American Journal of Pathology, Vol. 143, No. 6, December 1993

Immunohistochemical Localization of Advanced Glycosylation Endproducts in Coronary Atheroma and Cardiac Tissue in Diabetes Mellitus

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Advanced glycosylation end products (AGEs) accumulate on long-lived extracellular matrix proteins and have been implicated in the micro- and macrovascular complications of diabetes mellitus. Within the arterial wall, AGE-modified proteins increase vascular permeability, inactivate nitric oxide activity, and induce the release of growth-promoting cytokines. Recently developed anti-AGE antibodies were used in an immunohistochemical analysis of coronary arteries obtained from type II diabetic and nondiabetic patients. High levels of AGE reactivity were observed within the atherosclerotic plaque present in vessels from selected patients with diabetes. Considered together with the pathological effects of AGEs on vascular wall homeostasis, these data support the role of advanced glycosylation in the rapidly progressive atherosclerosis associated with diabetes mellitus. (Am J Pathol 1993, 143:1649–1656)

The high prevalence of cardiovascular disease in patients with diabetes mellitus has been attributed to an atherosclerotic process that is more extensive and develops at an earlier age in diabetic than in nondiabetic individuals.[1,2] Pathological changes that occur early in diabetic vessels include functional abnormalities in vascular wall permeability, upregulation of procoagulant activity, and impairment in endothelial-dependent relaxation.[1,3–6] The deposition of serum proteins and lipoproteins produces a thickening in the arterial basement membrane and medial layers. With time, fatty streaks appear, and endothelial disruption occurs. This is followed by myointimal proliferation and the formation of complex intraluminal plaques that constitute the hallmark lesions of atherosclerosis.[2,7,8]

Among the biochemical abnormalities that accompany diabetes mellitus is an increase in the formation of protein-linked advanced glycosylation end products (AGEs).[9–11] Advanced glycosylation is a posttranslational modification process that results from the spontaneous covalent reaction of circulating glucose with protein amino groups. Rearrangement reactions then occur to produce fluorescent, cross-linking moieties that remain irreversibly bound to proteins. AGEs primarily affect proteins within the extracellular insulin-independent tissue compartments and accumulate in increased amounts on long-lived basement membrane and matrix proteins. Within the vascular wall, collagen-linked AGEs cross-link, or "trap" plasma proteins, inactivate nitric oxide activity, and interact with specific cell surface receptors to induce cytokine and growth factor release.[6,12–14] Plasma low density lipoprotein (LDL) also undergoes an increase in advanced glycosylation in diabetes. Once attached to the lipid component of LDL, AGEs initiate oxidative reactions that promote the formation of oxidized LDL.[15] This modified form of LDL is cleared predominantly by tissue macrophages, which, in the process, are transformed into the lipid-laden foam cells that accumulate in atherosclerotic vessels.[2] Numerous experimental studies, therefore,

Supported by NIH grant AG-9453 and the American Diabetes Association.

Accepted for publication August 6, 1993.

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support the concept that advanced glycosylation contributes significantly to the atherogenicity of the diabetic milieu.

For many years, investigation of the pathogenic effects of advanced glycosylation has been hampered by the lack of specific and sensitive methods for detecting AGE-modified proteins *in vivo*. Several structurally defined AGEs, such as FFI, pyrraline, AFGP, CML, and pentosidine, have been identified; however, tissue analyses indicate that these particular compounds comprise, at most, only a small fraction of the AGEs that form *in vivo*.[16–22] In more recent experimental approaches, AGEs that have been prepared under native physiological conditions *in vitro* have been used to elicit AGE-specific monoclonal and polyclonal antibodies.[23–25] The antibodies produced to these "native" AGE immunogens specifically recognize a large class of glucose-derived AGEs that form *in vivo*. An important feature of these antibodies is their recognition of an AGE epitope(s) that forms on diverse protein substrates. Thus, anti-AGE antibodies have been used successfully to identify and quantitate the AGE modifications that occur on collagen, hemoglobin, serum proteins, and the lipid and apoprotein B components of LDL.[15,23,26] Over the last year, AGE immunoassays have been applied to a number of studies aimed at evaluating the role of advanced glycosylation in diabetic and age-related complications and assessing the efficacy of aminoguanidine, a recently developed pharmacological inhibitor of advanced glycosylation.[15,26]

In the present study, we applied anti-AGE antibodies to an immunohistochemical analysis of coronary arteries obtained from type II diabetic and nondiabetic patients. AGE reactivity localized to the atherosclerotic lesions present within diabetic vessels. The most intense staining was within the fibrous acellular areas of intraluminal plaque, which contained extracellular matrix deposits. AGE reactivity also was observed in less advanced vascular lesions that predominantly showed myointimal proliferation. The myocardial tissue showed strong AGE staining within intracellular granular deposits. These lesions displayed the morphological features of basophilic degeneration.

Materials and Methods

Reagents

AGE-modified bovine serum albumin (AGE-BSA) and AGE-modified ribonuclease (AGE-RNAse) were prepared by incubating proteins (25–50 mg/ml) in 200 mmol/L sodium phosphate buffer, pH 7.4, with 500 mmol/L glucose for 12 weeks at 37 C. Rabbit anti-AGE-RNAse and anti-RNAse antibodies were produced and characterized as described previously.[23] Goat anti-rabbit IgG was purchased from Cappel (West Chester, PA), and proteinase-K was obtained from E. Merck AG (Germany).

Subjects

Twenty-five specimens of cardiac tissue and left main or left anterior descending coronary artery were obtained at autopsy from 11 noninsulin-dependent diabetic patients (nine males and two females; mean age, 68 ± 10.5 years; mean duration of diabetes, 13.6 ± 10.4 years) and 14 nondiabetic patients (10 males and four females; mean age, 70.1 ± 7.2 years). All subjects were free of clinically evident coronary artery disease. The proximate cause of death did not differ significantly between the diabetic and nondiabetic groups (Table 1).

Immunohistochemical Staining

Formalin-fixed and paraffin-embedded tissue were cut into 3-μm thick sections and deparaffinized by treatment with xylene (5 min, three times), followed by graded ethanol. Mounted tissues were incubated with 0.05% proteinase-K (E. Merck) in 10 mmol/L phosphate-buffered saline (PBS), pH 7.4, for 30 min at 37 C. These were then washed with PBS and dipped in 0.3% H_2O_2-100% methanol for 30 min to block endogenous peroxidase. After additional washes (in PBS, three times), 20% normal goat serum (Vector Laboratories, Burlingame, CA) was added for 10 min at room temperature to block nonspecific binding. Slides were incubated with rabbit anti-AGE-RNAse or control anti-RNAse antiserum (1:30 to 1:100 dilution in PBS) for 24 hours at 4 C. The control antiserum consisted of rabbit polyclonal antibody raised to the AGE carrier protein, RNAse (anti-RNAse). After washing with PBS three times, peroxidase-labeled goat anti-rabbit IgG (Cappel) was added for 2 hours at room temperature. The slides then were rinsed with PBS and incubated for 3 min with 0.02% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in 50 mmol/L Tris-HCl buffer (pH 7.6) containing 0.003% H_2O_2. After washing with PBS and ddH_2O, Meyer hematoxylin was added as counterstain. As an additional control for anti-AGE immunoreactivity, positively stained tissues were subjected

Table 1. *Summary of AGE Staining within Coronary Artery and Cardiac Tissue Specimens*

Patient No.	Age (years)	Sex	Diabetes duration (years)	Cause of death	AGE staining	
					Coronary artery	Cardiac muscle
Nondiabetic patients						
1	57	F		Lung carcinoma	–	–
2	61	M		Lung carcinoma	–	
3	64	M		Renal failure	–	+
4	64	M		Hepatocellular carcinoma	–	–
5	64	M		Submandibular cancer	–	–
6	67	M		Tuberculosis	–	–
7	69	M		Lung carcinoma	–	–
8	70	M		Pulmonary fibrosis	–	–
9	75	M		Gastric cancer	–	–
10	76	M		Gastric cancer	–	–
11	77	F		Congestive heart failure	–	+
12	78	M		Pneumonia	–	+
13	79	F		Cervical cancer	–	+
14	80	F		Cervical cancer	–	+
Diabetic patients						
1	52	F	4	Renal cell carcinoma	–	–
2	57	M	20	Congestive heart failure	–	+
3	59	M	10	Liver cirrhosis	–	–
4	61	M	18	Gallbladder carcinoma	–	+
5	62	M	10	Gastric cancer	–	–
6	64	M	10	Shy-Drager syndrome	–	+
7	72	M	13	Renal failure	+	+
8	77	F	6	Pneumonia	–	–
9	79	M	5	Congestive heart failure	+	–
10	81	M	41	Esophageal cancer	+	+
11	84	M	9	Colon cancer	+	+

to blocking experiments in which anti-AGE antiserum was first preincubated with AGE-BSA (0.01 to 1 mg/ml) for 1 hour at 37 C. Sections that were stained with anti-AGE antiserum preincubated in this manner were uniformly negative.

Results

Preliminary studies showed that optimal tissue staining for AGEs was obtained by first treating tissue sections with proteinase-K. An enhancement of antibody reactivity after mild proteolysis is consistent with biochemical analyses showing that protein-bound AGEs account in large part for the age- and diabetes-associated increases in connective tissue cross-linking.[11,13,27] Proteolytic digestion serves to expose cross-linking AGE moieties that otherwise are sterically inaccessible to antibody binding. Figure 1 shows a representative study of atherosclerotic plaque present within the coronary artery of a patient with a 13-year history of type II diabetes. Hematoxylin-eosin staining revealed a markedly narrowed arterial lumen and a large intraluminal plaque that showed features of extracellular matrix deposition, cellular proliferation, cholesterol clefts, and calcification (Figure 1A). The arterial intima is irregularly thickened and disrupted by dense plaque material. Immunoperoxidase staining with anti-AGE antibody is shown in Figure 1B. There is positive AGE staining throughout much of the plaque; however, the most intense areas of AGE reactivity are in the regions of fibrous extracellular protein deposits that surround cholesterol clefts. The specificity of the anti-AGE reaction was verified in control experiments, in which it was observed that AGE reactivity was inhibited completely by preincubation of the anti-AGE antibody with the antigen AGE-BSA (not shown). Control sections that were incubated with anti-RNAse antibody, an antibody reactive with the carrier protein for the AGE immunogen, also showed no positive staining (Figure 1C).

Figure 2 shows sections from the abdominal aorta of the same case as that studied in Figure 1. This atheromatous lesion is less advanced and shows a smaller amount of intraluminal protein and lipid deposition than in the coronary atheroma. A prominent degree of endothelial disruption and myointimal proliferation is apparent. There is diffuse staining of AGEs throughout the lesion; however, the greatest density of AGEs appear in areas of matrix deposition. The regions of AGE reactivity virtually delineate the atheromatous area.

AGE immunoreactivity equivalent to that shown in Figure 1 was observed in atherosclerotic plaque that was present in four of 11 autopsied diabetic

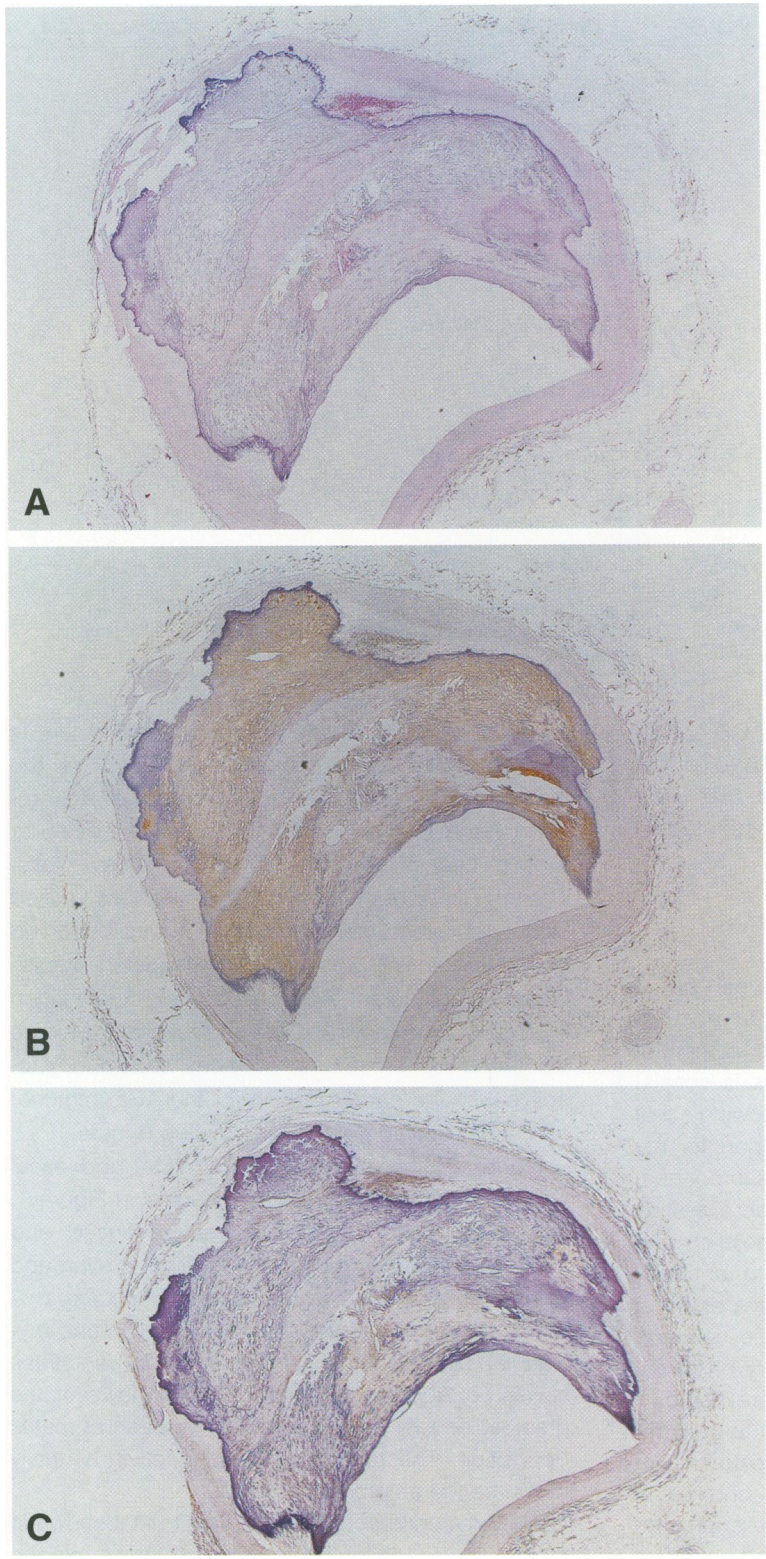

Figure 1. *Transverse sections of left anterior descending coronary artery obtained from a 72-year-old type II diabetic patient (duration of diabetes, 13 years).* A: *Hematoxylin and eosin stain (×10).* B: *Immunohistochemical staining of AGEs in a consecutive section (×10). The specimen was stained with rabbit antibody against AGEs (anti-AGE-RNAse), followed by peroxidase-conjugated anti-rabbit IgG, as described in Materials and Methods.* C: *A consecutive section stained with control rabbit antibody (anti-RNAse). Sections stained with anti-AGE-RNAse antibody that had been preincubated with the antigen AGE-BSA showed the same level of background staining as in* C.

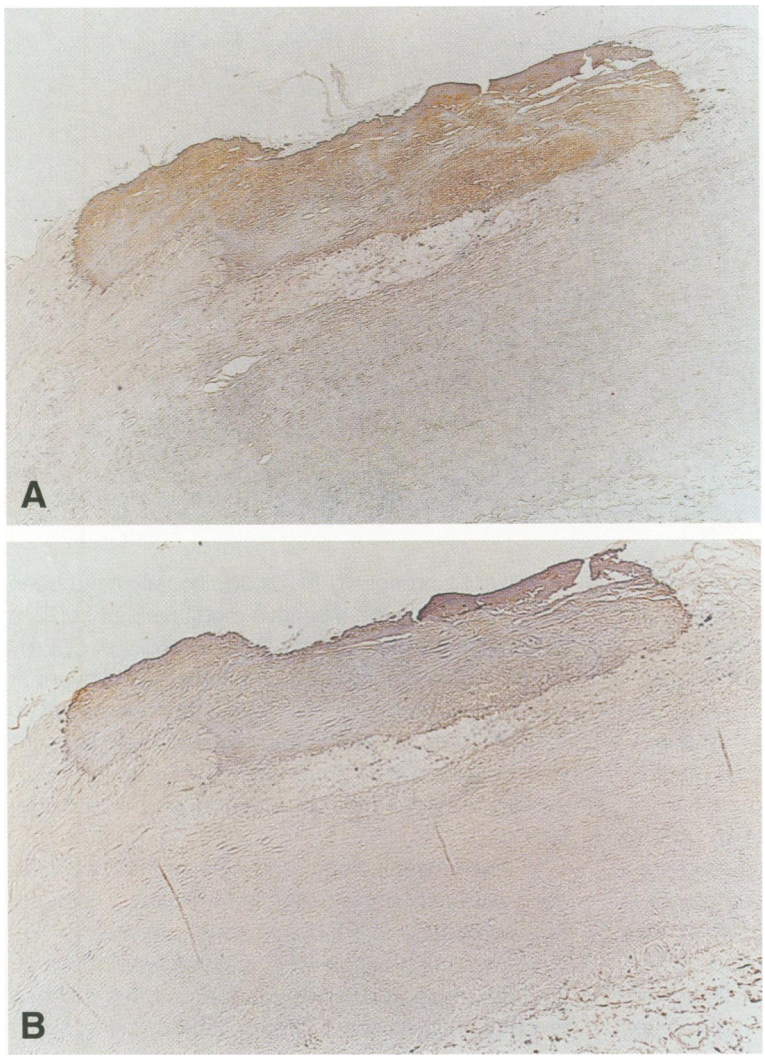

Figure 2. *Immunohistochemical analysis of a proliferative myointimal lesion from a diabetic abdominal aorta.* **A:** Anti-AGE (*anti-AGE-RNAse*) *antibody staining* (×*100*). **B:** *Control antibody* (*anti-RNAse*) *staining* (×*100*).

coronary arteries (Table 1). Interestingly, the positive group represented specimens obtained from individuals who, on the average, were older (79 ± 5 years) and suffered from diabetes for a longer duration of time (17 ± 8 years) than the specimens that showed little or no AGE immunoreactivity (61 ± 8 and 11 ± 3 years, respectively). A survey of vascular lesions identified in the coronary arteries of 14 nondiabetic patients showed no apparent AGE immunoreactivity. Because advanced glycosylation affects matrix proteins ubiquitously as a function of age and ambient glycemia,[11,27] it is likely that the negative tissue staining was the result of tissue-bound AGE levels below the level of detection of immunohistochemical techniques.

Cardiac muscle sections from both diabetic and nondiabetic patients revealed discrete regions of AGE reactivity that localized to intracellular granular deposits present within myocardial fibers (Figure 3). The deposits were generally perinuclear, appeared blue-gray with hematoxylin and eosin, and stained positively with periodic acid-Shiff reagent. These morphological features are consistent with basophilic degeneration. The etiology and clinicopathological significance of basophilic degeneration are unknown, but it is commonly found in cardiac tissue from older individuals.[28] AGE-reactive basophilic degeneration was observed in six of 11 diabetic specimens and five of 14 nondiabetic cardiac specimens (Table 1).

Discussion

The chronic hyperglycemia of diabetes increases the formation of protein-bound AGEs.[9–11] AGEs remain irreversibly bound and, once attached, impart on the protein new structural and functional proper-

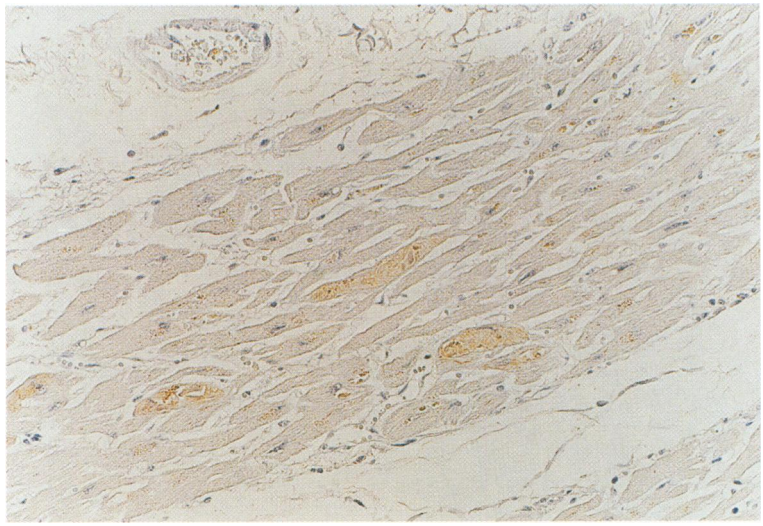

Figure 3. *Cardiac muscle obtained from an 81-year-old man with a 41-year history of diabetes mellitus. Basophilic degeneration of myocardial fibrils was strongly stained with anti-AGE antibody (×250).*

ties. AGEs progressively cross-link connective tissue collagen and contribute to the age-related increase in arterial and connective tissue rigidity that occurs at an accelerated rate in diabetes.[27] AGEs are chemotactic for monocytes and the receptor-mediated uptake of AGEs initiates cytokine-mediated processes that remove senescent macromolecules and promote tissue remodeling.[14] Occupancy of endothelial cell receptors by AGEs increases vascular permeability, down-regulates expression of the anti-coagulant factor thrombomodulin, and increases cell surface expression of the procoagulant tissue factor.[29] Protein-bound AGEs contribute to diabetes-related vascular dysfunction by acting directly to chemically inactivate endothelial-derived relaxing factor (nitric oxide).[6] Aminoguanidine, a pharmacological inhibitor of advanced glycosylation, has been shown in recent studies to ameliorate many of the pathological complications associated with long term diabetes mellitus.[30–33] AGEs, therefore, have been proposed to play a central role in the development of diabetic micro- and macrovascular disease and to contribute to the long term complications of retinopathy, nephropathy, and atherosclerosis.

A role for advanced glycosylation in the initiation and progression of atherosclerosis has been supported further by recent observations that AGEs covalently modify both the phospholipid and the apoprotein components of LDL.[15] Immunochemical measurements of LDL isolated from patients with diabetes have revealed four-fold increases in the level of phospholipid-linked AGEs and two-fold increases in the level of apoprotein B-linked AGEs compared to LDL obtained from normal nondiabetic controls. Phospholipid AGEs additionally have been shown to initiate the oxidative modification of LDL, a process that increases the atherogenicity of LDL by promoting its clearance by macrophage scavenger receptors.[2] Oxidized LDL levels are increased in patients with diabetes and correlate significantly with both lipid and apoprotein B AGE levels, supporting a mechanism for AGE-induced lipid oxidation *in vivo*.[15]

In the present study, anti-AGE antibodies were used in an immunohistochemical analysis of coronary arteries obtained from diabetic (type II) and nondiabetic patients. Positive AGE staining was observed throughout atherosclerotic plaque, with the most intense staining present in areas of fibrous extracellular protein deposits. Intraluminal protein deposition and hyalinization are common features of the atherosclerotic process.[1] The pattern of AGE reactivity that was observed in plaque is consistent with previous observations that AGEs form slowly (days to weeks) and achieve the highest specific activity of modification on long-lived proteins present in the extracellular matrix.[20,23,27] AGEs may accumulate in fibrous areas from advanced glycosylation reactions that occur *in situ* or, alternatively, from the covalent trapping of AGE-modified serum proteins and lipoproteins.[11,13] Both processes may occur simultaneously. The identification of an AGE-modified lipid component on LDL together with the enhanced rate of formation of lipid AGEs compared to protein AGEs allow for the possibility that a significant portion of the AGE reactivity present within atheroma may arise from lipid AGE deposition.[15] Once trapped, lipid AGEs may contribute to pathological effects that have been attributed to protein-linked AGEs. An increase in the subendo-

thelial density of lipid-AGEs would enhance protein deposition and cross-linking, stimulate macrophage-monocyte chemotaxis, and inactivate the vasodilatory effects of nitric oxide (endothelial-derived relaxing factor).[6,12–14,34] Lipid AGEs also may function to stimulate the AGE-specific receptor systems present on macrophages and endothelial cells.[35] Additional effects would then ensue, such as enhancement of vascular permeability and stimulation of local cytokine and growth factor release.[14,29]

An incidental finding in the present study was the observation that within cardiac myofibrils, AGE reactivity localizes to areas of basophilic degeneration. Basophilic degeneration describes a finely granular, basophilic material that occurs within the myocardial cytoplasm. These lesions increase with age and have been identified in varying amounts in 88% or more of individuals over the age of 11 years.[28] The pathological significance of basophilic degeneration is unknown, but it has been reported to be present in greater amounts in patients with hypothyroidism, certain forms of myoclonic epilepsy, and idiopathic myocardiopathy.[28,36] The chemical nature of basophilic degeneration also remains unknown, although histochemical studies suggest that it represents fibrillar material of polysaccharide origin.[28] The present findings suggest that AGEs account for one component of basophilic degeneration and that the inherent cross-linking activity of AGEs may contribute to the insoluble aggregated nature of these deposits.

Taken together with the diverse pathological effects of AGEs, the immunohistochemical identification of AGEs within vascular atheroma support the role of advanced glycosylation in the rapid progression of diabetic vasculopathy. Immunohistochemical analyses of vascular wall AGEs may prove useful in evaluating the role of advanced glycosylation inhibitors, such as aminoguanidine, in ameliorating both diabetic and nondiabetic vascular disease.

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