

Increased Accumulation of the Glycooxidation Product N^ε-(carboxymethyl)lysine in Human Tissues in Diabetes and Aging

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Abstract

N^ε-(Carboxymethyl)lysine (CML), a major product of oxidative modification of glycated proteins, has been suggested to represent a general marker of oxidative stress and long-term damage to proteins in aging, atherosclerosis, and diabetes. To investigate the occurrence and distribution of CML in humans an antiserum specifically recognizing protein-bound CML was generated. The oxidative formation of CML from glycated proteins was reduced by lipoic acid, aminoguanidine, superoxide dismutase, catalase, and particularly vitamin E and desferrioxamine. Immunolocalization of CML in skin, lung, heart, kidney, intestine, intervertebral discs, and particularly in arteries provided evidence for an age-dependent increase in CML accumulation in distinct locations, and acceleration of this process in diabetes. Intense staining of the arterial wall and particularly the elastic membrane was found. High levels of CML modification were observed within atherosclerotic plaques and in foam cells. The preferential location of CML immunoreactivity in lesions may indicate the contribution of glycooxidation to the processes occurring in diabetes and aging. Additionally, we found increased CML content in serum proteins in diabetic patients. The strong dependence of CML formation on oxidative conditions together with the increased occurrence of CML in diabetic serum and tissue proteins suggest a role for CML as endogenous biomarker for oxidative damage. (*J. Clin. Invest.* 1997. 99:457–468.) Key words: glycation • oxidative stress • complications of diabetes • N^ε-(carboxymethyl)lysine • atherosclerosis

Introduction

With advancing age many organs such as the lungs, arteries, skin, joints, and bones lose their elasticity. These alterations are accelerated in diabetic patients suffering from reduced lung elasticity, limited joint mobility, and particularly from arterial stiffness and premature atherosclerosis. The observation that the extracellular matrix of the affected tissues in diabetic patients—mainly consisting of long-lived collagens—exhibit

increased nonenzymatic glycosylation (glycation) has provided the basis of an interesting hypothesis linking hyperglycemia with the development of late complications (1–4). This hypothesis became even more attractive when it could be demonstrated that the extent of glycation of the tissues correlated with the extent of late complications the patients had suffered from (4). However, there was only a marginal age-dependent increase in glycation of connective tissue proteins, e.g., in aorta or tendon (5), human skin collagen (6–7) and human lens proteins (8) in nondiabetic individuals suggesting that glycation is unlikely to be directly involved in the development of age-related diseases. Later on, it became apparent that the glycation reaction may proceed further to initiate a complex series of oxidative, cleavage, and rearrangement reactions which lead to the accumulation of brown and fluorescent products, termed advanced glycosylation end (AGE)¹ products, in diabetes and aging (9–17). The identification of individual AGE products has led to the isolation and characterization of the fluorescent pentosine and pyrroline (18,19). Furthermore, using the model compound fructose-lysine (FL), it could be shown that the carbohydrate moiety of glycated proteins is oxidatively cleaved between carbon 2 and 3 yielding N^ε-carboxymethyllysine (CML) and erythronic acid (20) (see Fig. 1). The reaction (glycooxidation) proceeding only in the presence of oxygen was shown to occur by a free radical mechanism involving superoxide radicals and hydrogen peroxide (21). Since the formation of CML modification in proteins is irreversible, it has been suggested that CML may be an integrative biomarker for the accumulated oxidative stress the respective tissue had been exposed to (22, 23).

Using specific and sensitive assays based on selected ion monitoring gas chromatography, mass spectrometry, CML has been quantitated in hydrolyzed samples of human skin and lens (6–8), and in rat (24) and human urine (25). Both CML and pentosidine accumulate naturally in skin and lens tissue proteins with age and at an accelerated rate in diabetes (6–8, 26). Increases in age-adjusted levels of both CML and pentosine were correlated with the severity of retinopathy and nephropathy in diabetes suggesting that glycooxidation reactions and oxidative stress may be important mechanisms in the pathogenesis of diabetic complications (7, 27–29) and possible age-related degenerative processes. To investigate the effect of age and diabetes on CML-accumulation and localization in human tissues we produced CML antisera in rabbits and characterized their specificity by *in vitro* experiments and by immunohistochemical analysis. Thereby, this specific antiserum was applied to localize CML in skin, lung, heart, kidney, intestine,

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1. Abbreviations used in this paper: AGE, advanced glycosylation end; CML, N^ε-(carboxymethyl)lysine; FL, fructose-lysine; KHL, key-hole limpet hemocyanin.

arteries, and intervertebral discs obtained from diabetic and nondiabetic individuals of different ages. Furthermore, we developed an ELISA system for the determination of CML concentrations in human serum and found elevated CML values in diabetic patients.

Methods

Materials. Na(CN)BH₃, 3-amino-9-ethyl-carbazole, glyoxylic acid, BSA, desferrioxamine, aminoguanidine, vitamin E, glycine, alanine, sarcosine, and lysine were purchased from Sigma Chemical Co. (Deisenhofen, Germany). Bovine catalase, bovine superoxide dismutase, and keyhole limpet hemocyanin (KHL) were from Calbiochem (Bad Soden, Germany). FL was prepared as described recently (30) and CML was a gift from Dr. Erbersdobler (University of Kiel, Kiel, Germany).

Synthesis of CML-modified proteins. HSA, BSA, and KHL were carboxymethylated as described previously (31). Briefly, 10 mg HSA, BSA (66 kD) or KHL (9–27 × 10⁶ kD) were dissolved in 2 ml 0.3 M sodium tetraborate pH 9.2 at 0°C and 1 mg Na(CN)BH₃ was added. 12 μmoles glyoxylic acid were then added every 6 min. After 60 min the reaction mixture was stirred for further 30 min and the proteins were extensively dialyzed before use. The extent of CML modification was estimated as described (31).

Preparation of antisera and antibodies and characterization by ELISA. Antisera against CML-modified BSA or CML-KHL were raised in rabbits as described recently (31). For preparation of antibodies the CML antiserum (KHL) was passed through a column containing CML-BSA coupled to Sepharose. Accordingly, the CML antiserum (BSA) was applied to a column loaded with CML-KHL. CML antibodies (KHL) or CML antibodies (BSA) were eluted with 0.2 M glycine buffer, 2.5 pH, neutralized, and dialyzed. Positive antisera or immunopurified antibodies against both CML-modified proteins were analyzed by enzyme-linked immunoassay. Therefore, 100 μl of CML-BSA or HSA-CML (20 μg/ml) was dispensed in each well of a microtiter plate as coating reagent and incubated overnight at 4°C. After washing three times with PBS/Tween, 200 μl of 2.7% crotein (Boehringer, Mannheim, Germany) were applied into each well and incubated for 1 h. After further washings, 100 μl of antiserum (1:1,000) or purified antibody (1:80) was applied to each well and incubated for 2 h at room temperature. Subsequently, three washings with PBS/Tween were performed. Then, 100 μl of goat anti-rabbit IgG conjugated with peroxidase (1:8,000) was added to each well and incubated for 2 h. After eight washings 100 μl of phenylenediamine substrate solution (1.68 mg/ml) was added and the plate was incubated for 30 min in the dark. The absorbance was measured at 450 nm by a microplate reader (model MR 600; Dynatech Laboratories, Inc., Chantilly, VA) using 650 nm CML-antiserum as reference wavelength. The specificity of the antisera or immuno-purified antibodies was analyzed by competitive ELISA. The antiserum/antibody was preincubated with increasing amounts of CML-HSA or free hapten or with CML analogues for 1 h at room temperature. Solid phase-bound rabbit IgG was quantified as described above.

Quantification of CML formation by ELISA. Wells of microtiter plates were coated with 5 μg of the CML-modified protein in 0.1 ml carbonate buffer and incubated overnight at 4°C. After several washings the microtiter plates were blocked as described before and 0.1 ml CML antiserum (1:1,000) was added and incubated for 2 h at room temperature. Quantification of bound rabbit IgG was determined as described before.

Time course of CML-formation. Solutions of 2.5 ml HSA or lens crystallin (20 mg/ml) were incubated with 5.5, 22, and 55 mM D-glucose in 5 ml 0.14 M NaCl, 5 mM phosphate buffer, pH 7.4 (PBS), containing penicillin (100 U/ml) and streptomycin (0.1 μg/ml). The solutions were sterilized by filtration through 0.2 μm pore membrane into sterile conical tubes and incubated at 37°C in the dark for the time periods indicated. Incubation with 55 mM sorbitol was used as control. Oxygen-free conditions were produced by bubbling the final reaction

mixture with argon gas for 5 min and then sealing under a stream of argon. Test samples were taken at various time points as indicated and stored at –20°C until analysis.

Effect of various compounds on the formation of CML modification. A solution of 20 ml HSA (50 mg/ml) and 555 mM glucose in PBS were incubated at 37°C under oxygen-free conditions (see above). After 7 d the glycated HSA was dialyzed under argon atmosphere and diluted yielding a HSA solution of 20 mg/ml. 0.5 ml of the test agent solved in PBS was added to an equal volume of glycated HSA (20 mg/ml) and incubated at 37°C. Samples were removed at various time points and stored at –20°C until analysis.

Tissue samples. For the immunohistochemical analysis, we used formalin-fixed and paraffin-embedded tissues that had been obtained either at autopsy or as biopsy material for routine histopathological investigation. As normal tissue, we analyzed in this study skin, lung, heart, kidney, bone, and intervertebral discs from otherwise healthy fetal (22–30 wk of gestation), juvenile (8 yr), and adult patients (30–82 yr). In addition, liver, spleen, intestine, pancreas, aorta, and skeletal muscle samples were investigated from juvenile (8 yr) and various adult patients (30–82 yr), but not from fetal controls, whereas we also analyzed placental tissue from the fetuses. Only patients without any indication for hyperglycemic conditions in their clinical records were included. As a further study group, we similarly analyzed kidney, aorta, and skin tissue samples from eight diabetic patients with long-standing diabetes mellitus. The data from this patient group are given in detail in Table III.

Immunohistochemical methods. The immunohistochemical localization of CML-modified proteins was performed using the avidin-biotin, complexed peroxidase staining method (ABC method) for paraffin-embedded tissue as established (32). 3 μm tissue sections were deparaffinized and incubated with 0.1% pronase in PBS at 37°C for 15 min and for further 15 min at room temperature. Subsequently, the endogenous biotin was blocked by 5% H₂O₂ for 10 min. After washing steps and further blocking with normal goat serum (1:50 for 30 min), the slides were incubated overnight with a 1:300 dilution of CML-antiserum in a humid chamber. After further washings, the slides were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Inc., Burlingame, CA) for 30 min, rinsed with PBS, and incubated for 30 min at room temperature with avidin biotinylated horseradish peroxidase H reagent (Vectastain, Elite ABC kits; Vector Labs). A final PBS washing was performed. The color reaction was carried out by incubating the slides with freshly prepared 3-amino-9-ethyl-carbazole reagent. Finally, the nuclei were counterstained with hematoxylin. For competitive blocking experiments a 1-h preincubation period by addition of CML-BSA (5 μg/ml) to the CML-antiserum was carried out before application to the tissue sections. In some tissues, the immunostaining was quantified by evaluating the positively stained area of the sections at the same conditions of light intensity of the microscope using a semiautomatic procedure with an image analyzer (Vidas 25; Kontron Elektronik, Munich-Eching, Germany). Accordingly, five randomly selected areas of dermal connective tissue, blood vessel walls, and intervertebral discs of each case were analyzed.

Determination of CML in human serum by competitive ELISA. The assay was performed in analogy to the procedure described recently (33). Microtiter plates were coated with 0.1 ml CML-HSA (0.5 μg/ml) in 0.1 M carbonate buffer, pH 9.6, and incubated overnight at 4°C. Wells were washed with PBS/0.5% Tween 20 and blocked with 1% BSA in PBS for 1 h at room temperature. After washing 0.05 ml of the 1:5 diluted samples and 0.05 ml of 1:1,500 diluted rabbit antiserum were added to the wells. Plates were incubated for 2 h at room temperature and overnight at 4°C. Wells were washed with PBS/Tween and developed with a peroxidase-conjugated anti-rabbit IgG using *o*-phenylene diamine as substrate. For calibration serial dilutions of CML-modified HSA was used (31). 35 sera from diabetic individuals without any indication of nephropathy and 21 age- and sex-matched control sera were provided by the diabetes ambulatory unit. Furthermore, 25 sera from uremic patients (13 diabetic and

12 nondiabetic) were analyzed. HbA1c was determined by the Diamat system (BioRad, Munich, Germany).

Results

Characterization of CML-antisera. Our analysis clearly shows that the rabbits immunized with CML-modified BSA or CML-modified KHL produced specific antibodies to CML-modified proteins (Fig. 1). The evaluation of both antisera indicated that the immunization with both antigen preparations yielded antisera with comparable titers (Fig. 2A). Quantitative comparison of protein-bound and free CML (Fig. 2B) revealed that ~50-fold higher concentration of the free hapten is needed to obtain similar competition values as with HSA-bound CML indicating that the antisera recognize protein-bound CML with high affinity. The specificity of both CML-antisera was assessed by preincubation with increasing amounts of free hapten or structurally related compounds (Fig. 3; Table I). Clearly, CML competed for the binding of both CML-antisera to CML proteins at CML concentrations below 1 mM, while lysine and FL had no effect. Because glycine and particularly sarcosine—but not the stereoisomer alanine—showed minor but significant competition, it appears that the presence of the amino-carboxymethyl-structure is absolutely necessary for binding. Obviously, alkylation of the amino group increases the cross-reactivity substantially. Both antibodies obtained by immunosorption cross-reacted with sarcosine and glycine with higher affinity than the corresponding CML-antisera (Table I). Because of the lower stability of the immunoabsorbed antibodies we used the CML-antisera, particularly the CML-antiserum (KHL) for the investigation of the glucoxidation reaction in model systems and for the localization and quantification of CML-modified proteins in human tissues and serum samples.

Formation of CML in glycated proteins in the presence and absence of oxygen. The time-dependent formation of CML is shown in Fig. 4. Obviously, slow but significant, time-dependent formation of CML may be observed when HSA is incubated with 55 mM glucose under physiological (aerobic) conditions. Lower CML-formation is found when incubation was performed with 22 mM glucose. In the presence of normal glucose concentrations (5.5 mM) no significant formation of CML could be detected after 14 wk. Since incubation under an argon

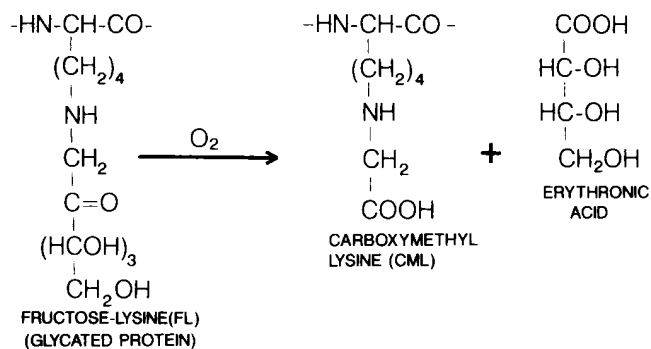


Figure 1. Formation of N^ε-(carboxymethyllysine)-modified proteins. In the presence of oxygen the FL moiety of glycated proteins is slowly oxidized yielding CML and erythronic acid. Because of the role of glucose and oxygen in their formation, CML is a glycoxidation product (see Fig. 4) and considered as biomarker for the oxidative damage to proteins.

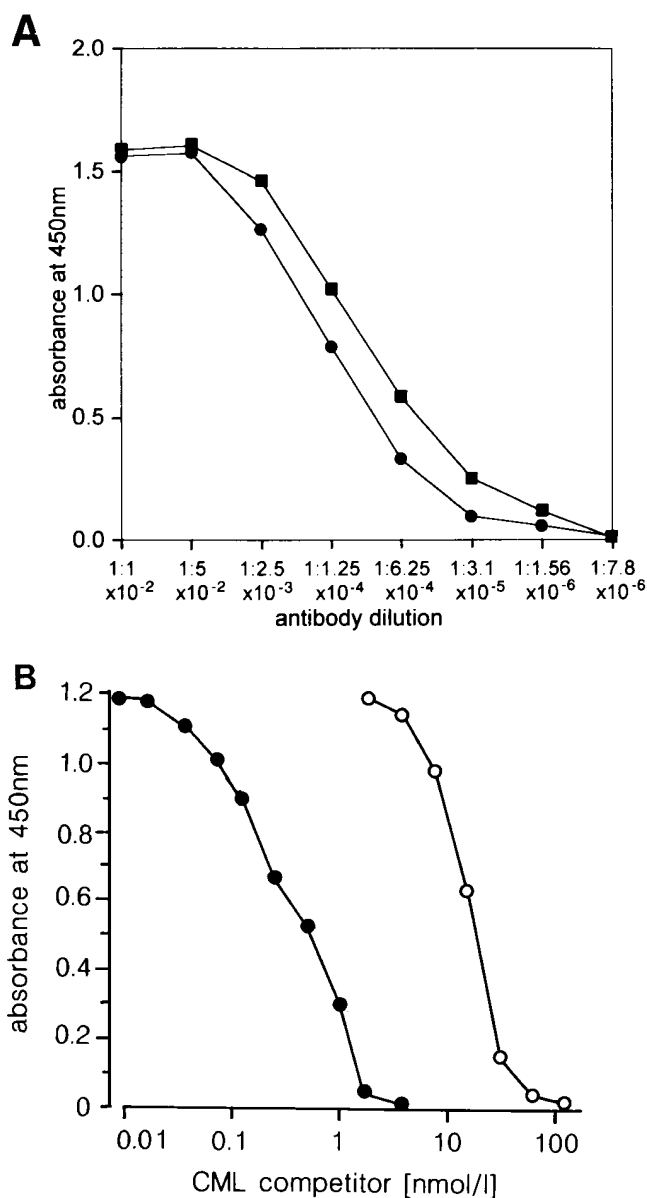


Figure 2. Titer curves of CML-antiserum (A) and specificity of the antiserum for protein-bound CML (B). (A) CML-antisera were raised in rabbits by immunization with CML-KHL (closed circles) or CML-BSA (closed squares). Binding of antisera to CML-modified proteins was determined as described in the method section. (B) Increasing amounts of free (open circles) or HSA-bound CML (closed circles) are added in the competition ELISA as described in Methods.

atmosphere or incubation with 55 mM sorbitol did not lead to formation of CML within 14 wk, this result suggests that both the presence of glucose and oxygen is necessary for the formation of CML. Similar results were obtained with lens crystallins (data not shown) indicating a minor influence of the matrix protein on the formation of CML.

Effect of various inhibitors on the formation of CML-modified proteins. Furthermore, we analyzed the formation of CML from preglycated protein in the absence of glucose to exclude the possibility that the formation of CML is derived from reactive intermediates formed by the reaction of oxygen with glucose. These glucose oxidation products may yield CML

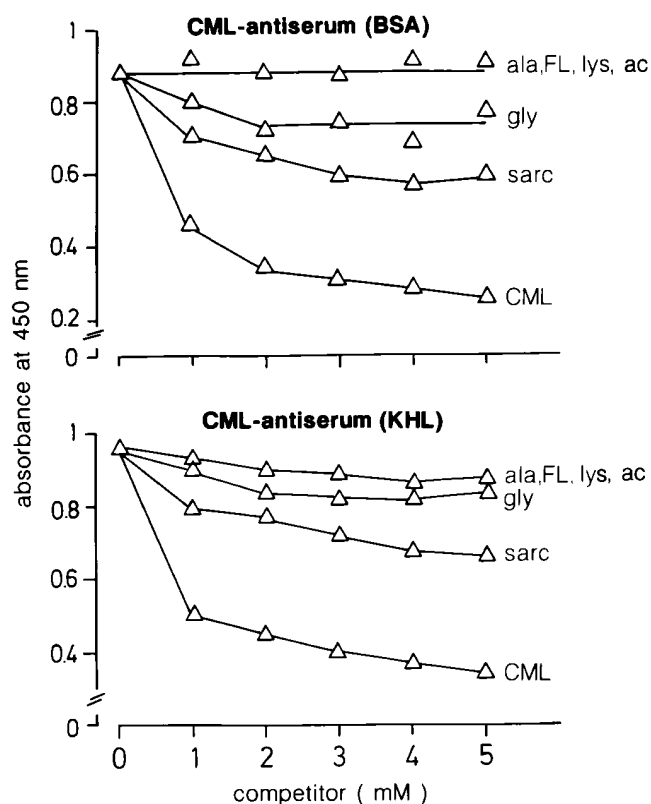


Figure 3. Characterization of CML-antisera. The ELISA curves represent the ability of CML and structural analogues to inhibit binding of the CML-antiserum (KHL) to immobilized CML-BSA and CML-antiserum (BSA) to CML-KHL, respectively. CML, carboxymethyl-lysine; ala, alanine; gly, glycine; sarc, sarcosine; lys, lysine; ac, acetate; FL, fructose-lysine.

upon direct reaction with the amino group of lysine (34). In addition, we used high glucose concentrations, short incubation times, and anaerobic conditions to minimize the CML formation during preparation of glycated HSA. As seen with incuba-

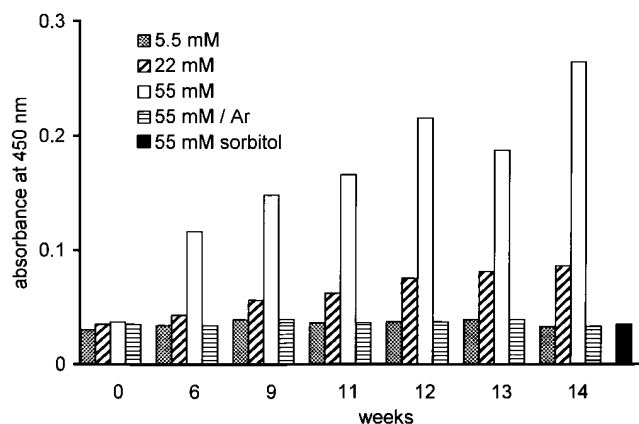


Figure 4. Time-dependent formation of CML-HSA in the presence of glucose under aerobic and anaerobic conditions. Formation of CML in HSA incubated under physiological conditions with glucose at normal (5.5 mM) and elevated (22 and 55 mM) concentrations. Furthermore, incubation with 55 mM was also performed in the absence of oxygen, e.g., under argon atmosphere. Incubation with 55 mM sorbitol was used as control. The HSA concentration was 20 mg/ml. The ELISA was performed as described in the method section.

tions of HSA with glucose (Fig. 4) the time-dependent formation of CML was also obtained upon aerobic incubation of preglycated HSA (Fig. 5). Presence of reducing agents had different effects on the formation of CML. While addition of α -lipoic acid or aminoguanidine decreased formation of CML by $\sim 30\%$ vitamin E was much more efficient; the presence of 20 and 50 $\mu\text{g/ml}$ vitamin E reduced the formation of CML by 70 and 89%, respectively. Catalase and superoxide dismutase decreased the rate of formation of CML by $\sim 50\%$ and desoxyferamine blocked the formation of CML completely (Fig. 5).

Localization of CML-modified proteins in normal tissues. Initial immunohistochemical experiments were performed without proteolytic pretreatment. Using a pronase treatment we obtained similar staining results but with better contrast. Therefore, this procedure was used throughout this study. The

Table I. Specificity of CML-antisera and CML-antibodies

Competitor	Structure	CML antisera		CML antibodies	
		(KHL)	(BSA)	(KHL)	(BSA)
CML	NH_2 $^9\text{O}_2\text{C}-\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}-\text{CH}_2-\text{C}-\text{O}^9$ H	100	100	100	100
Sarc	H-CH ₂ -N-CH ₂ -C-O ⁹ H	48	44	87	100
Gly	H-N-CH ₂ -C-O ⁹ H	20	18	58	84
Ala	H-N-CH-C-O ⁹ CH ₃	12	0	0	0
Acetate	H-CH ₂ -C-O ⁹ O	0	0	0	0
Lys	NH ₂ $^9\text{O}_2\text{C}-\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}-\text{H}$ H	12	0	0	0
FL	NH ₂ $^9\text{O}_2\text{C}-\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}-\text{CH}_2-\text{C}-\text{O}^9$ H	12	0	0	0

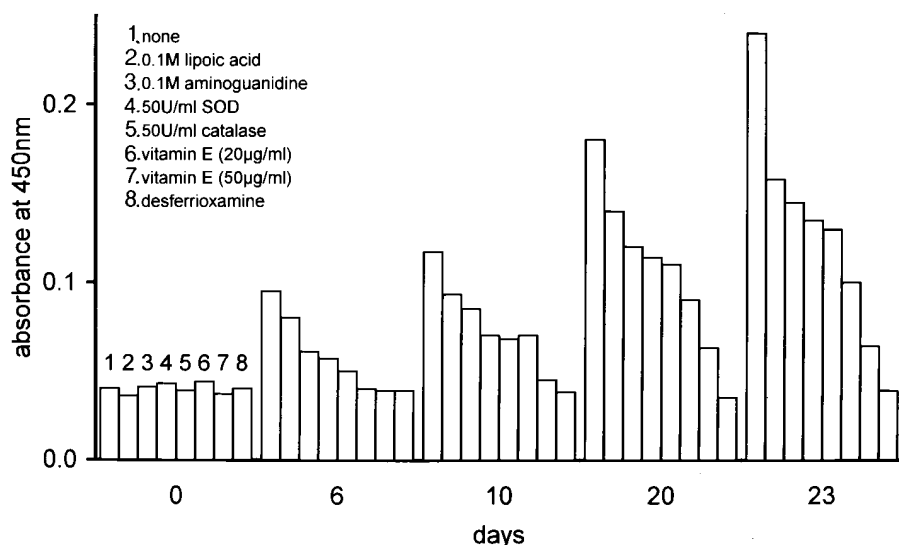


Figure 5. Effect of various compounds on the oxidative formation of CML in gly-cated HSA. HSA was preglycated under argon atmosphere for 7 d and dialyzed against glucose-free buffer, 7.4 Ph. Test agents were added and samples were taken at the time points indicated. HSA bound CML content was determined by ELISA.

immunolocalization of CML-modified proteins in normal fetal, juvenile, and adult tissues revealed a clearly age-dependent staining pattern (Figs. 6–8). Significant positive results were only seen in tissues from adults. The competitive inhibition of the staining by preincubation of the CML-antiserum (KHL) with CML-BSA completely abolished the staining reaction thereby suggesting specific staining (Fig. 6, C and G). All fetal tissues analyzed—small and larger arteries, lung, heart, kidney, bone, cartilage, anulus fibrosus and nucleus pulposus of intervertebral discs (Table II)—as well as placenta were completely negative. Similar negative staining results were seen (Table II) in the juvenile samples of dermis, small, and larger arteries including aorta (Fig. 7 A), lung, heart, kidney, bone, cartilage, anulus fibrosus and nucleus pulposus (Fig. 8 A), liver, spleen, and skeletal muscle (not shown). Tissue specimens from adults of younger age (30–49 yr), in contrast, showed a mild to moderate positive staining which was most evident in small and medium-sized arterial blood vessels mostly associated with elastic membrane material and/or vascular musculature (Fig. 6 E)—with significant increase of the staining intensity in areas of arteriosclerotic intimal thickening (see below). Furthermore, we observed in those patients a slight diffuse positive staining of the dermal collagenous connective tissue (Fig. 6 A) and a mild positive staining around adipocytes. The dermal capillaries were only faintly stained (Fig. 6 A). Interestingly, the cytoplasm of keratinocytes of different layers was strongly labeled. Significant staining was also seen in the interalveolar walls of the lung (not shown). In contrast, heart, skeletal muscle, bone, and cartilage remained negative. Similarly, the normal anulus fibrosus and the nucleus pulposus were negative while a slight to moderate positive staining was seen for degenerated intervertebral disc tissue (not shown). Tissue samples from patients of advanced age (> 50 yr) revealed a significantly stronger staining reaction for CML-modified proteins. Thus, we found a strong staining in medium-sized and larger arteries, again mostly associated with elastic fibers and musculature (Figs. 6 F and 7 F). The aorta mostly showed arteriosclerotic thickening with strong staining (see below) and also small capillaries in the dermis and inner organs were positively labeled. In the dermal connective tissue particularly the elastic fibers were stained (Fig. 6 B). Positive staining was also seen in

the portal connective tissues of the liver, the trabeculas of the spleen and the interstitium of the kidneys (not shown), such as major staining was also seen in all intervertebral disc tissues (anulus fibrosus and nucleus pulposus; Fig. 8 B). Bone, pancreatic, skeletal, and heart muscle, in contrast, remained almost completely negative (Table II).

Immunohistochemical analysis of arteriosclerotic vessel walls. In our study population, we observed in those patients of advanced age (50–82 yr) mild to strong arteriosclerotic intimal lesions of the aorta or smaller arteries and arterioles (like in the kidney or the dermis). While arterial walls of fetal, juvenile (Fig. 7, A and E), and younger adult cases were negative for CML-modified proteins, we observed in patients of more than 55 yr of age a positive staining which was more pronounced in areas of major atherosclerosis (Fig. 7 B). In those areas, we observed a positive CML staining not only in the connective tissue and the amorphous atheroma material, but also very pronounced in the cytoplasm of foamy macrophages (Fig. 7 D). Similarly, in renal arteries with intimal thickening, the vessel wall was significantly labeled with a maximal staining of the lamina elastica interna, some staining of the fibrotic intimal, and medial connective tissue (Fig. 7 F).

Table II. Distribution of CML-modified Proteins in Selected Normal Human Tissues

	Fetal (22–30 wk)	Juvenile (8 yr)	Young adults (30–54 yr)	Old adults (55–82 yr)
Arteries	–	–	+	+++
Capillaries	–	–	–	+
Dermal connective tissue	nd	–	+	++
Lung	–	–	++	nd
Heart	–	–	–	nd
Bone	–	–	–	–
Cartilage	–	–	+	++
Nucleus pulposus	–	–	–/+	+++
Anulus fibrosus	–	–	–	++

nd, not determined; – absent, + mild, ++ moderate, +++ strong staining.

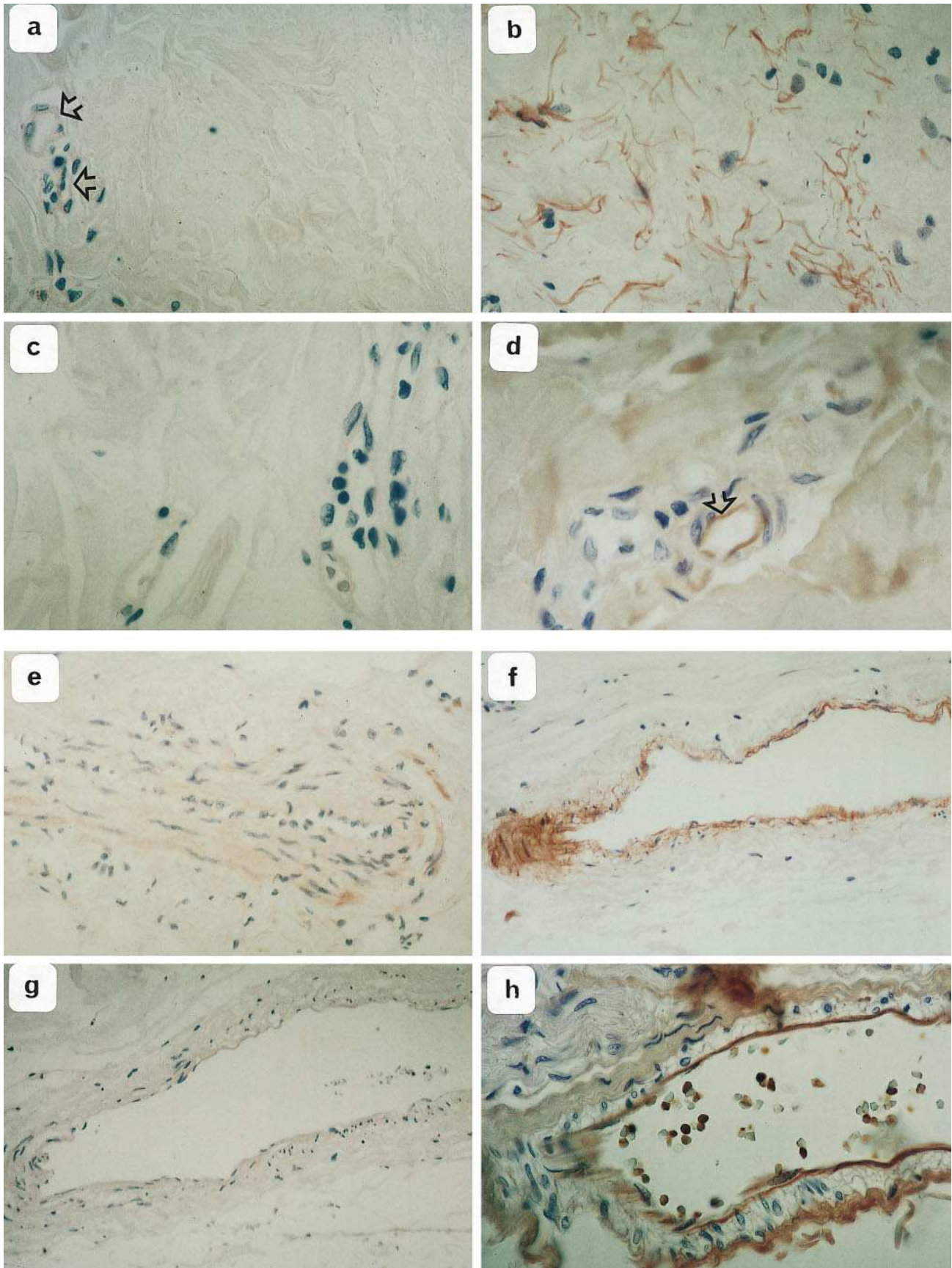


Figure 6. Localization of CML-modified proteins in human dermis and small arteries. (a) Dermal connective tissue of a 30-yr-old individual shows only a very faint positive staining. Note the slight staining of capillary walls (arrows). (b) In the dermis of a 82-yr-old individual the strong

Table III. Distribution of CML-modified Proteins in Diabetic Renal Tissue

	Age	Sex	Type/duration of d.m.	Staining for CML-modified proteins					
				Renal arteries	Stained area (%)	Renal arterioles	Glomeruli	Tubuli	Interstitium
<i>Controls</i>									
fetal	22 wk	F	–	–	< 0.5	–	–	–	–
juven.	8 yr	F	–	–	0.5	–	–	–	–
adult	50 yr	M	–	+		–	–	+	+
adult	68 yr	M	–	+++*	24.9	–	–	++	++
adult	82 yr	F	–	+++*	37.7	–	–	+	++
<i>Diabetics</i>									
1	70 yr	M	II/nr	+++	52.6	–	–	–	+/+++
2	82 yr	F	II/nr	+++	34.7	+	–	+	–
3	72 yr	F	II/nr	++	33.4	–	–	+	+
4	82 yr	M	II/> 10 yr	+++	49.9	–/+	–	++	–
5	73 yr	M	II/22 yr	++	37.7	–/+	–	+	+/+++
6	65 yr	M	II/> 15 yr	++	34.6	–/+++	–	+	++
7	63 yr	M	II/16 yr	++	20.4	–	–	++	+
8	84 yr	F	I/> 15 yr	++	43.8	–	–	++	++

Nr, not recorded; – absent, + mild, ++ moderate, +++ strong staining; *marked arteriosclerosis.

Localization of CML-modified proteins in tissues from diabetic subjects. To find out whether there exist differences in the localization and staining intensity between tissues from nondiabetic and diabetic subjects, we immunohistochemically analyzed skin, aorta, and kidney tissue for the occurrence of CML-modified proteins. When compared to age-matched controls, we observed an enhanced CML staining in dermal connective tissue (Fig. 6 D), small blood capillary walls (Fig. 6 D), and the vessel walls of arterioles (Fig. 6 H) and arteries in diabetics (Fig. 7 G). The aortal tissue from our diabetic patients showed significant atherosclerosis with a CML staining comparable to that of nondiabetic atherosclerotic aorta (Fig. 7 C). In the diabetic kidneys (Table III), arterial thickening was also associated with enhanced CML staining. A much more variable CML staining pattern was seen for arteriolo-hyalinosis with occasionally very strong labeling of the hyaline deposits (Fig. 7 H). The diabetic glomeruli with either diffuse or nodular glomerulosclerosis, in contrast, were negative (see Fig. 7 H). The interstitial connective tissue showed in some cases a significant positive staining.

Quantification of CML immunostaining. To quantify CML staining we performed a morphometric analysis. Representative data from dermal connective tissues, vessel walls, and intervertebral discs (nucleus pulposus) confirm the semiquantitative evaluation (Fig. 9). While the CML content of all tissue studied was below detection limit in fetal or juvenile tissues the stained area in dermal connective tissues from old adults was

increased sevenfold in nondiabetic and 20-fold in diabetic patients when compared to young adults. Similarly, the stained area of arterial walls was significantly enhanced in old adults when compared to young adults. However, the increase in the stained area of diabetics was less pronounced when compared to other tissues. Accordingly, we observed only a marginal difference in arteriosclerotic arteries in old diabetic and nondiabetic patients (Table III). Noteworthy, the stained area in the nucleus pulposus showed the most significant increase with age. We had no opportunity to study CML immunostaining in intervertebral discs from diabetic subjects.

Immunochemical determination of CML-modified proteins in sera from normal and diabetic individuals. Because of the low CML content in human sera a highly sensitive, competitive ELISA procedure was developed for the quantitation of CML in human serum proteins. When compared to normal individuals (range: 4.1–11 pmol/mg protein; mean = 7.3) 17 out of 35 diabetic patients (range: 6.2–19.6; mean = 11.6) showed elevated CML values (Fig. 10 A). CML values of both groups were unrelated to age and no correlation with duration of diabetes was observed. No change in CML content was found after dialysis of the samples. A weak correlation between CML values and HbA1c was observed in 29 diabetic patients (Fig. 10 B). Preliminary studies with sera from uremic patients demonstrated elevated values (~ two- to threefold) in both diabetic and nondiabetic patients (data not shown).

CML-staining is particularly associated with the elastic fibers. (c) Dermal tissue of the 82-yr-old patient from (b) after preincubation with the antigen. Note the complete suppression of staining. (d) Dermis from a 65-yr-old patient with longterm diabetes mellitus (type 2). Note the diffusely enhanced staining of the dermal connective tissue and the significant staining of the capillary wall (arrow). (e) Small artery in the subcutis from a 30-yr-old individual showing slight positive staining associated with smooth muscle cells. The inner elastic membrane here remains unstained. (f) Small artery in the subcutis from an 82-yr-old individual with a strong positive staining along the inner elastic lamina. (g) The same area as in f after preincubation with the antigen shows virtually no positive staining. (h) Small dermal artery from a patient with longterm diabetes mellitus (same as d). Note the strongly positive labeling of the inner and outer elastic membrane. (a–d: original magnification $\times 400$; e–g: $\times 250$; h: $\times 400$).

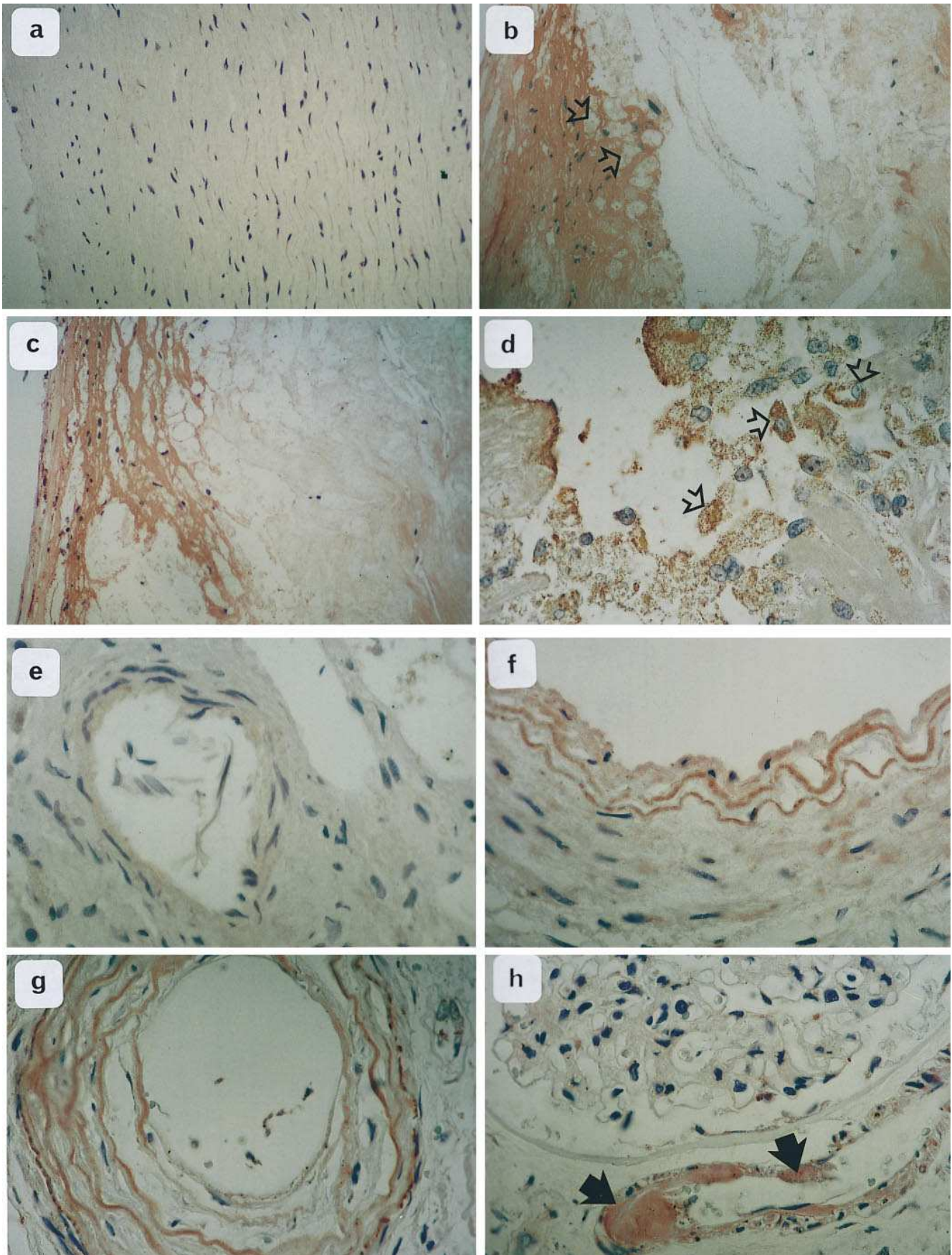


Figure 7. Immunolocalization of CML-modified proteins in normal and atherosclerotic blood vessels and in diabetic renal tissue. (a) A cross-section through the aorta from an 8-yr-old infant shows no positive CML staining. (b) The atherosclerotic intima from a 62-yr-old individual re-

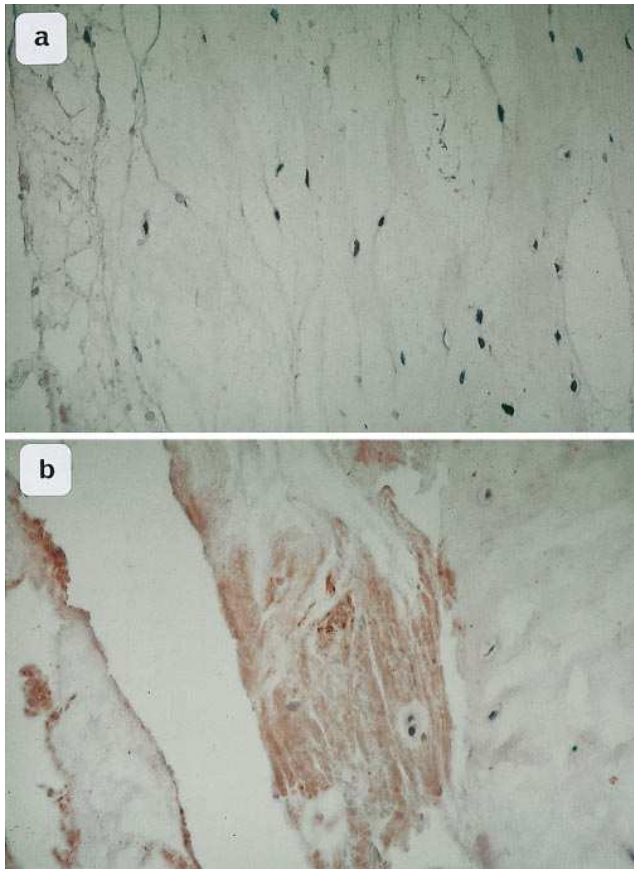


Figure 8. Detection of CML-modified proteins in the nucleus pulposus of intervertebral discs. (a) The nucleus pulposus of a 13-yr-old patient shows no significant positive staining. (b) In contrast, the nucleus pulposus tissue from a 77-yr-old male shows a diffuse CML-staining with focal strongly labeled areas at the margins of a tissue cleft. (a and b: $\times 400$).

Discussion

Production of CML-antiserum. One aim of our study was the development of antibodies to the well-defined glycoxidation product CML for immunohistochemical localization of CML in tissues from normal and diseased subjects. Six observations indicate that the antibodies specifically recognize CML modification in proteins. First, although very different CML-modified proteins were used for immunization, both antisera yielded very similar competition curves for the free hapten CML. Second, only CML but not structurally related compounds competed for the epitope binding. Furthermore, the finding that protein-bound CML competes at much lower levels than free CML and proteolytic pretreatment is not necessary for epitope

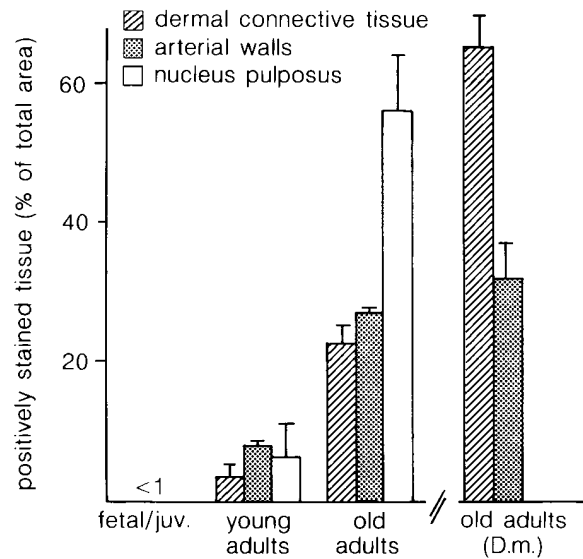


Figure 9. Quantitative evaluation of CML-stained tissue areas. Tissue samples obtained from patients described in Table II were analyzed. The bars (mean \pm SE) represent morphometric quantification of CML staining per tissue area obtained as described in the method section.

recognition, indicates a high affinity of the antiserum to protein-bound CML. A similar decrease in immunoreactivity (100-fold) of a monoclonal AGE-antibody (recognizing the CML epitope) to low molecular weight CML has previously been observed (35). Third, the antibody recognized the formation of CML in different glycosylated proteins only under aerobic conditions while no significant CML formation was observed in the absence of oxygen. Fourth, reduced reactivity was found when known inhibitors of the glycoxidation reaction were present during aerobic formation of CML. Fifth, in tissue sections most of the immunoreactivity is localized to the extracellular matrix. This finding is reasonable since the glycoxidation reaction is slow and is likely to be detected only in proteins with slow turnover. Furthermore, staining could be completely inhibited by preincubating the CML-antiserum with low concentration of CML-modified protein. Sixth, we found a clear age-dependent increase in CML content of human skin similar to results of Baynes et al. (6, 7) who used the highly specific and sensitive gas chromatography/mass spectrometry procedure for the determination of CML. Taken together, these data indicate that the obtained antisera specifically recognize CML modifications in proteins in solution or in tissue sections with high affinity.

Occurrence of CML modification in normal and pathological human tissues. In accordance with the slow *in vitro* formation of CML no immunoreactivity could be detected in tissue

veals a significant staining for CML in the superficial fibrous cap, in the fibrotic connective tissue surrounding an atheroma and in foam cells (arrows). (c) Atherosclerotic aortal intima from a diabetic individual similarly shows a strong CML staining as in nondiabetic atherosclerosis (b). (d) A detail photograph from an atherectomy specimen from a 58-yr-old nondiabetic subject shows the strongly positive CML staining in multiple foam cells (arrows) at the margins of an atheroma. (e) The small renal artery from an 8-yr-old infant lacks any specific positive CML staining. (f) A renal artery from a 50-yr-old nondiabetic patient with slight arteriosclerotic intimal thickening shows a mild to moderate staining of the internal elastic lamina. (g) Cross-section through the renal artery from a longterm diabetic (65-yr-old, type 2 dm) with significant CML staining of the sclerotic vessel wall. (h) The focal strongly positive reaction of arteriolo-hyalinosis in a diabetic kidney (arrows) strongly contrasts to the absent CML staining of the diffusely enlarged glomerular mesangium. (a-c: $\times 160$; d-h: $\times 400$).

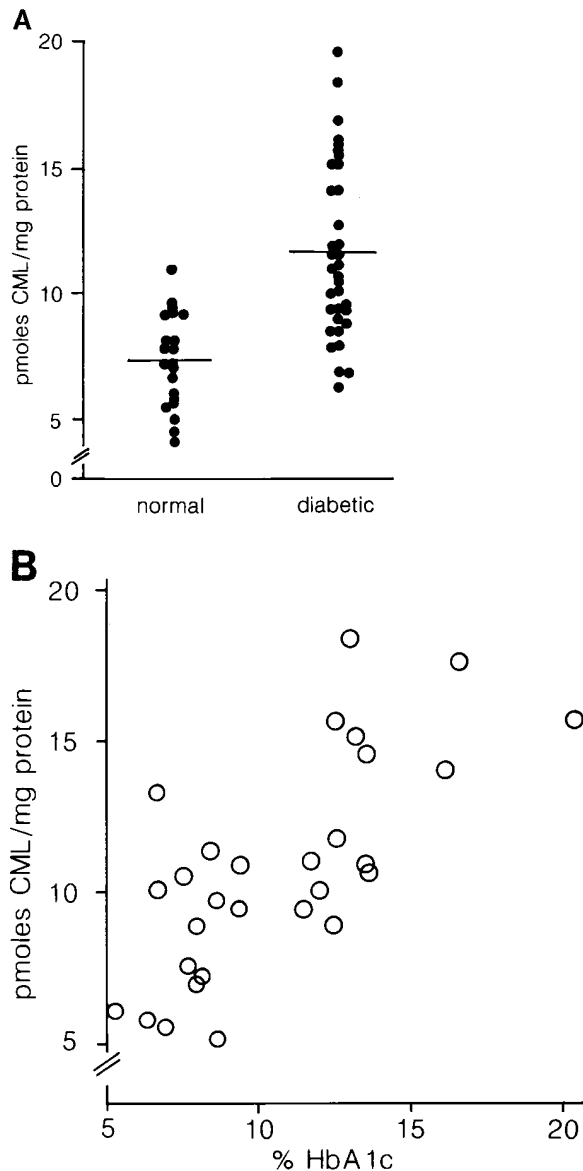


Figure 10. CML serum concentrations in normal and diabetic subjects. (A) CML-serum levels were determined in 21 apparently healthy individuals and in 35 age- and sex-matched diabetic patients without any sign of nephropathy. Serum protein bound CML was quantitated by competitive ELISA as described. (B) Correlation of CML serum levels from 29 diabetic patients with HbA1c values; $r = 0.70$; $P < 0.01$.

samples from fetuses or juvenile subjects (< 9 yr). Clearly, immunoreactivity of dermal connective tissue, cartilage, and blood vessels increased with advancing age. This finding is in good agreement with earlier quantitative determinations of CML in human skin collagen and lens proteins showing that CML is barely detectable at birth and increases linearly with age (6–8, 35). However, our studies reveal a significantly uneven distribution of CML in the tissues studied. While elastic fibers and elastic membranes in skin and blood vessel walls were intensely stained, the surrounding tissue appeared significantly less stained. These results indicate that the CML content of individual skin proteins may even be much higher than previously estimated in hydrolyzed skin collagen samples (6, 7). The

preferred localization of CML in the extracellular matrix is not unexpected because of the slow formation rate of CML (see Fig. 4) so that it accumulates only in proteins with slow turnover.

In addition to normal tissues, we studied two conditions in which chronic oxidative stress may be expected. In atherosclerosis we observed a significantly enhanced CML staining in areas of intimal fibrosis and atheromatous plaque formation which was particularly pronounced when compared to vascular segments with less severe alterations. Interestingly, in addition to the positive reaction of the extracellular matrix proteins in atheromatous plaques we observed a strong cytoplasmic staining of foamy macrophages. One explanation may be that these cells on the surface of which AGE receptors have been characterized (37) readily take up and subsequently degrade AGE-modified matrix proteins from the plaques. This interpretation is supported by our findings that intestinal macrophages were also positively stained with the CML antiserum (data not shown). In this case, the AGE-products may be of exogenous origin, e.g., food or beverages. However, the unexpectedly high intracellular CML staining in macrophages may also be explained by the formation of CML via a lipid peroxidation reaction since in a recent publication the formation of CML from unsaturated fatty acids (38) has been reported. In this reaction pathway glyoxal generated during metal-catalyzed oxidation of polyunsaturated fatty acids is converted to CML in the presence of protein. Noteworthy, incubation of HSA with glyoxal readily forms an immunoreactive product when tested with our CML-antisera (Schleicher, E.D., and E. Wagner, unpublished observation). For these reasons, it is more likely that the CML found in foam cells and atheromas is formed by lipid peroxidation; therefore, in these locations, CML may be a marker for lipid peroxidation.

The second condition under investigation was long-term diabetes. We observed an enhanced staining for CML particularly in vessel walls and interstitial connective tissues, like the dermis, when compared to age-matched nondiabetic individuals resembling a CML staining intensity of older individuals. Additionally, in diabetes the CML staining appeared much more evenly distributed in the extracellular matrix than in nondiabetic subjects of old age. Atherosclerotic lesions in diabetics were, however, not apparently different from nondiabetic lesions with respect to extent and distribution of enhanced CML staining. To quantify the immunostaining, a morphometric analysis was performed. With this procedure a strong dependence of CML staining in dermal connective tissue or nucleus pulposus on age and diabetes was confirmed. The quantitative evaluation of the data indicates that the enhancing effect of diabetes on CML staining in arterial vessel walls is less apparent than in dermal connective tissue although a clear age dependence was observed in both tissues. The reason for this difference is not readily obvious. One explanation may be the different mechanism of CML formation in these tissues as discussed below. Our recent finding that CML is qualitatively and quantitatively increased in retinas of streptozotocin-diabetic rats furthermore supports the enhancing effect of diabetes on CML accumulation (Hammes, H.P., and E. Schleicher, unpublished data). Taken together, these observations in atherosclerosis and diabetes indicate an enhanced CML staining when compared to age-matched normal tissues which may be due to enhanced oxidative stress in these conditions.

Recently, an immunohistochemical analysis studying the distribution of pyralline, a Maillard product supposed to be

formed by nonoxidative mechanism, was reported (39). Well in agreement with our observations the staining occurred preferentially in the extracellular matrix of the tissue investigated, in particular, in skin collagen and vascular walls. Furthermore, increased staining intensity was observed with age and in diabetic patients. However, intense immunoreactivity in sclerosed glomeruli from diabetic patients was seen, at variance with our findings. If this difference in immunoreactivity—concordant staining of both antisera in some tissues like vessel walls and discordant staining in other tissues like in the expanded mesangial matrix—reflects a different exposition to oxidative stress may not be deduced from these studies. Further studies—possibly using the specific antisera to well defined Maillard products—may reveal if different Maillard products are colocalized or not colocalized in diabetes and with advancing age. Anti-AGE antibodies were applied in two immunohistochemical analyses for the detection of AGE products in human coronary atheroma and atherosclerotic aortic lesions. In one study a polyclonal anti-AGE antiserum was used (40). High levels of AGE reactivity were observed within the arteriosclerotic plaque present in the vessels from selected patients with diabetes. Although raised against AGE-modified proteins the staining pattern appeared similar to what we see with our CML antiserum. In another study a monoclonal anti-AGE antiserum was used to detect AGE products in atherosclerotic lesions of human aorta (41). In this extensive investigation strong intracellular staining in intimal lesions was found primarily in macrophages again in line with our observations. Recently, the authors demonstrated that the monoclonal antibody used in their immunohistochemical studies specifically recognizes CML-modified proteins while a polyclonal AGE-antiserum contained at least two populations, one population recognizing CML modification, and the other one still undefined AGE structures (38). Assessing the specificity of the AGE-antiserum which Reddy et al. (42) had obtained by immunization with AGE-KHL it was found that CML-BSA could completely inhibit the antigen-antibody formation indicating that CML is an important/unique epitop recognized by these AGE-antisera. These results would explain why no obvious difference in staining pattern of the immunohistochemical studies using AGE-antibodies and our study with CML-antibody could be found. However, another group reports on a substantially decreased immunoreactivity of their anti-AGE antibodies if AGE-proteins are acid-hydrolyzed, possibly indicating that the acid-stable CML is not a major antigenic determinant for this AGE antibody preparation (43). Taken together, these data suggest that currently existing AGE antisera should be reevaluated for their epitop specificity.

Mechanism of CML formation. Based on in vitro experiments Ahmed (20) originally described CML as a product of metal ion-catalyzed oxidative fragmentation of FL. Detailed kinetic analysis revealed that ~ 40% CML is formed from fructose-lysine under physiological conditions (21, 34). In both studies CML formation, which was determined by chemical methods, was decreased by desferrioxamine, catalase, superoxide dismutase, anaerobic conditions and aminoguanidine (21) and chelators, sulfhydryl compounds, and antioxidants (44) similar to our results obtained by using the CML antiserum for the determination of CML. Together these results again emphasize the essential role of reactive oxygen species in the formation of CML in glycated proteins. To exclude that CML is primarily formed by oxidation of free glucose we used

low phosphate concentrations, a condition known to favor the formation of CML via the FL intermediate (34). Additionally, in one experiment we used preglycated HSA to exclude the glucose oxidation pathway in the formation of CML. Although the in vitro experiments clearly identify CML as a glycoxidation product, it is difficult to unequivocally prove the mechanism of CML formation in tissues. The formation of CML modification in proteins is dependent on both the level of glycemia and the levels of reactive oxygen species (44). Since the steady state concentration is dependent of both formation and degradation, the protein turnover also influences the CML tissue levels. In nondiabetic subjects only a marginal age-dependent increase in glycation of long-lived proteins has been found (4, 6–8). Assuming that this little increase in FL content reflects unaltered protein turnover, the age-dependent accumulation of CML may possibly indicate increased oxidative stress. The increased CML accumulation found in age-matched diabetic patients may originate from increased glycation, enhanced oxidative stress, or both. As discussed earlier, CML may also be formed from metal-catalyzed oxidation of polyunsaturated fatty acids recently identified as another source of CML formation (38). In this context it is important to note that anti-AGE antibodies detect an increase in AGE epitopes in atherosclerotic plaques in normoglycemic animals supporting the view that these epitopes—possibly including CML epitopes—may be formed by lipid peroxidation (45). Taken together these data indicate that CML formation in tissue may result from both glycoxidation and lipid peroxidation, in any case an oxidative reaction is involved.

CML content of serum protein in normal and diabetic subjects. In accordance with the slow formation of CML, the extent of CML modification in serum proteins is present in low levels, only detectable by the very sensitive competitive ELISA. As observed for the glycation of serum proteins CML modification was not age dependent. Because increased CML content in serum proteins was detected in diabetic patients and since these increases correlated with HbA1c (e.g., glycemic control) these CML elevations may be derived from increased glycation and/or from increased oxidative stress. Further investigations on the extent of serum glycation, oxidative stress, and influence of antioxidants may reveal the origin of CML in serum proteins.

In summary, we produced a specific antiserum recognizing the CML modification of proteins. The use of the antiserum revealed an age-dependent CML accumulation in various tissues and a significant variation of staining intensity within the tissue samples. Accordingly, particular anatomical subsettings seemed to be more readily prone to undergo CML modification. This holds particularly true for the aging arterial wall and is also seen in other interstitial connective tissues in aging. A very strong CML modification is particularly seen in arteriosclerotic lesions which strongly reacted with our CML-antiserum. Similarly, a high level of CML modification occurs in long-term diabetes apparently effecting extracellular matrix proteins in a more even pattern which is in line with the presumed process of CML formation. As observed semiquantitatively in the tissue samples the CML content of serum proteins was also increased in nearly half of diabetic patients. If this elevation in CML content of serum proteins represents a medium-term integrator of oxidative stress remains to be shown. In general, our observations strongly suggest that the estimation of CML may represent a possible marker for chronic oxidative stress. To further

support this notion we are currently investigating the effect of antioxidant treatment on the CML content in normal and diabetic subjects. These studies may elucidate the possible value of CML determination for the assessment of endogenous oxidative stress and for the efficiency of therapeutic approaches for limiting oxidative reactions in human subjects.

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