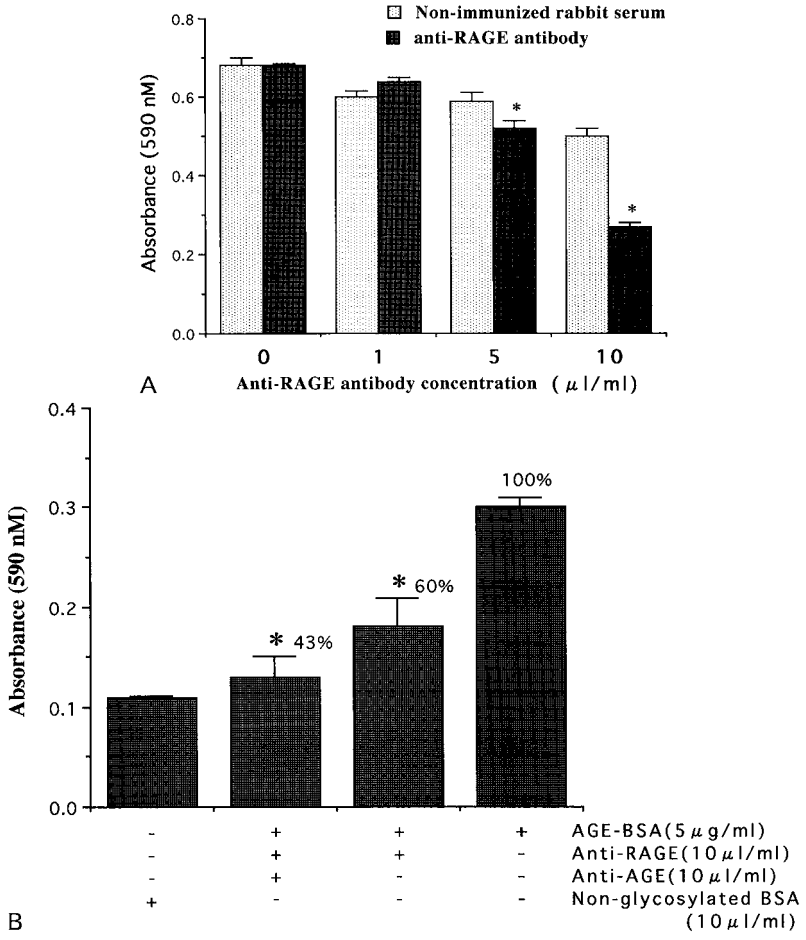

Fig. 5 Inhibitory effect of antibodies against RAGE and AGE on enhanced VSMC growth

Inhibitory effect of anti-RAGE antibody on enhanced VSMC growth by 10% diabetic rat serum 20 weeks after STZ treatment (A). Results are expressed as percent of the controls, in which the same concentrations of non-immunized rabbit sera as those of anti-RAGE antibody were used. Values are the means ± SE of 3 independent experiments. *$p<0.05$ vs. the control sera.

Inhibitory effect of anti-AGE and anti-RAGE antibodies on enhanced VSMC growth by 5 μg/ml of AGE-BSA (B). BSA: non-glycated BSA 5 μg/ml, AGE-BSA: AGE-BSA 5 μg/ml, Anti-RAGE: anti-RAGE antibody 10 μl/ml, Anti-AGE antibody (6D-12) : 10 μl/ml. Values are the means ± SE of 3 independent experiments. *$p<0.05$ vs. AGE-BSA (+).

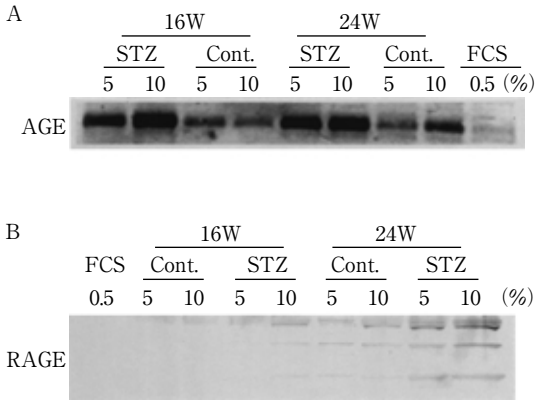

Fig. 6 Western blotting analysis of AGE and RAGE in cultured VSMC
Western blot analysis of AGE (A) and RAGE (B) was performed in VSMC cultured for 72 hours with 5% and 10% diabetic rat sera 16 weeks and 24 weeks after STZ treatment. AGE was significantly more accumulated in VSMC cultured for 72 hours with diabetic rat serum 16 weeks or 24 weeks after STZ treatment than with the control non-diabetic serum (A). Although RAGE expression in VSMC cultured with the diabetic rat serum 16 weeks after STZ treatment and with the control serum was not different, the diabetic serum 24 weeks after STZ treatment induced RAGE expression in VSMC (B). FCS; fetal calf serum.

Discussion

We confirmed that the rat sera after STZ treatment enhanced the growth of VSMC prepared from non-diabetic rats. This effect depended on the period of hyperglycemia and the serum concentration in culture media (**Fig. 1, Fig. 3**). Since age of the animals at sacrifice was identical, the cause promoting VSMC proliferation by the sera was most likely derived from the diabetic condition rather than from aging. We previously reported that insulin like growth factor-1 (IGF-1) increased in the STZ-treated rat sera, which enhanced expression of basic fibroblast growth factor (bFGF), bFGF receptor and IGF receptor in VSMC. We proposed that these growth factors and receptors are probably related to the VSMC proliferation[26]. In the present study, we attempted to elucidate that AGE-RAGE interaction mediates the stimulatory effect of diabetic sera to VSMC proliferation.

The monoclonal anti-AGE antibody (6D12) used in the ELISA could recognize a Ne-(carboxymethyl) lysine (CML)-protein adduct[27], which appears in AGE preparations obtained from bovine or human serum albumin, human hemoglobin and collagen, polylysine, lysine, lysine derivatives and monoaminocarboxylic acids, but not in early stage products of the Maillard reaction and AGE structures including 2-(2-furoyl)-4-(5)-(2-furanyl)-1 H-imidazole (FFI), pyrraline and pentosidine[28,29].

Based on the ELISA, the serum levels of AGE increased in diabetic sera until 20 weeks after STZ injection (**Fig. 2**). However, AGE-BSA prepared *in vitro* also exhibited a stimulatory effect on VSMC growth (**Fig. 4**), and 6 D 12 significantly inhibited this effect (**Fig. 5B**). These findings appear to suggest that AGE including CML may cause the enhanced effect of diabetic rat sera on VSMC proliferation. However, the serum levels of AGE in diabetic sera (approximately from 10 to 18 ng/m*l*) were markedly less than those of AGE-BSA (5 and 10 μg/m*l*) which exhibited significant stimulatory effect on VSMC growth (**Fig. 2, Fig. 4**). In addition, the magnitude of the VSMC growth-enhancement by the diabetic sera was more intense than that by the AGE-BSA solution (**Fig. 3, Fig. 4**) and it did not parallel the AGE levels of diabetic sera (**Fig. 2**). Therefore, AGE could contribute to the enhanced effect of diabetic rat sera on VSMC proliferation as one of many factors including cytokines and growth factors.

We demonstrated the endocytic uptake of AGE after culture with STZ treated rat serum by Western blotting and immunocytochemistry (**Fig. 6A, Fig. 7 A, B**). The expression of RAGE in was revealed to be enhanced with diabetic serum by Western blotting (**Fig. 6B**) and immunocytochemistry (**Fig. 7D, E**). Although the reason for this discrepancy is unclear, RAGE expression in VSMC might be up-regulated by AGE or other ligands like in vascular endothelial cells [19,23,30]. A specific anti-RAGE antibody significantly interrupted the promoting effects of diabetic sera and AGE-BSA on VSMC growth incompletely and completely, respectively (**Fig. 5 A, B**). While Higashi et al. reported that VSMC uptake AGE via an unknown AGE receptor, which is distinct from RAGE or macrophage scavenger receptor[31], the present findings

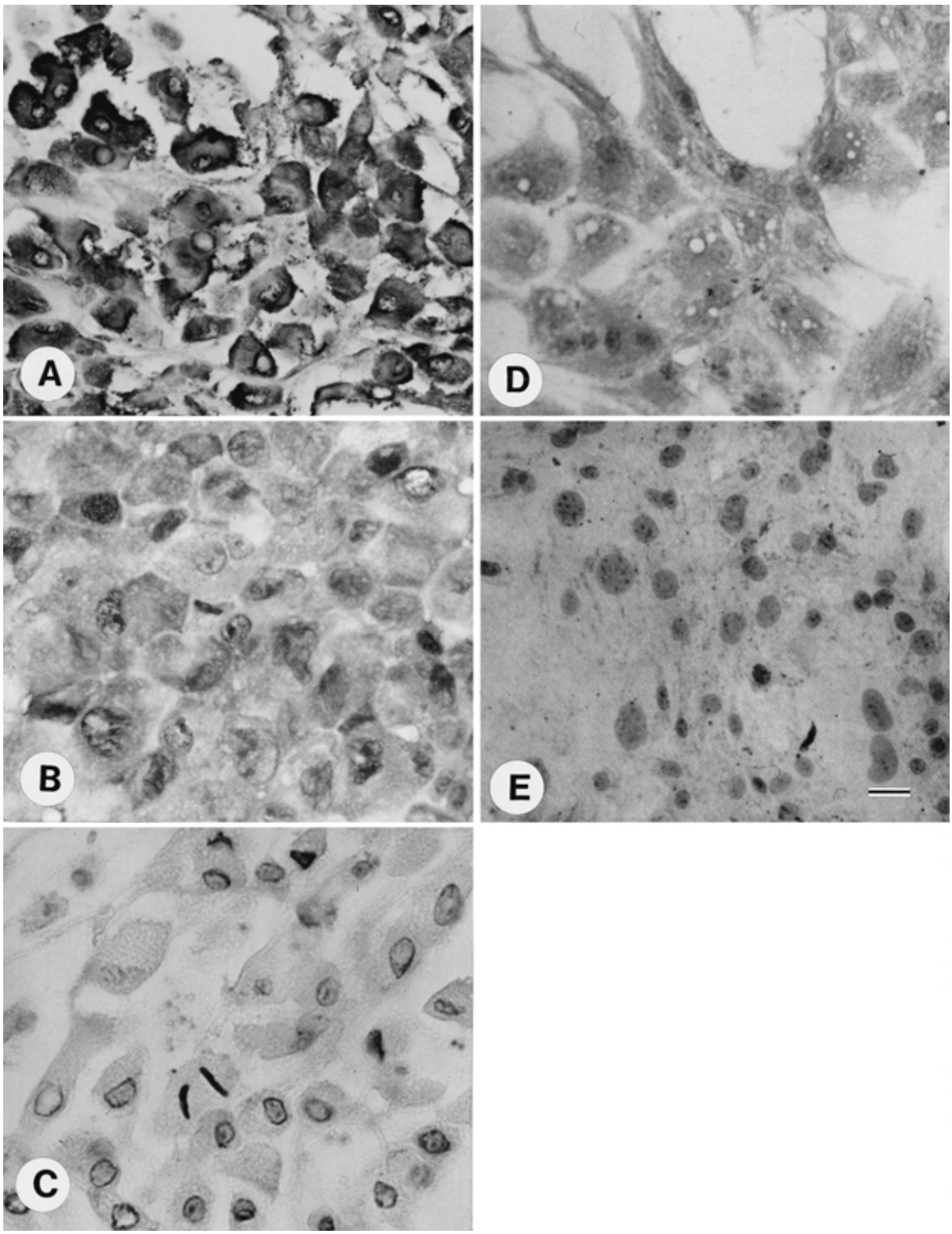

Fig. 7 Immunohistochemistry of AGE and RAGE in cultured VSMC
AGE and RAGE were immunocytochemically detected in cultured VSMC. A; AGE in VSMC cultured with 10% diabetic rat serum at 20 weeks after STZ treatment. B; AGE in VSMC cultured with the control rat serum. C; VSMC with normal mouse IgG instead of the primary antibody, showing no immunoreactivity. D; RAGE in VSMC cultured with 10% diabetic rat serum at 20 weeks after STZ treatment. E; RAGE in VSMC cultured with the control rat serum. Scale bar: 10 μm.

indicate that the interaction between AGE and RAGE participated in the VSMC proliferation process depending on the RAGE expression levels as well as the AGE amount.

Signal pathways continued after AGE binding to RAGE was not clarified in the present study. However, some studies have reported the AGE-RAGE interaction enhanced mitogen-activated protein (MAP) kinase activity or activate p 21^{ras} and nuclear factor-κB (NF-κB) in various SMC[32,33]. In addition, it was suggested that plural signal transmission pathways existed following AGE-RAGE binding, Some could be directlty connected with the promoting or cell-cycle and others could induce gene expression including growth factors via transcription factors like NF-κB. AGE induces various types of cells, including endothelial cells, SMC and macrophages, to synthesize growth factors and secrete them into serum[10,14,17,23,26], and simultaneously, AGE could directly stimulate proliferation not in endothelial cells but in SMC[32]. In conclusion, the present study revealed that the AGE-RAGE interaction in VSMC as well as growth factors induced by AGE contributes to the stimulatory effect of diabetic sera on VSMC proliferation. It is very important for diabetic macroabgiopathy because VSMC proliferation in the intima accelerates atherosclerosis.

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