Effect of glycated collagen on proliferation of human smooth muscle cells in vitro

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Summary While non-enzymatic glycation of long-lived tissue proteins such as collagen has been implicated in chronic complications of diabetes mellitus, its role in the aetiology of diabetic macroangiopathy has not been elucidated. To test the hypothesis that glycation of collagen abolishes the inhibitory effect of native collagen on the proliferation of human smooth muscle cells, we obtained smooth muscle cells from human gastric arteries and cultured them on dishes coated with glycated or non-glycated collagen. The proliferation of human smooth muscle cells in the presence of 10 % fetal calf serum or platelet derived growth factor-BB (10 ng/ml) was inhibited by type 1 collagen coated on the dishes. Glycation of collagen with glucose 6-phosphate for 7 days abolished the growth-inhibitory effect of native collagen. Succinylation of collagen, which like glycation blocked the

lysyl residues in collagen, also abolished the growth-inhibitory effect. Adhesion of human smooth muscle cells to collagen-coated dishes was not affected by glycation of collagen. Addition of glycated albumin to the medium did not affect the growth of human smooth muscle cells on plastic dishes. The inhibition of human smooth muscle cell proliferation by collagen was not reversed by the glycation of collagen in the presence of aminoguanidine. Results suggest that early glycation abolishes the inhibitory effect of collagen on human smooth muscle cell proliferation and may thus participate in the progression of macroangiopathy in diabetes. [Diabetologia (1996) 39: 800–806]

Keywords Smooth muscle cell, collagen, aminoguanidine, glycation, atherosclerosis

Macroangiopathy progresses rapidly in patients with diabetes mellitus. While its mechanism is not well understood, it is thought that the smooth muscle cells (SMC) may be involved. Accelerated growth

of aortic SMC from diabetic animals has been reported [1–3]. These cells migrate from the media to the intima of the aorta and proliferate in response to growth factors such as platelet derived growth factor (PDGF) and basic-fibroblast growth factor (b-FGF) [4]. Type 1 collagen, which is abundant in the artery, inhibits the proliferation of SMC [5, 6]. Recent studies postulate that excessive glycation of proteins is a cause of the vascular complications of diabetes. Advanced glycation end products (AGE) induce the production of interleukin-1 and tumour necrosis factor- α [7] by macrophages. These cytokines have been reported to stimulate the proliferation of SMC [8]. Non-enzymatic glycation alters the properties of collagen for fibroblasts [9, 10]. To determine whether the inhibitory effect of collagen on the growth of SMC may be abolished in the diabetic state, we investigated the effect of collagen

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Abbreviations: BSA, Bovine serum albumin; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; TCA, trichloroacetic acid; H-SMC, human smooth muscle cell; PDGF, platelet derived growth factor; b-FGF, basic-fibroblast growth factor; AGE, advanced glycation endproduct; G6P, glucose 6-phosphate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; 5-HMF, 5-hydroxymethylfurfural.

glycation on the proliferation of human-SMC (H-SMC) in response to fetal calf serum (FCS) or PDGF in vitro.

Materials and methods

Sterile purified type 1 collagen and culture dishes were purchased from Iwaki Glass (Funabashi, Japan). Dulbecco's modified Eagle's medium (DMEM) and FCS were purchased from Gibco (New York, N. Y., USA) and Nipro (Osaka, Japan), respectively. PDGF-BB, aminoguanidine hydrochloride, succinic anhydride, and all other drugs were purchased from Wako Chemicals (Osaka, Japan). Bovine serum albumin (BSA) (Sigma A0281) was purchased from Sigma Chemical Company (St. Louis, Mo., USA). Glucose 6-phosphate (G6P) was purchased from Oriental Yeast (Osaka, Japan). ^3H -methyl thymidine (80.0 Ci/mmol) was purchased from Amersham (Tokyo, Japan).

Human smooth muscle cell culture. H-SMC were obtained from gastric arteries removed from the stomachs of patients undergoing resection for early gastric cancer. None of these patients had hypertension, diabetes, hyperlipidaemia, or cardiovascular disease. H-SMC were cultured by the explant method in DMEM (pH 7.4) containing 10% FCS. Five to ten passages were performed for each experiment. H-SMC were identified by their hill and valley appearance and a positive immunofluorescence for α -actin [11]. After H-SMC became confluent in a Lab-Tek chamber slide, immunofluorescence staining was performed using mouse monoclonal antibody (ASM-1) for α -smooth muscle isoform with actin as the first antibody and then with FITC-conjugated goat antimouse IgG as the second antibody, according to Skalli et al. [11]. All cells were reacted using this antibody to α -actin.

Glycation of type 1 collagen and albumin. Purified type 1 collagen (0.3 mg/ml) was non-enzymatically glycated by incubation in phosphate buffered saline (PBS) at 37°C for 1 week in the presence of 20 mmol/l G6P, with or without 4 mmol/l aminoguanidine under sterile conditions. As a control, collagen was incubated for 1 week without G6P. The collagen suspensions were subsequently dialysed for 12 h three times with 0.15 mol/l saline to remove free G6P. We used G6P, instead of glucose, to accelerate the process of non-enzymatic glycation [12, 13]. The concentration of aminoguanidine was determined so as to prevent the formation of AGE. For 20 mmol/l G6P, at least 4 mmol/l aminoguanidine was required to prevent the AGE formation as measured by fluorescence. BSA (40 mg/ml) was also incubated in PBS for 1 week at 37°C in the presence or absence of 20 mmol/l G6P, and then dialysed. We used a sterile preparation of collagen. The albumin and all reagents were passed through a 0.22 μm filter. These samples were then dialysed with autoclaved 0.15 mol/l saline and a sterile dialysis membrane.

Succinylated collagen. Type 1 collagen was succinylated with succinic anhydride by the method of Wang et al. [14]. The extent of succinylation of ϵ -NH₂ groups of lysyl residues in collagen was determined by the method of Kakade and Liener [15]. Briefly, 1 ml of 0.1% 2,4,6-trinitrobenzenesulfonic acid (TNBS) was added to 1 ml of sample in 4% NaHCO₃ (pH 8.5). After incubation at 40°C for 2 h, 3 ml of concentrated HCl was added and autoclaved at 120°C for 1 h, and then 5 ml of distilled water was added and extracted twice with ethyl ether. The absorbance was read at 346 nm for the aqueous

solution. The blank was carried through the same procedure except that HCl was added to the samples prior to addition of TNBS. The amount of 'available' lysine (lysyl residues in which the ϵ -amino group are free) was calculated from a standard curve obtained with ϵ -trinitrophenyl-L-lysine. The extent of succinylation of ϵ -NH₂ groups of lysyl residues in collagen was determined by calculating occupied lysine as percentile of available lysine in native collagen.

Extent of glycation and 'available' lysine in native and glycated collagen with or without aminoguanidine. The extent of early glycation was determined by the method of Elder and Kennedy using thiobarbituric acid [16]. After adding 0.5 ml of 1 mol/l oxalic acid to 1 ml of sample (0.3 mg), the mixtures were autoclaved at 121°C for 1 h, cooled and mixed with 0.5 ml of 40% trichloroacetic acid (TCA). Samples were centrifuged at 300 g for 15 min and 0.5 ml thiobarbituric acid (0.05 mol/l) was added to the supernatant. The tubes were incubated at 40°C for 30 min and then at room temperature for 15 min. The absorbance was measured at 443 nm against blank employing 5-hydroxymethylfurfural (5-HMF) as standard. The degree of AGE modification was measured with a fluorometer (650–10 M; Hitachi, Tokyo, Japan, 370 nm excitation; 440 nm emission). PBS was used as blank for fluorometer evaluation of AGE. We corrected the fluorescence values for the hydroxyproline content of collagen and for the milligram of albumin. The 'available' lysine of native and glycated collagen with or without aminoguanidine was determined by the method of Kakade and Liener [15], as described above. The hydroxyproline content was determined by the chloramine-T assay after acid hydrolysing with 6 mol/l HCl for 24 h in a 110°C oven [17]. The protein concentration was determined by the Coomassie brilliant blue method (Bio Rad protein assay kit; Hercules, Calif., USA).

Coating of culture dishes with modified collagen. We coated 24-well flat-bottom culture dishes with 15 μg of collagen, with or without prior glycation, then dried them in air for 3 h. To reconstitute the collagen fibres, the dishes were incubated with 10% DMEM + FCS for 1 h. The amounts of the unmodified and modified collagen adhering to the culture dishes were determined using hydroxyproline assay [17]. After coating with collagen, the dishes were incubated with PBS at 37°C for 1 h. After aspiration of PBS, the hydroxyproline in collagen remaining on each dish was assayed after acid hydrolysis. There was no difference in the amount of hydroxyproline in any of the dishes coated with collagen, glycated collagen, or collagen glycated with aminoguanidine (92.8 ± 5.7 , 96.7 ± 4.2 , 95.0 ± 3.0 %, respectively, $n = 4$). Therefore, the adhesive property of collagen was not altered by glycation.

Adhesion of H-SMC to collagen-coated dishes. H-SMC were subcultured for 24 h on plastic dishes that were either uncoated or coated with type 1 collagen or glycated collagen. Thereafter, the number of non-adherent cells present in the medium was counted.

Assay of proliferation of H-SMC. For measurement of cell number, H-SMC were plated on wells at a density of $2 \times 10^4 \cdot \text{ml}^{-1} \cdot \text{well}^{-1}$. After reaching subconfluence of cell growth in 4 days, the cells were trypsinized and the cell number was counted with a counting chamber (Erma, Tokyo, Japan).

For measurement of DNA synthesis, H-SMC were plated on wells at a density of $2 \times 10^4 \cdot \text{ml}^{-1} \cdot \text{well}^{-1}$. After the subconfluent stage of cell growth was attained in 3 days, the medium was exchanged for serum-free DMEM for the next 24 h. After being stimulated with 10% DMEM + FCS or DMEM +

PDGF-BB (10ng/ml) for 24 h, the cells were incubated with 1 μ Ci of 3 H-methyl thymidine for the last 4 h of incubation, then were washed twice with PBS and incubated with 10 % ice cold TCA for 20 min. The cells were washed with 10 % TCA and rinsed twice with PBS. Finally, the cells were dissolved in 200 μ l of 0.1N NaOH. The cell lysate (100 μ l) was placed in a scintillation counter after being neutralized with 0.1N HCl. The glucose concentration during the 'stimulation' period was 5.5 mmol/l in all experiments.

Statistical analysis

Data are expressed as mean $\pm$ SD. Comparisons between groups were made with Student's *t*-test or one-way Anova. A level of *p* less than 0.05 was considered as statistically significant.

Results

Extent of glycation and 'available' lysine in native and glycated collagen with or without aminoguanidine. The extent of early glycation of collagen was determined by the thiobarbituric acid assay [16]. The amount of Amadori products in each collagen was determined as follows; native collagen: 0.15 ± 0.01 nmol 5-HMF/ μ g hydroxyproline, glycated collagen: 0.23 ± 0.01 , collagen glycated with aminoguanidine: 0.21 ± 0.03 . The extent of glycation of collagen was 1.53-fold compared to native collagen, which is similar to the results of in vivo experiments reported in a previous investigation [18]. The amount of Amadori products was similar between glycated collagen and collagen glycated with aminoguanidine. Glycated collagen showed an increase in fluorescence intensity that was completely abolished by aminoguanidine (Table 1). We measured the 'available' lysine as free ϵ -NH₂ groups of lysyl residues in collagen incubated with G6P. The 32 % of total 'available' lysyl residues in native collagen was modified by G6P and the modification was not affected by G6P in the presence of aminoguanidine.

Degree of AGE modification of albumin. Fluorescence intensity of glycated albumin was increased compared to normal albumin, and was almost completely abolished by aminoguanidine (normal albumin: 0.660 ± 0.003 fluorescence/mg albumin, glycated albumin: 0.783 ± 0.005 , albumin glycated with aminoguanidine: 0.672 ± 0.005 , $p < 0.001$, $n = 6$).

Effect of glycated collagen on adhesion of H-SMC. To investigate the possibility that glycation of collagen affects the adhesion of H-SMC to culture dishes, H-SMC was subcultured for 24 h on plastic dishes that were either uncoated or coated with type 1 collagen or glycated collagen. Thereafter, the number of non-adherent cells present in the medium was counted. No significant differences were observed in the adhesiveness of

Table 1. Determination of AGE formation by fluorometry in native and modified collagen

	Fluorescence/ μ g hydroxyproline
Collagen	0.100 ± 0.003
Collagen glycated	0.315 ± 0.005^a
Collagen glycated with aminoguanidine	0.088 ± 0.002

Values are means $\pm$ SD ($n = 8$)

^a $p < 0.001$ vs collagen and collagen glycated with aminoguanidine

Table 2. The effect of glycated collagen on H-SMC adhesion to dishes

	Cell number 10^4 /ml
Collagen (-)	0.30 ± 0.48
Collagen (+)	0.33 ± 0.50
Collagen glycated	0.33 ± 0.50
Collagen glycated with aminoguanidine	0.38 ± 0.51

Values are means $\pm$ SD ($n = 10$)

H-SMC (2×10^4 /ml) were seeded on dishes coated without collagen or with collagen, collagen glycated, and collagen glycated with aminoguanidine. Number of non-adherent cells in the culture media were counted after 24 h

H-SMC cultured on uncoated dishes or on dishes coated with native or glycated collagen (Table 2).

Effect of type 1 collagen on H-SMC proliferation. We coated dishes with collagen at increasing amounts (0–50 μ g), as shown in Figure 1. Native collagen significantly inhibited 3 H-thymidine uptake in a dose-dependent manner by H-SMC stimulated by 10 % FCS; 60 % inhibition was achieved when the amount of collagen reached 50 μ g/well ($p < 0.001$, $n = 8$) (Fig. 1).

Effect of glycated collagen on 3 H-thymidine uptake by H-SMC stimulated by 10 % FCS or PDGF-BB. The inhibitory effect of native collagen on 3 H-thymidine uptake by H-SMC stimulated by 10 % FCS was abolished by collagen glycation ($p < 0.005$, $n = 11$) (Fig. 2). Aminoguanidine did not reverse the effect of glycated collagen. 3 H-thymidine uptake by H-SMC stimulated with PDGF-BB (10 ng/ml) was also inhibited by native collagen, but not by glycated collagen ($p < 0.05$, $n = 12$) (Fig. 3).

PDGF-BB (10 ng/ml) stimulated the proliferation of H-SMC by 92 % of stimulation with 10 % FCS.

Effect of glycated collagen on H-SMC proliferation determined by cell number. The increase in the number of H-SMC stimulated by 10 % FCS was inhibited by native collagen, and was also abolished by glycation of collagen ($p < 0.05$, $n = 8$). The aminoguanidine added to the incubation mixture of collagen with G6P

Fig. 1. Dose dependent inhibition by native collagen of ³H-thymidine uptake into H-SMC stimulated by 10 % FCS. We coated native collagen on the 24-well dishes at increasing doses (0–50 µg). The data indicate the percent of ³H-thymidine taken up by cells cultured on plastic dishes ($n = 8$). *N.S.; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.001$

Fig. 2. Effect of glycated collagen on ³H-thymidine uptake into H-SMC stimulated by 10 % FCS. Native collagen inhibited H-SMC proliferation on plastic dishes. Glycated collagen abolished the inhibitory effect of native collagen. Aminoguanidine did not prevent the effect of glycated collagen ($n = 11$). *N.S.; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.05$

did not reverse the stimulating effect of glycated collagen on the growth of H-SMC (Table 3).

Effect of succinylated collagen on ³H-thymidine uptake by H-SMC stimulated with PDGF-BB. The extent of succinylation of ϵ -NH₂ groups in lysyl residues of succinylated collagen was 61 %. Succinylated collagen (50 µg/well) abolished the inhibitory effect of native collagen (50 µg/well) on ³H-thymidine uptake by H-SMC stimulated with PDGF-BB (10 ng/ml) to the level of control without collagen ($p < 0.005$, $n = 8$) (Fig. 4).

Effect of glycated albumin on ³H-thymidine uptake by H-SMC cultured on uncoated plastic dishes. The addition of 1 mg/ml of albumin into the medium had no effect on ³H-thymidine uptake by H-SMC stimulated with 10 % FCS ($p < 0.005$, $n = 8$) (Fig. 5). Also, 1 mg/ml of albumin glycated with or without aminoguanidine showed no effect on H-SMC growth. There was no difference in H-SMC growth between the native albumin and the glycated albumin in the media.

Discussion

SMC may play an influential role in the progression of diabetic macroangiopathy. Accelerated growth of aortic SMC from diabetic animals has been reported [1–3]. In atherosclerosis, the SMC migrates from the media to the intima of the aorta and proliferates in response to growth factors such as PDGF and b-FGF [4].

Collagen acts as a substrate for SMC and influences activities such as cell morphology, growth, and differentiation [19]. Cells from an atherosclerotic vessel synthesize type 1 collagen in preference to type 3 collagen, unlike the cells of normal arteries [20–22]. Thie et al. [5, 6] reported that type 1 collagen inhibits proliferation of SMC. A contractile phenotype of SMC does not respond to serum mitogens [23]. The proliferation of SMC in a three dimensional culture system of collagen matrix was slowed down, as compared with that in monolayer cultures, even though SMC were in an anabolic state [5]. An increased cross-linking of type 1 collagen has been observed in animals and humans with diabetes [24–26].

The effect of non-enzymatic glycation on the physical and chemical properties of collagen has been studied extensively [9, 10, 27]. A decreased adhesion of aortic endothelial cells and an increased adhesion of human monocytes to collagen layers has been reported after non-enzymatic glycation of collagen [28–30]. The growth of mesangial cells is affected by the glycation of extracellular matrices [31, 32]. In these studies, the proliferation of mesangial cells was inhibited by glycated matrices. Since matrices instead of collagen were glycated with 200 mmol/l G6P for 2 weeks, the AGE formation in these matrices would be further advanced compared to ours with 20 mmol/l G6P for 1 week.

Since SMC in an atherosclerotic vessel appear to function like fibroblasts [33], we investigated the effect of glycation of collagen on proliferation of SMC stimulated by FCS or PDGF-BB. A recent study demonstrated an effect of AGE on SMC proliferation

Fig. 3. Effect of glycated collagen on ³H-thymidine uptake into H-SMC stimulated by 10 ng/ml of PDGF-BB. Glycated collagen abolished the inhibitory effect of native collagen. ** $p < 0.05$, $n = 12$; * $p < 0.001$

Fig. 5. Effect of albumin and glycated albumin on the proliferation of H-SMC stimulated by 10 % FCS. There were no differences in ³H-thymidine uptake into H-SMC ($n = 8$).

Table 3. The effect of glycated collagen on H-SMC proliferation stimulated by 10 % FCS

	Cell number (10^4 /ml)
Collagen (-)	18.1 ± 5.3^a
Collagen (+)	12.7 ± 3.5
Collagen glycated	21.0 ± 8.7^a
Collagen glycated with aminoguanidine	18.5 ± 5.9^a

Values are means $\pm$ SD ($n = 8$)

H-SMC (2×10^4 /ml) were seeded on dishes coated without collagen or with collagen, collagen glycated, and collagen glycated with aminoguanidine. After 4 days culture with 10 % FCS + DMEM, cell number was counted

^a $p < 0.05$ vs collagen (+)

Fig. 4. Effect of succinylated collagen on H-SMC growth. In contrast to native collagen (50 μ g/well), succinylated collagen (50 μ g/well) did not inhibit ³H-thymidine uptake into H-SMC stimulated by PDGF-BB (10 ng/ml). ** $p < 0.005$, $n = 8$; * $p < 0.001$

[34]; collagen-bound AGE were found to block the cytostatic effect of nitric oxide on aortic SMC. These results differ from the direct inhibitory effect of collagen on SMC growth found in our study in that they showed an inhibitory effect of AGE in basement membrane on the cytostatic effect of nitric oxide derived from endothelial cells. The reason for the discrepancy between our data and theirs might be due to the difference in experimental conditions. First, they used a higher concentration of collagen

(5 mg/ml) and G6P (500 mmol/l) than we did and the incubation time was much longer than ours (60 days). Second, their incubation time for cell culture was only 48 h, which was shorter than our 5 days. We think this was too short, since at least 4 days was required to produce the effect of glycated collagen on growth of SMC in our study. Although AGE may modulate macrophage and endothelial cell function, [7, 35–37], AGE albumin had no effect on proliferation of SMC in our study.

Blocking collagen fibril formation by succinic anhydride suppresses the inhibition of fibroblast proliferation by native collagen [38]. It is also known that modification of lysyl residues of laminin abolishes neurite outgrowth [39]. The ϵ -amino groups in lysyl residues of collagen, which are considered to be essential for the interaction with fibroblasts, are modified by the succinylation [38]. In our study, the modification of lysyl residues of collagen by succinic anhydride suppressed the inhibition of H-SMC proliferation by native collagen. To ascertain the importance of lysyl residues of type 1 collagen for the inhibitory effect of proliferation of H-SMC, we measured the

'available' lysine as free ϵ -NH₂ groups of lysyl residues in collagen incubated with G6P. The 32 % of 'available' lysyl residues in native collagen was modified by G6P, which included the new modification not only by Amadori formation (ketoamine) but also by formation of schiff base and AGEs. Therefore, inhibition of proliferation of SMC by type 1 collagen in the intact arterial matrix could be impaired by early glycation of collagen. This may make the SMC susceptible to serum mitogens. The mechanism by which collagen inhibits H-SMC proliferation in response to growth factors has not been elucidated. Lin et al.[40] reported a decrease in the level of PDGF-stimulated receptor autophosphorylation by fibroblasts in collagen matrices. Native collagen may inhibit the growth factor inducing cascade either at or downstream from the receptor site.

Aminoguanidine inhibits cross-linking of glycated collagen, but not the formation of Amadori products[41–43]. In our study, the extent of Amadori product formation was same between glycated collagen and collagen glycated with aminoguanidine. Treatment of glycated collagen with aminoguanidine did not reduce the effect of glycated collagen on proliferation of H-SMC. Recently, the influence of Amadori glucose adducts on cell growth has been reported [44, 45]. A block of lysyl residues essential to inhibit SMC proliferation abolished the physiological function of native collagen in our study. Therefore, early glycation is sufficient to impair the normal function of collagen.

Our results indicate that the suppressive action of native collagen fibril on the proliferation of H-SMC was abolished by the glycation of collagen. Early glycation that blocks lysyl residues of collagen may be sufficient to abolish the effect of collagen. The glycation of collagen caused by hyperglycaemia may accelerate diabetic macroangiopathy via an enhanced growth of SMC.

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