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Original Articles

Increased Levels of Advanced Glycation Endproducts in the Lenses and Blood Vessels of Cigarette Smokers

Iain D. Nicholl,[1] Alan W. Stitt,[2] Jonathan E. Moore,[3] Andrew J. Ritchie,[4] Desmond B. Archer,[2] and Richard Bucala[1]

[1]The Picower Institute for Medical Research, Manhasset, New York, U.S.A.
[2]The Queen's University of Belfast, Department of Ophthalmology, Belfast, U.K.
[3]The Eye Department, The Royal Victoria Hospital, Belfast, U.K.
[4]Cardiothoracic Surgery Unit, Papworth Hospital, Papworth Everard, Cambridge, U.K.

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Abstract

Background: Advanced glycation endproducts (AGEs) arise from the spontaneous reaction of reducing sugars with the amino groups of macromolecules. AGEs accumulate in tissue as a consequence of diabetes and aging and have been causally implicated in the pathogenesis of several of the end-organ complications of diabetes and aging, including cataract, atherosclerosis, and renal insufficiency. It has been recently proposed that components in mainstream cigarette smoke can react with plasma and extracellular matrix proteins to form covalent adducts with many of the properties of AGEs. We wished to ascertain whether AGEs or immunochemically related molecules are present at higher levels in the tissues of smokers.

Materials and Methods: Lens and coronary artery specimens from nondiabetic smokers and nondiabetic nonsmokers were examined by immunohistochemistry, immunoelectron microscopy, and ELISA employing several distinct anti-AGE antibodies. In addition, lenticular extracts were tested for AGE-associated fluorescence by fluorescence spectroscopy.

Results: Immunoreactive AGEs were present at significantly higher levels in the lenses and lenticular extracts of nondiabetic smokers ($p < 0.003$). Anti-AGE immunogold staining was diffusely distributed throughout lens fiber cells. AGE-associated fluorescence was significantly increased in the lenticular extracts of nondiabetic smokers ($p = 0.005$). AGE-immunoreactivity was significantly elevated in coronary arteries from nondiabetic smokers compared with nondiabetic nonsmokers ($p = 0.015$).

Conclusions: AGEs or immunochemically related molecules are present at higher levels in the tissues of smokers than in nonsmokers, irrespective of diabetes. In view of previous reports implicating AGEs in a causal association with numerous pathologies, these findings have significant ramifications for understanding the etiopathology of diseases associated with smoking, the single greatest preventable cause of morbidity and mortality in the United States.

Introduction

It is estimated that smoking causes 3 million deaths per year worldwide, and this figure is expected to rise 3-fold in the next century, primarily as a consequence of the increased use of tobacco-related products in developing countries (1). Exposure to cigarette smoke has been linked epidemiologically to the increased incidence in smokers of many degenerative conditions including cataract, cardiovascular disease, chronic

Address correspondence and reprint requests to: Dr. Iain D. Nicholl, The Picower Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, U.S.A. Phone: (516)562-9494; Fax: (516)365-5286; E-mail: inicholl@picower.edu

obstructive pulmonary disease, and certain cancers (2–4). The mechanisms by which the chemical constituents of mainstream cigarette smoke compromise cellular and tissue function remain poorly defined at the molecular level, and are likely to result from the interplay of numerous factors (see refs. 5–10 and references therein).

We recently suggested that tobacco products may be a source of reactive glycation products capable of promoting advanced glycation endproduct (AGE) formation in vivo (11). AGEs originate from the spontaneous, nonenzymatic reaction of reducing sugars with the amino groups of macromolecules (i.e., proteins, DNA, lipids). Amadori-type early glycosylation products form first, but additional dehydration, rearrangement, and oxidation reactions occur over time to produce reactive carbonyl-containing compounds that irreversibly cross-link amino groups and form complex heterocyclic structures that are often yellow-brown in color (12,13). AGEs accumulate upon aging, most notably on proteins with relatively long half-lives such as basement membrane collagen, red cell hemoglobin, and lens crystallins (14). AGEs also form in increased amounts as a consequence of hyperglycemia and have been implicated in the pathogenesis of many of the complications associated both with aging and diabetes, including cataract, atherosclerosis, and renal dysfunction (12,13).

In the present study, we attempted to ascertain whether AGEs accumulate in tissues as a consequence of smoking and examined lens and coronary artery specimens from nondiabetic smokers and nonsmokers by fluorescence spectroscopy, immunohistochemistry, immunoelectron microscopy, and ELISA employing several distinct anti-AGE antibodies.

Materials and Methods

Immunoelectron Microscopy of Lenses

Cataractous lenses from nondiabetic individuals were removed by extracapsular cataract extraction. All cataractous lenses exhibited nuclear opacification. Lenses were sectioned in half through the equatorial plane. Sectioned lenses were fixed in 4% paraformaldehyde/0.5% glutaraldehyde in phosphate-buffered saline (PBS; pH 7.4) for 2 hr at 4°C, washed in ice-cold PBS, quickly dehydrated, and embedded in Unicryl resin. Ultrathin sections were cut and mounted on nickel grids. After hydration in PBS and blocking in normal goat serum, sections were incubated with anti-AGE-RNase A sera or anti-RNase A sera (both 1:1000 dilution) overnight at room temperature, washed, and the primary antibody visualized with 15 nm gold conjugated anti-rabbit secondary antibody. Sections were counterstained with lead citrate. Electron micrographs were obtained using a Hitachi H7000 electron microscope. In control studies, anti-AGE antibody reactivity was found to be abolished by preincubating the anti-AGE antibody with the AGE ligand attached to diverse carrier proteins (AGE-RNase A or AGE-BSA) (15).

AGE Levels in Cataractous Lenses from Smokers and Nonsmokers

Cataractous lenses were obtained from nondiabetic smoking (9 males and 8 females; mean age, 72 ± 10 years) and nondiabetic nonsmoking (10 males and 10 females; mean age, 79 ± 11 years) individuals. Lenses were individually homogenized (9000 rpm/10 sec) in 5 ml of ice-cold buffer containing 50 mM sodium phosphate, pH 7.4, 50 mM dithiothreitol (DTT), and 5 mM EDTA. The homogenate was centrifuged at 30,000 × *g* for 30 min at 4°C to remove insoluble proteins and membrane remnants, and the supernatant decanted. The water-soluble fraction (WSF) protein concentration was determined using the BIORAD protein assay according to the manufacturer's directions and employing BSA as the protein standard. WSF protein concentrations were normalized to 0.1 mg/ml with PBS/2 mM EDTA. AGE levels in WSFs normalized for protein concentration were determined using a competitive ELISA employing AGE-BSA as the coating and competing protein as previously described (16,17). Competitive ELISA values were converted to AGE Unit values as described (17). The significance of differences in AGE levels in smokers and nonsmokers was calculated using the Mann-Whitney U test.

Fluorescence Measurements

AGE-associated fluorescence was measured at 440 nm emission with excitation at 370 nm (excitation slit, 5 nm; emission slit, 10 nm) using an LS 50B fluorescence spectrometer (Perkin Elmer) (11). Fluorescence values were measured at a protein concentration of 0.1 mg/ml in PBS/2 mM EDTA solution. Values are in arbitrary units.

Immunohistochemical Analysis of Coronary Arteries

Vascular specimens were fixed in formalin and embedded for wax histology. Sections were dewaxed and rehydrated in PBS and the endogenous peroxidase activity quenched in 3% hydrogen peroxide. Sections were washed in PBS and blocked in 5% normal goat serum (20 min) in an antibody diluent buffer (PBS containing 1% BSA, 0.01% Triton X-100). The anti-AGE antibodies (mAb and pAb) were diluted in antibody diluent (1:200) and added to sections overnight at 4°C in a humidified chamber. For controls, mouse IgG (Sigma Chemical Co.) was added at similar concentrations to the primary antibody or the primary antibody was omitted. Anti-RNase A antibody was used as a control for the polyclonal anti-AGE antibody. After extensive washing, biotinylated anti-rabbit or anti-mouse antibody (F'ab fragment; Dako) was added to the sections at a 1:100 dilution for 1 hr, followed by washing in PBS. Strepavidin was added in the form of the ABC complex (Vector Laboratories) for 1 hr and subsequent detection was carried out by development in 3-amino-9-ethylcarbazol (AEC; Vector Laboratories) which yielded a red reaction product after 15 min at 37°C. After stopping the reaction development by immersion in water, the sections were briefly washed, counterstained with 0.02% fast green, and mounted in Glycermount (Dako). AGE-immunoreactivity (AGE-IR) was scored in a blind fashion by an independent investigator according to an arbitrary scoring system (− = no staining; +, ++, or +++ = little, moderate, and intense staining, respectively). The significance of differences in AGE levels in smokers and nonsmokers was calculated using the Mann-Whitney U test and corrected for ties. In all statistical analyses a value of $p < 0.05$ was considered significant.

Results

To address the hypothesis that AGEs accumulate in vivo as a result of exposure to tobacco smoke (11), we examined cataractous lenses from nondiabetic smokers and nondiabetic nonsmokers for immunoreactivity against AGE epitopes. The lens is an ideal tissue with which to examine AGE formation because the major protein components of lenticular cells, the crystallins, are synthesized from birth and persist over the lifetime of the individual (18). The exceptionally long half-life of these proteins facilitates the characterization of post-translational modifications that may accumulate in situ over prolonged periods (18,19). Consequently, the lens can be regarded as an "integrator" of protein modification and thus an ideal system with which to examine the accumulation of AGEs. The lens crystallins have been shown previously to exhibit increased glycation as a consequence of diabetes and aging (19–23).

Human cataractous lenses were obtained by extracapsular extraction, fixed in a paraformaldehyde/glutaraldehyde mixture, and processed for immunoelectron microscopy, using a previously characterized polyclonal anti-sera raised to AGE-modified ribonuclease A (RNase A) (16). Specimens obtained from smokers (Fig. 1C, D) consistently exhibited greater reactivity to the AGE-RNase A antisera than specimens from nonsmokers (Fig. 1A, B). Sections from smokers failed to react with the control antibody (Fig. 1E, F), or with anti-AGE antibody that had been preadsorbed with the AGE-immunogen, indicating that immunoreactivity is specific for proteins containing AGE moieties. Immunoreactivity was broadly distributed throughout the lenticular cells, with no preferential localization at subcellular structures.

To place the analyses of AGE levels in lenses on a quantitative footing, water-soluble fractions were prepared from cataractous lenses (18) and analyzed by a previously described competitive ELISA for AGEs (16,17). We utilized three different AGE-specific antibodies for the analysis; a polyclonal antibody raised against AGE-modified RNase A, and distinct polyclonal and monoclonal antibodies raised against AGE-modified keyhole limpet hemocyanin (KLH) (16,17). It has been previously demonstrated that these antibodies do not react with Amadori products or other early glycation products, but are reactive with different AGE-epitopes that form in vivo, such as cypentidone and a covalent, imidazole-based arginine-lysine cross-link (17,24). The water-soluble fractions were prepared by briefly homogenizing lenses in a PBS-based buffer containing high levels of the reducing agent, DTT, and the cation chelator EDTA to minimize activation of lens calcium-activated proteases. The use of reducing agents decreases the level of high-molecular-weight protein aggregates characteristic of cataractous lenses, facilitating the analysis of proteins that have undergone post-translational modification (25). Indeed, aggregate formation has been suggested to be a consequence of an increased susceptibility of lens proteins to sulphydryl oxidation after nonenzymatic glycation

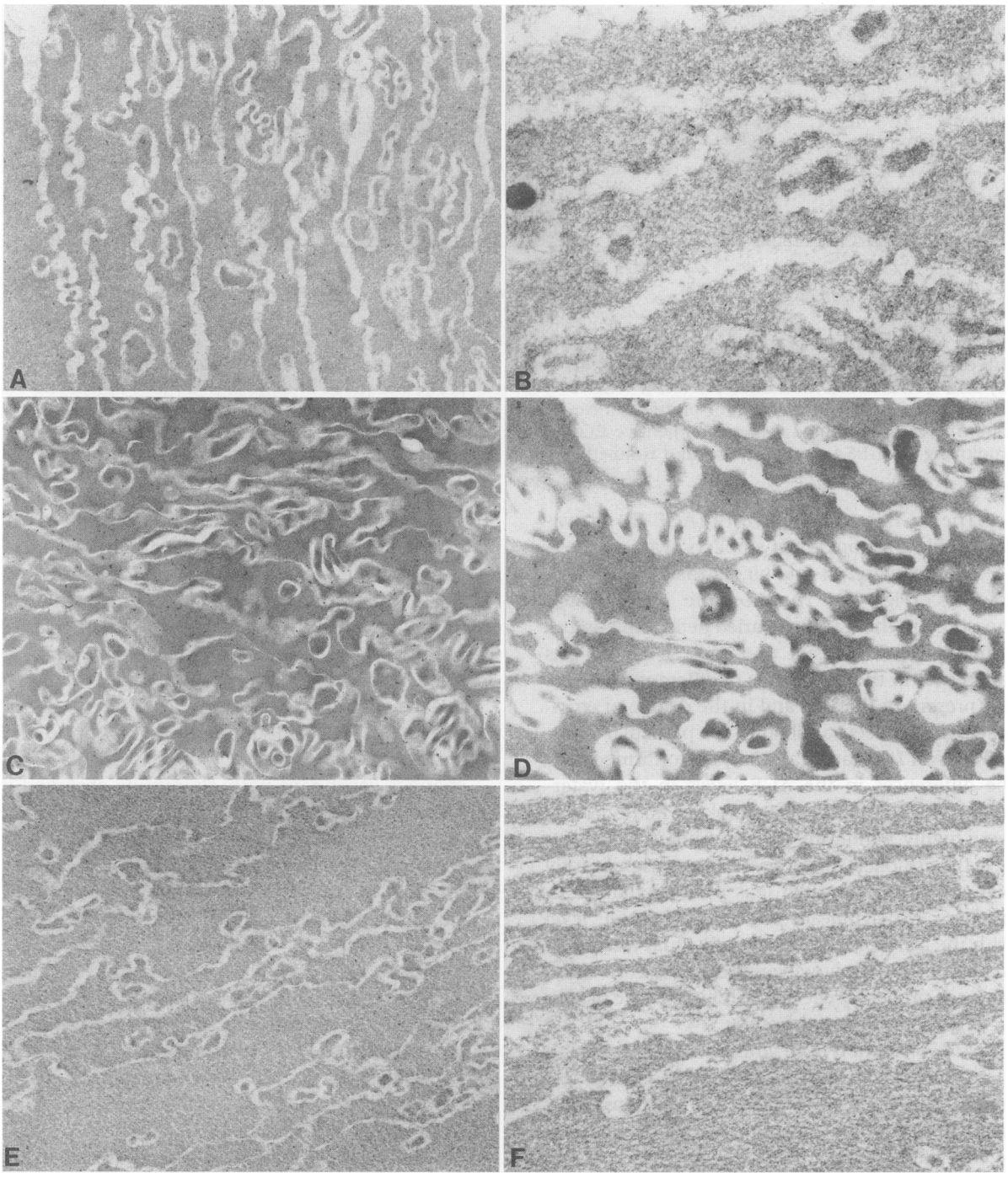

Fig. 1. Immunoelectron microscopy of AGEs in cataractous lenses obtained from smokers and nonsmokers. Processing of lenses for electron microscopy is described in Materials and Methods. **(A, B)** Lens sections from nondiabetic nonsmokers. **(C, D)** Lens sections from nondiabetic smokers probed with the polyclonal anti-AGE-RNase A serum. **(E, F)** Sections from nondiabetic smokers probed with an anti-RNase A control serum (reacting only with the AGE-carrier protein). Magnification, A, C, E: ×9000; B, D, F: ×15,000.

(25), and tryptophan fluorescence and circular dichroism studies have confirmed that substantial conformational changes occur in lens crystallins upon glycation (26,27).

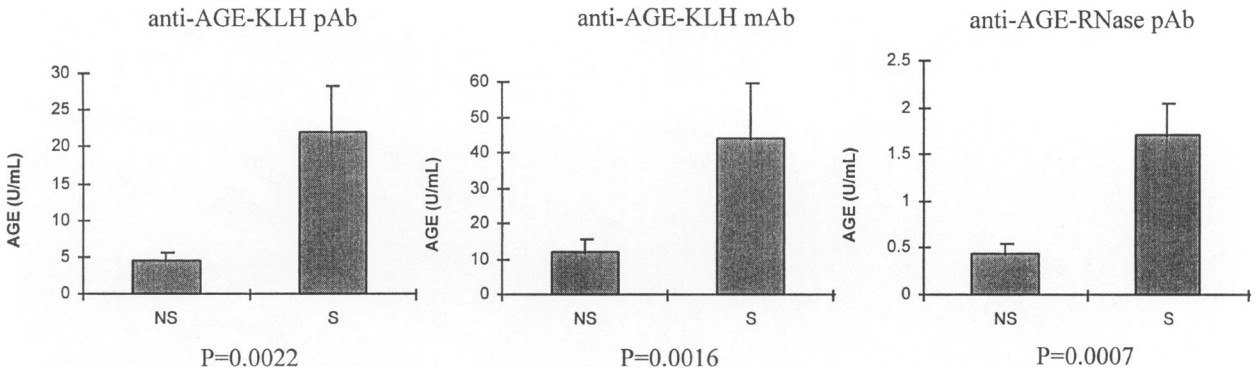

Fig. 2. AGE content in extracts prepared from the cataractous lenses of nondiabetic smokers (S) and nondiabetic nonsmokers (NS). AGE levels in supernatants (normalized for protein concentration) were determined using a competitive ELISA employing AGE-BSA as the coating and competing antigen and the values obtained converted to AGE Units as previously described (17). Values are expressed as mean ± SEM (n = 20 for nondiabetic nonsmokers; n = 17 for nondiabetic smokers). The p value compares AGE levels in extracts from nonsmokers versus smokers.

The AGE-immunoreactivity (AGE-IR) of water-soluble fractions from smokers was, on average, approximately 4-fold higher than that of nonsmokers (Fig. 2). The absolute AGE values measured in the fractions varied depending on the antibody used, a result that most likely reflects differences in the prevalence of different AGE species present in the lens and in the degree of cross-reactivity between antibodies for particular AGE-epitopes (17). Statistical analysis confirmed that the differences in the AGE levels in the water-soluble fractions from nonsmokers and smokers were highly significant (Fig. 2). These results suggest that smokers have accumulated significantly higher levels of glycated lens proteins than nonsmokers. No significant correlation in AGE level with the age or gender of an individual was noted (data not shown); however, the lack of correlation with age probably reflects the limited range of subject age examined in the study.

AGE formation from glucose is associated with increased protein (non-tryptophan based) fluorescence (12,14). The water-soluble fractions (normalized for protein concentration) were thus analyzed further by fluorescence spectroscopy. Extracts from smokers exhibited significantly higher AGE-associated fluorescence than nonsmokers (95 ± 20.9 units versus 77 ± 20.5 units; Mann-Whitney U test, p = 0.005)—further, independent evidence that smokers' lenses contain substantially higher levels of AGEs or AGE-like molecules than the lenses of nonsmokers.

AGE deposition on vascular connective tissue has been proposed to play an important role in the accelerated development of atherosclerosis in diabetics (12,28). In support of this hypothesis, AGE products have been localized to the vascular wall matrix and to the apolipoprotein-B (ApoB) and phospholipid components of low-density lipoproteins (LDL) where they have been found to delay LDL clearance from plasma (15,29). AGEs can also quench nitric oxide activity, thus detrimentally modulating endothelial relaxation (30). Furthermore, administration of exogenous AGE-modified proteins to nondiabetic rats and rabbits induces vascular wall changes consonant with alterations observed in diabetes and aging, including significant increases in vascular permeability and mononuclear cell migratory activity (31). The marked increase in lenticular glycation in smokers, together with the likelihood that lenticular AGE levels reflect an individual's lifetime AGE burden, thus prompted us to examine coronary arteries from nondiabetic smokers and nonsmokers for the presence of AGEs by immunohistochemistry. It is worth noting that anti-AGE antibodies have been used successfully in the past to identify AGE-IR in human coronary, carotid, renal, and retinal vasculature (15,32,33). AGE-IR was significantly higher in age-matched coronary arteries from nondiabetic smokers (p = 0.015), with immunoreactivity concentrated in the intima and in the endothelial cells of the vessel walls (Fig. 3).

Discussion

These findings markedly extend prior observations suggesting that cigarette smoke is an exogenous source of reactive glycation products (11).

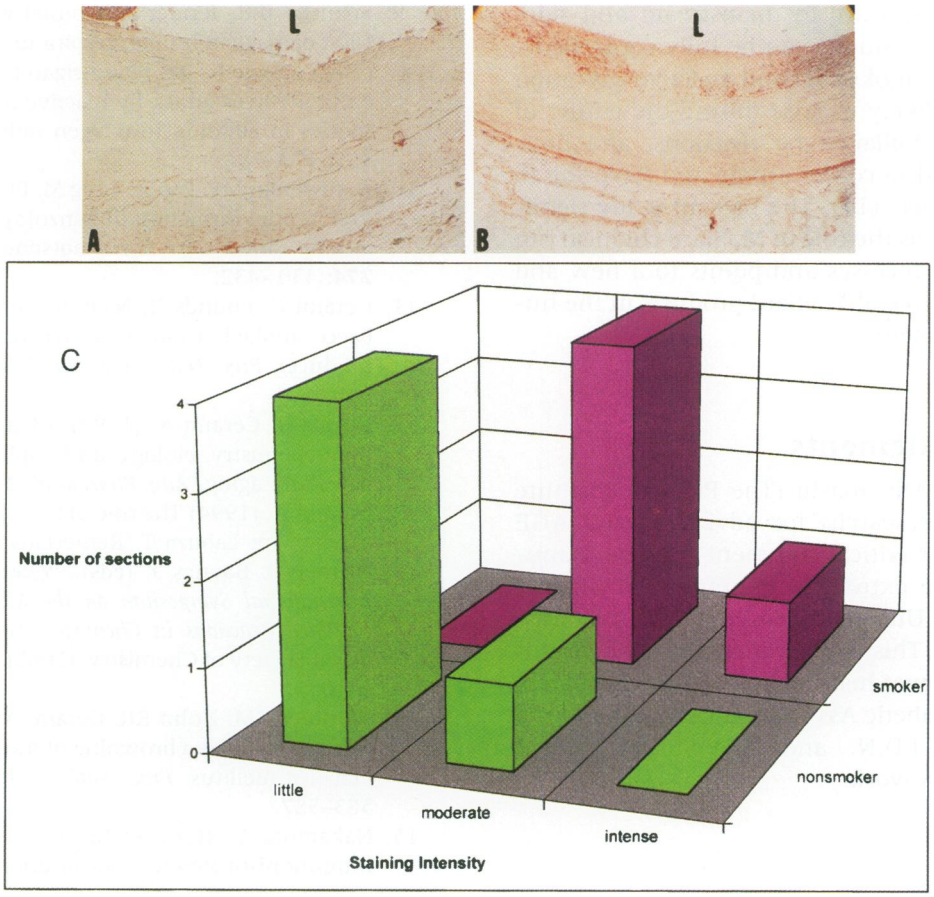

Fig. 3. AGE-immunoreactivity (AGE-IR) is increased in the coronary arteries of nondiabetic smokers. Processing of arterial sections for anti-AGE immunohistochemical analysis is described in Materials and Methods. Anti-AGE- and AEC-developed sections were examined by light microscopy. Representative results of AGE-IR in a nondiabetic nonsmoking patient **(A)** and a nondiabetic smoking patient **(B)** are shown. The lumen (L) of the vessel is indicated. Neither patient had a history of diabetes. There is extensive immunoreactivity (shown as a red reaction product) within the vessel wall of the coronary artery from the smoker. AGE-IR of the coronary artery from the nonsmoking patient is greatly reduced compared with the smoker. Magnification, ×400. **(C)** Composite AGE-IR in sections of coronary arteries stained with anti-AGE antibodies. Sections were from nondiabetic nonsmoking patients (n = 5), and nondiabetic smoking patients (n = 5). AGE-IR was graded per section in a blind fashion by an independent investigator. AGE-IR was significantly elevated in the sections from smokers compared with nonsmokers (p = 0.015).

As glycation compromises the chaperone function of α-crystallin, which is believed to be critically important in maintaining lenticular transparency (34,35), cigarette smoke–mediated AGE formation in α-, β- and γ-crystallins may act in concert with oxidative stress (2,3,5,6) and heavy metal deposition (36,37) to contribute to the higher incidence of cataract noted in smokers. Further work will be necessary to characterize the molecular structure of the AGE or AGE-like species that are formed on macromolecules as a consequence of exposure to cigarette smoke. Pertinent to this point is the observation that the increase in AGE-associated fluorescence noted in extracts from smokers is less than the increase in AGE levels as assayed by ELISA. This indicates that the major AGE species present in the lenticular extracts are probably not strongly fluorogenic.

In view of reports indicating that AGE accumulation may be causally related to atherosclerosis (reviewed in ref. 38), we hypothesize that the increased AGE-IR observed in the vasculature of smokers may contribute to the accelerated development of atherosclerotic lesions in this patient population. Further study will be necessary to fully test this hypothesis, most likely in animal model systems where duration and

intensity of exposure to mainstream and sidestream cigarette smoke can be fully controlled.

Cigarette smoke–derived reactive glycation products are likely to arise during the curing of tobacco via Maillard-type reactions, and have been proposed to confer aroma and flavoring to tobacco products (11). The present study significantly broadens the role of Maillard chemistry in pathological processes and points to a new and significant source of Maillard products in the human environment.

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