Advanced Glycosylation End Products in the Mesenteric Artery

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We measured advanced glycosylation end products in the mesenteric artery of 37 patients (ages 29–82 years), 34 of whom were nondiabetic. Samples of arterial tissue were obtained during bowel resectioning. Advanced glycosylation end products were measured as collagen-linked fluorescence (excitation wavelength 370 nm, emission wavelength 440 nm) after collagenase digestion of tissue samples. Mean fluorescence of the arterial samples was 15 U/mg (range 5.3–27). Collagen fluorescence correlated with patients' age (r = 0.57; P < 0.001). No difference in the collagen-linked fluorescence was observed between men and women (P = 0.63), hypertensive and normotensive patients (P = 0.44), smokers and nonsmokers (P = −0.52), and patients with and without symptomatic coronary heart disease (P = 0.7). This study demonstrates, for the first time, the relationship between collagen-linked fluorescence and patients' age in human arterial tissue ex vivo.

Additional Keyphrases: age-related effects · collagen · fluorescence

Age increase and diabetes mellitus are associated with changes in the physicochemical properties of collagen: the mechanical stiffness of collagen increases and it becomes more resistant to proteolytic degradation (1, 2). These changes result from progressive collagen crosslinking (3), which at least partly results from the formation of advanced glycosylation end products (AGE).³ AGE are end products of the Maillard (browning) reaction (4, 5), and most are fluorescent. Measurements of collagen-linked fluorescence (CLF) are thought to reflect AGE accumulation. AGE formation on collagen has been demonstrated in animals (6) and humans (7). Clinical studies of AGE have measured CLF of skin biopsy specimens. However, because in vitro experiments suggest that the accumulation of AGE may play a role in the pathogenesis of vascular complications of diabetes and in atherogenesis (4, 5), the direct measurement of AGE in arterial tissue is important.

Here we measure the fluorescence of collagen in human arterial tissue obtained ex vivo and study the relationship between fluorescence of arterial collagen and patients' age.

Materials and Methods

We obtained mesenteric arteries from 37 patients (18 men, 19 women), ages 29–82 years (mean 62.5), who underwent elective surgical large bowel resection. The bowel pathology in these patients included malignancy (n = 26), inflammatory bowel disease (n = 9), and diverticular disease (n = 2). Three of the six patients known to be hypertensive had left ventricular hypertrophy. Two of the hypertensive patients received calcium-channel blocking agents, one received a β-adrenoceptor antagonist, and three had no therapy. Ten patients had ischemic heart disease documented by preoperative electrocardiogram. Three patients had type 2 (non-insulin-dependent) diabetes (duration 7, 10, and 14 years); two required oral hypoglycemic drugs and one was controlled by diet alone. Ten patients smoked cigarettes. There was no significant abnormality in the preoperative hematological or biochemical indices. All nondiabetic patients had plasma glucose concentrations (checked at random) <6.7 mmol/L. This study was approved by the Stobhill General Hospital Medical Ethics Committee.

Immediately after surgical resection, mesenteric arteries with external diameter 3–4 mm were dissected at a site distant from the primary pathology. The arteries were stored in physiological saline at −20 °C until analysis. We prepared arterial collagen by the modified method (8; J. Bell, N. H. Cox, S. K. Jones, M. H. Dominiczak, unpublished abstract, 1989) of Monnier et al (9). We dissected the artery from the surrounding tissue and cut it into small pieces. We suspended the pieces in 10 mL of phosphate buffer (pH 7.4), homogenized them for 30 s (IKA homogenizer; IKA Labortechnik, F.R.G.), and then centrifuged the homogenized sample at 4 °C at 1300 × g for 15 min (Sorvall RT-6000 centrifuge, Du Pont, Stevenage, U.K.). We removed the supernate; washed the pellet three times with distilled, filtered water; and extracted lipids by adding 5 mL of chloroform/methanol (2/1 by vol) with gentle shaking in a cooling bath (4 °C for 24 h). We centrifuged the sample (1300 × g, 4 °C, 15 min), removed the supernate, and washed the pellet twice with ice-cold methanol; three times with distilled, filtered water; and twice with 0.02 mol/L 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer, pH 7.5. For collagen digestion, we suspended the pellet in 1 mL of 0.02 mol/L HEPES buffer containing 250 U of Clostridium histolyticum collagenase (EC 3.4.24.3; Sigma, Poole, U.K.) and added chloroform/toluene (1/1 by vol; 4 µL) to prevent bacterial contamination. A blank tube containing collagenase in 0.02 mol/L HEPES buffer was included with each batch of samples. After overnight incubation at 37 °C, we centrifuged the samples at 10 000 × g for 5 min at 4 °C and

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Nonstandard abbreviations: AGE, advanced glycosylation end products; CLF, collagen-linked fluorescence; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid and aifu, arbitrary fluorescence units.

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then measured fluorescence and hydroxyproline concentration of the supernate, which contained the digested collagen. Hydroxyproline was measured colorimetrically according to Stegemann and Stalder (10). Collagen concentration was calculated by assuming a 14% hydroxyproline content (1).

CLF was measured in the collagen digest at an excitation wavelength of 370 nm and an emission wavelength of 440 nm, with an LS3B scanning fluorescence spectrometer (Perkin-Elmer Corp., Beaconsfield, U.K.). The fluorescence values detected in the samples were corrected against the collagenase blank. In vitro browned collagen was included as a control. The results were expressed in arbitrary fluorescence units per milligram of collagen (afu/mg). Triplicate measurements were made for each sample. The within-batch imprecision (CV) of fluorescence measurement was 10.1% for a fluorescence of 16.8 afu/mg and 11.0% for 22.7 afu/mg. The between-batch imprecision for a fluorescence of 12.8 afu/mg was 10% and for 15.4 afu/mg was 7.8%. Statistical analysis included linear-regression analysis (least-squares method), multiple regression, Student’s t-test, and the Mann–Whitney test, as appropriate (STATGRAPHICS, Version 2.6; Statistical Graphics Corp. Inc., Rockville, MD).

Results

At the excitation wavelength (370 nm), the emission spectrum of the arterial-tissue collagenase digests was similar to the spectrum of native calf-skin type I collagen. As shown in Figure 1, the in vitro browning of native collagen for 14 days in the presence of 250 mmol/L glucose 6-phosphate, which is known to lead to AGE accumulation, resulted in an increase in collagen fluorescence.

The mean fluorescence of the mesenteric artery collagen was 15 afu/mg (range 5.3–27). We observed an increase of CLF that was related to patients’ age ($r = 0.57, P < 0.001$) (Figure 2). The generation of CLF appeared to accelerate in individuals older than 60 years. Fitting the data into an exponential model yielded a correlation coefficient ($r = 0.61; P < 0.0001$) similar to that obtained above with linear-regression analysis (data not shown).

CLF was similar in hypertensive and normotensive patients ($P = 0.44$), in patients with inflammatory bowel disease, in patients with malignancy, and in patients with and without symptomatic ischemic heart disease ($P = 0.7$). CLF was not affected by sex ($P = 0.63$) or cigarette smoking ($P = 0.52$).

Discussion

Our study demonstrates for the first time an age-related increase in CLF in human arterial tissue ex vivo. Substantial evidence indicates that CLF reflects the accumulation of AGE, stable intraprotein crosslinks (4, 5, 11–14).

Measuring AGE is difficult because of their heterogeneity. AGE compounds identified to date may be only a fraction of those occurring in vivo (5, 15). Although CLF has been measured in most clinical studies, measurement of protein-linked fluorescence is not absolutely specific for AGE: most but not all AGE are fluorescent (4, 5), and protein conformation and oxidation may affect fluorescence patterns (16, 17). Alternative methods of AGE measurement are now being developed. 2-(2-Furyl)-4-(5)-(2-furanyl)-1-H-imidazole was the first AGE to have its structure identified (12). A radioimmunoassay for this compound has been developed (18), but its clinical utility appears to be limited because of data suggesting that it may be formed as an artifact during hydrolysis (19). Sell and Monnier (20) measured

![Fig. 1. Collagen-linked fluorescence from human mesenteric arteries (---), native calf-skin type I collagen (---), and calf-skin type I collagen incubated in vitro with glucose 6-phosphate (250 mmol/L) for two weeks at 37 °C (---). Excitation at 370 nm](image)

![Fig. 2. Relationship between collagen-linked fluorescence in the mesenteric arteries and patients’ ages ($r = 0.57; P < 0.001$)](image)

○, Nondiabetic patients; ●, diabetic patients
pentosidine, which may be partly responsible for collagen fluorescence. However, pentosidine may constitute <1% of all the AGE present on a protein molecule (15). Nevertheless, the pattern of age-related pentosidine accumulation on human skin collagen is similar to the pattern we observed here by measuring CLF (13), and pentosidine may be a useful marker of AGE accumulation. The most recent promising development was the introduction of the radioreceptor assay based on AGE binding to specific membrane receptor on macrophage-derived cells (21, 22). This assay appears to be more specific and sensitive than CLF measurement; it is based on a biological activity of AGE, rather than on structural information, and standardization remains empirical. Data for comparing the radioreceptor assay, pentosidine measurement, and CLF measurement are still limited (22); at the time of this study, the radioreceptor assay was not available to us. Our earlier experiments indicated that the fluorescence spectrum at excitation wavelength 370 nm and emission wavelength 440 nm reflects the accumulation of AGE (Bell et al., unpublished abstract, 1989). We show here that the emission spectrum of arterial tissue fluorescence is equivalent to that of collagen incubated with glucose 6-phosphate in vitro.

We measured CLF in human vascular tissue because some data indicate that AGE may be involved in the mechanisms of aging, in the vascular complications of diabetes, and possibly in atherosclerosis (4). AGE interact with macrophages and with endothelial cells; tissue concentrations of AGE may be partially regulated by the activity of the specific macrophage receptor (23). The uptake of AGE-modified proteins by this receptor stimulates the secretion of interleukin 1 and tumor necrosis factor (24) and increases the transcription of insulin growth factor 1 (25). These data suggest that AGE may induce processes that lead to vascular tissue remodeling, which occurs during diabetic complications and in atherosclerosis. Moreover, the exposure of bovine endothelial cell monolayer to AGE-modified fibronectin increases the permeability of the monolayer and induces the procoagulant state, on the surface of endothelial cells (26).

We studied small mesenteric arteries because they are easily obtained untraumatized during bowel surgery and may play an important role in regulating the cardiovascular system, particularly systemic blood pressure (27). Although the arteries studied were taken from patients with underlying bowel disease, histological examination showed no involvement by the primary pathology. Malignancy is unlikely to affect the structure of collagen crosslinks, and we were unable to detect any difference in CLF between patients with and without neoplasia. The arterial CLF in our patients increased slowly up to age 60 years and increased rapidly thereafter. This is similar to the exponential pattern of the pentosidine accumulation in the human skin from individuals between ages 10 and 100 years (13, 28). A similar pattern of CLF increase was observed in aging nondiabetic and streptozotocin-diabetic rats (29). The age-related increase in CLF in human skin and dura mater was previously reported (30, 31). Clinical studies of CLF measurements on skin biopsy collagen suggest a relationship between AGE accumulation and the development of diabetic complications. The increase in CLF in type 1 diabetic patients correlated with the presence (8, 9) and severity (9) of diabetic retinopathy; correlation between CLF and both systolic and diastolic blood pressure was also observed but did not persist upon multiple-regression analysis. Limited data on arterial AGE concentrations are available from studies of human autopsy material. Sell and Monnier (13) reported the presence of pentosidine in human aorta, and Makita et al. (22), using a radioreceptor assay, observed an increase in arterial AGE concentration in type 1 diabetic patients and in nondiabetic individuals with renal failure. Our patients were nondiabetic, and we were unable to demonstrate a relationship between arterial CLF and hypertension, smoking, or symptomatic coronary heart disease; studies involving detailed analysis of the severity of these factors are needed.

The mechanism of age-related AGE accumulation in tissues is not known. Excess AGE accumulation in diabetes may result from prolonged hyperglycemia. Similarly, AGE accumulation with advancing chronological age in nondiabetic individuals may result from slow deterioration of glycemic control with age (32). However, Eble et al. (33) observed that glucose-induced protein crosslinking, once initiated, can progress independently of the glucose concentration; this suggests a self-perpetuating mechanism of AGE accumulation and has not been verified in humans.

Although our primary purpose was to study the effect of chronological age on the arterial AGE concentrations in nondiabetic patients, we had an opportunity to measure CLF in the arteries of three individuals with type 2 diabetes. All three diabetic patients had more arterial CLF than did age-matched nondiabetic individuals. To our knowledge these are the first measurements of AGE-related fluorescence in the arterial tissue of diabetic patients ex vivo. This observation, although anecdotal, supports the findings of Sell and Monnier (13) and Makita et al. (22) and indicates the need for further studies.

The importance of our finding is the direct demonstration of age-related increases in AGE concentrations in human arterial tissue ex vivo. The age-related increase in the arterial-wall AGE content supports the hypotheses relating AGE accumulation to vascular pathology. The mesenteric artery used is a good model for studies of advanced glycosylation in human arterial tissue.

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References
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