Rapid Publications

Nonenzymatic Glycosylation Products on Collagen Covalently Trap Low-Density Lipoprotein

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SUMMARY

Advanced nonenzymatic glycosylation products capable of cross-linking proteins accumulate on collagen in vivo in proportion to time-averaged blood glucose concentration. In this report, we have evaluated the ability of advanced nonenzymatic glycosylation products formed on collagen in vitro to covalently bind low-density lipoprotein (LDL) in a manner similar to that which occurs in human atherosclerotic lesions. At constant LDL concentration, covalent trapping increased linearly with the extent of advanced glycosylation product formation, from 1.42 ± 0.15 to 4.46 ± 0.36 μg LDL protein/mg collagen. At a constant level of collagen advanced glycosylation product, LDL binding increased as a function of increasing LDL concentration. At an LDL-cholesterol level of 103 mg/dl, covalent trapping of LDL by nonenzymatic glycosylation products on collagen averaged 3.2 times as much as control ($P < 0.01$).

These data indicate that LDL is bound specifically by reactive products generated by nonenzymatic glycosylation of collagen, and suggest that excessive LDL trapping by hyperglycemia-induced advanced glycosylation endproducts may contribute to the accelerated development of atherosclerosis in patients with diabetes mellitus. DIABETES 1985; 34:938–41.

Accelerated atherosclerotic disease in coronary, cerebral, and peripheral arteries occurs frequently in patients with diabetes mellitus, even though most of these patients have plasma cholesterol and low-density lipoprotein (LDL) levels that are within the normal range.[1] This effect of diabetes has been shown by epidemiologic data to be independent of all other known cardiovascular risk factors as well.[2]

The diabetes-associated excessive attachment of glucose to extravascular matrix proteins such as collagen could be the biochemical link between excessive LDL accumulation in the arterial wall and persistent hyperglycemia. It has been known for some time that glucose forms chemically reversible Schiff base and Amadori product adducts with proteins in proportion to glucose concentration.[3] Equilibrium is reached after several weeks, however, and further accumulation of these early nonenzymatic glycosylation products does not continue beyond that time. More recent studies have demonstrated that under physiologic conditions, subsequent reactions of the Amadori product slowly give rise to irreversible advanced glycosylation endproducts (AGE), which accumulate on long-lived proteins such as collagen.[4] These advanced glycosylation endproducts are capable of reacting with additional amino groups on other proteins to form intermolecular cross-links. Examples that have been described include lens crystallins cross-linking with itself, collagen with albumin and IgG, and ribonuclease A with other molecules of RNase A.[5–7] In this report, we have evaluated the interaction of advanced glycosylation endproducts on collagen with LDL isolated from human plasma.

MATERIALS AND METHODS

Native type I collagen from calf skin (Elastin Products, Elastin Company, Pacific, Missouri) was immobilized on agarose by a previously described technique,[6] and incubated for 14 days at 44°C[7] in phosphate-buffered saline (pH 7.4) containing 0–500 μM of glucose-6-phosphate. Identical incubations were carried out using denatured type I calf skin collagen immobilized on agarose (Pierce Chemical Company, Indianapolis, Indiana), in order to avoid artifacts introduced by removal of non-resin-bound collagen components during subsequent washing with SDS. All solutions contained 3 mM NaN_3 to prevent bacterial growth. Nonenzymatically glycosylated and unmodified, immobilized collagen samples were then washed with 50 vol PBS before LDL-binding experiments. The amount of collagen bound to the resin (determined by Lowry protein analysis with a collagen standard after hydrolysis in hot trichloroacetic acid) was 17.2 mg/ml gel (native collagen) and 7.38 mg/ml gel (denatured collagen).[8]

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Advanced glycosylation endproducts were quantified by spectrofluorimetry after solubilization by enzymatic digestion.[9] Each sample was washed 6 times with PBS, and incubated with 5% (wt/wt) purified collagenase (type CLSPA, Worthington, Millipore, Bedford, Massachusetts) in 0.1 M $CaCl_2$/0.02 M Tris buffer (pH 7.55) for 24 h at 37°C with shaking. Sample pH was then adjusted to 8.0 with NaOH, 1 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, Indiana) was added to each sample, and the incubation was continued for 16 h. Enzymatic digests were analyzed by measuring fluorescence at 440 nm upon excitation at 370 nm, using a Perkin-Elmer fluorimeter model 204 (Norwalk, Connecticut). Measurements were made against a blank containing collagenase and proteinase K in sample buffer, and expressed as fluorescence/mg Lowry protein.[9] Selected samples of glycosylated collagen were reduced with sodium borohydride as described previously[10] before LDL-binding experiments to assess the stability of protein-trapping collagen glycosylation products.

LDL was prepared from pooled normolipidemic human serum by density gradient ultracentrifugation in a vertical rotor,[11] and iodinated with $Na^{125}I$ using the method of Fraker and Speck.[12] Specific activity was 2–4 × 10^4 cpm/μg. Covalent binding of LDL to immobilized collagen was evaluated after incubation of 4.4-mg aliquots of each collagen preparation with varying amounts of radioiodinated LDL for 48 h at 37°C. The resin was then washed exhaustively, first with PBS and then with 4% SDS, until no further radioactive material could be eluted. The amount of radioiodinated LDL bound to immobilized collagen was determined by counting the resin in a Packard Tricarb Scintillation Counter (Model 3002, Packard Instrument Company, Inc., United Technologies, Downers Grove, Illinois).

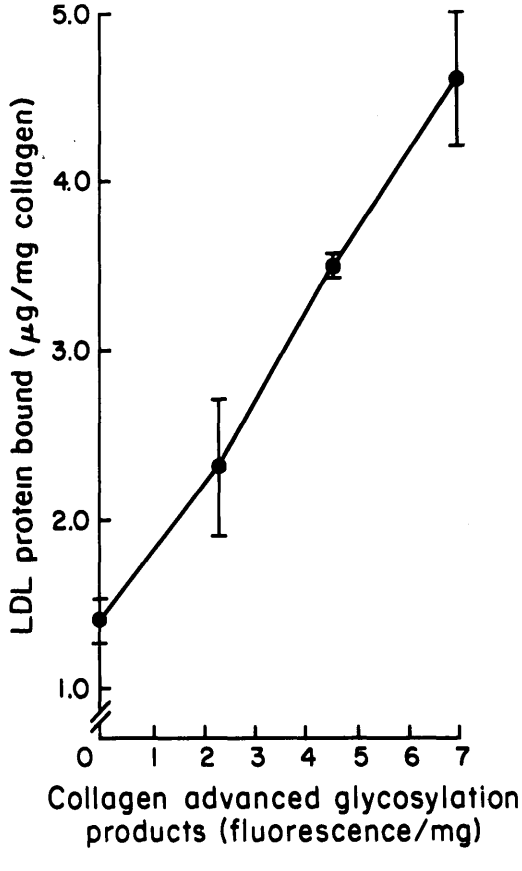

FIGURE 1. Covalent binding of ^{125}I-LDL by immobilized collagen as a function of collagen advanced nonenzymatic glycosylation product formation. Data are expressed as the mean ± SEM of three experiments.

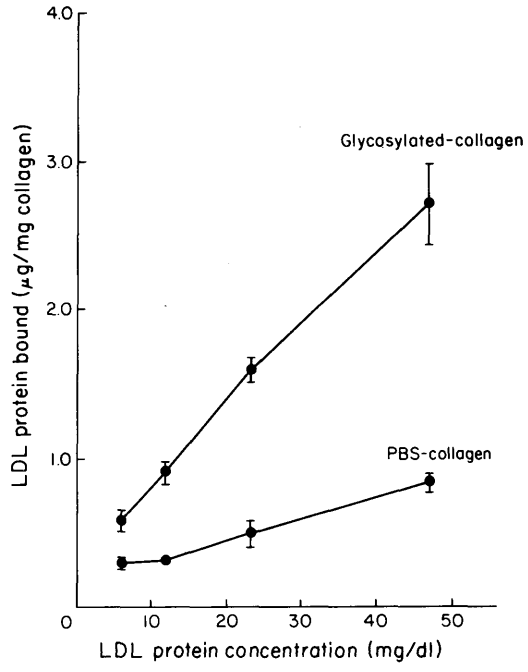

FIGURE 2. Covalent binding of ^{125}I-LDL by nonenzymatically glycosylated and control collagen as a function of LDL protein concentration. Data are expressed as the mean ± SEM of three experiments.

RESULTS

Formation of advanced glycosylation products on collagen was proportional to sugar concentration in the incubation medium, with no significant differences between native and denatured collagen. Denatured collagen was selected as the model system for LDL binding to avoid artifacts introduced by removal of non-resin-bound collagen components during subsequent washing with SDS. At constant LDL concentration (77 mg protein/dl), covalent trapping increased linearly with the extent of advanced glycosylation product formation, from 1.42 ± 0.15 to 4.46 ± 0.36 μg LDL protein/mg collagen (Figure 1). At a constant level of collagen advanced glycosylation product formation (6.8 U/mg), LDL binding increased as a function of increasing LDL concentration (Figure 2). At an LDL protein concentration equal to a normal plasma LDL-cholesterol level of 103 mg/dl, covalent trapping of LDL by nonenzymatic glycosylation products on collagen averaged 3.2 times as much as control ($P < 0.01$). The number of glucose-derived reactive binding sites involved in this LDL trapping cannot be <1/molecule of bound apolipoprotein B, but it may be much greater, since several lysine residues in 1 molecule of apolipoprotein B may simultaneously bind at separate reactive sites. The absolute number of these reactive sites, therefore, cannot be extrapolated from this type of experimental datum. Reduction of nonenzymatically glycosylated collagen with sodium borohydride appeared to have no significant effect on subsequent LDL binding (2.3 ± 0.01 μg/mg, reduced glycosylated collagen; 2.70 ±

0.28, nonreduced glycosylated collagen). This observation suggests that the borohydride-reducible Amadori products are not directly involved in the trapping of soluble lipoproteins by nonenzymatically glycosylated collagen. It seems likely that reactive groups are instead generated during formation of borohydride-stable, nonreducible advanced glycosylation endproducts, such as the one recently isolated from glycosylated albumin,[13] 2-(2-furoyl-4[5]-2-furanyl)-1*H*-imidazole, but this has not yet been demonstrated.

DISCUSSION

The data presented in this report indicate that LDL is bound specifically by reactive products generated by nonenzymatic glycosylation of collagen. In vivo, fibrous plaque lipid also appears to be chemically attached to components of the arterial wall. While LDL in normal intima can be readily removed from tissue samples by electrophoresis, immobilized plaque lipoprotein can only be released from lesions by treatment with proteolytic enzymes.[14] Most of the accumulated lipid in these lesions is extracellular, not intracellular.[15] Enhanced extracellular immobilization of plasma lipoproteins by advanced glycosylation endproducts on collagen or other long-lived vessel wall proteins would promote excessive fibrous plaque lipid accumulation, even at normal levels of plasma LDL, by preventing lipoprotein diffusion out of the intima, and perhaps also by restricting interactions with endothelial cells necessary for subsequent LDL clearance by scavenging macrophages.[16,17]

In vitro, glucose-6-phosphate was used instead of glucose to accelerate the formation of reactive advanced glycosylation endproducts over a short experimental period (2 wk), since the rate of nonenzymatic protein glycosylation increases significantly when G-6-P is substituted for glucose on an equimolar basis.[18] High sugar concentrations (100–500 mM) and slightly increased incubation temperature (44°C) were also chosen to accelerate the formation of reactive advanced glycosylation endproducts. These conditions are similar to those employed by Eble et al.[7] in their investigations of glucose-dependent cross-linking of protein. It is important to note, however, that covalent attachment of soluble proteins by nonenzymatically glycosylated native collagen has also been demonstrated after incubation with glucose at 37°C.[6] In vivo, with concentrations of glucose found in diabetic plasma (10–20 mM), this process would occur over a much longer period, since it is the integral of sugar concentration over time that determines the extent of advanced glycosylation endproduct accumulation.[4] In fact, an age-dependent linear increase in advanced glycosylation product accumulation has recently been demonstrated in collagen from nondiabetic subjects, and this process is accelerated in diabetic subjects.[9] Such a rate is consistent with the slow evolution of clinically demonstrable atherosclerotic disease, which occurs over a period of many years.

The relationship between increased LDL trapping and higher levels of collagen advanced glycosylation products demonstrated in Figure 1 suggests that the accelerated increase of advanced glycosylation endproduct accumulation observed in collagen from diabetic patients[9] results in excessive LDL trapping, thereby contributing to a more rapid development of macrovascular disease. At any given level of accumulated advanced glycosylation endproducts, moreover, higher LDL concentrations would result in more extensive lipoprotein trapping (Figure 2), due to the increased number of LDL amino groups potentially available to react with AGE on collagen. The experimentally observed correlation between amount of LDL bound to nonenzymatically glycosylated collagen and concentration of reactive LDL in the incubation medium closely resembles the in vivo relationship between arterial intima lipoprotein cholesterol concentration and plasma LDL.[19]

Excessive trapping of LDL by advanced glycosylation endproducts on collagen most likely acts in synergy with other pathogenetic mechanisms to produce accelerated large vessel disease in diabetes. Advanced glycosylation endproduct formation, itself, may further promote plaque formation in the diabetic vessel wall by reducing susceptibility of connective tissue elements, deposited LDL, and fibrin, to proteolytic degradation.[20,21] Diabetes-induced increases in platelet aggregability[22] might stimulate abnormal proliferation of arterial smooth muscle cells,[23] and diabetes-associated defects in cell-to-plasma cholesterol transport could contribute to progressive intracellular cholesterol accumulation.[24] The rate at which atherosclerosis develops in a given diabetic individual may reflect independent contributions from each of these causal factors.

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