Post-translational modification of apolipoprotein B by transglutaminases

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The major form of cross-link found in apolipoprotein B was identified as N^1N^{12} -bis-(γ -glutamyl)spermine, a product known to be formed through the catalytic action of transglutaminases (EC 2.3.2.13). N^1 -(γ -Glutamyl)spermine was present in a trace amount but ϵ -(γ -glutamyl)lysine cross-links, which are formed during fibrin formation in plasma, were not detected. In the presence of catalytic amounts of plasma Factor XIIIa (a thrombin-dependent extracellular transglutaminase) or cellular transglutaminase (a cytosolic enzyme), apolipoprotein B and other plasma apolipoproteins (A-I, A-II and C) underwent covalently bridged polymerization and served as amine acceptor substrates. These results suggest that transglutaminases may participate in the covalent modification of apolipoproteins, either in the physiological state or during pathogenesis.

INTRODUCTION

Apolipoprotein B is the major apolipoprotein constituent of chylomicrons, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL) (Jackson *et al.*, 1976; Osborne & Brewer, 1977; Scanu & Teng, 1979; Kane, 1983). It exists primarily in two forms: apolipoprotein B-48 and apolipoprotein B-100. Apolipoprotein B-48 is synthesized by the intestine and is a component of chylomicrons and their remnants. Apolipoprotein B-100 is synthesized by the liver and is the primary apolipoprotein of VLDL, VLDL remnants and LDL. Apolipoprotein B functions as the ligand for the removal of LDL from the circulation by receptor-mediated uptake into a variety of tissue and cell types (Pittman *et al.*, 1979; Brown *et al.*, 1981).

The molecular size of apolipoprotein B-100, which had once been controversial, has now been determined to be about 513 kDa using cDNA cloning methods (Chen *et al.*, 1986; Knott *et al.*, 1986).

Several years ago a report appeared (Shore & Shore, 1976) that seemed to shed some light on the probable cause of the extreme insolubility of apolipoprotein B-100 and the great discrepancy in the reported molecular masses. It stated that ϵ -(γ -glutamyl)lysine cross-links occur in apolipoprotein B. These cross-links are among the products formed by transglutaminases (EC 2.3.2.13), calcium-dependent enzymes that catalyse an acyl transfer reaction in which γ -carboxamide groups of peptide-bound glutamine residues and the primary amino groups of a variety of compounds react to form monosubstituted γ -amides of peptide-bound glutamic acid. Although we were unable to confirm the presence of ϵ -(γ -glutamyl)lysine cross-links in apolipoprotein B from human plasma LDL (Cocuzzi & Breckenridge, 1986), our data did not preclude this protein as a transglutaminase substrate.

Since a variety of transglutaminases with distinct characteristics (Chung, 1972; Folk, 1980) exists in mammals, and the biological role of each, especially the physiological role of an intracellular transglutaminase ubiquitous to all cells, is not known, there has been a search for substrates and products of transglutaminases. Factor XIII is present in plasma as a zymogen which is exclusively associated with fibrinogen (Chung *et al.*, 1979; Chung & Lewis, 1986), and is activated by thrombin, which is formed through the cascades of the activation scheme. Since fibrin was thought to be the main substrate for Factor XIIIa, a number of other plasma proteins have also been identified as substrates, e.g. α_2 -macroglobulin and fibronectin (Mosher, 1976), α_2 -plasmin inhibitor (Sakata & Aoki, 1980; Carmassi & Chung, 1983), Factor V (Francis *et al.*, 1986), von Willebrand factor (Hada *et al.*, 1986) and thrombospondin (Bale & Mosher, 1986). The reports that plasma factor XIIIa activity is significantly higher in hypertriglyceridaemic patients than in normolipidaemic controls (Cucuianu *et al.*, 1973, 1976), and that polyamines may be covalently incorporated into lipoproteins (Delcros *et al.*, 1987), prompted us to examine apolipoproteins as possible physiological transglutaminase substrates. We now report findings that suggest that apolipoprotein B is a transglutaminase substrate.

EXPERIMENTAL

Isolation of lipoproteins

VLDL, IDL and LDL were isolated from pooled fresh normal human serum or plasma (containing 1 mM-EDTA), donated by seven fasting volunteers (N.I.H. Blood Bank), by sequential flotation in a 50.2 Tl rotor at 16 °C in a Beckman model L5-50 ultracentrifuge, according to the standard methods (Hatch & Lees, 1968), using solid KBr for density adjustments. VLDL were

Abbreviations used: VLDL, very-low-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins.

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isolated at density 1.006 g/ml (16 h), IDL at densities between 1.006 and 1.019 g/ml (20 h) and LDL at densities between 1.019 and 1.063 g/ml (20 h). The respective lipoproteins were washed after the initial centrifugation by refloating them at a higher solvent density. Chylomicrons were judged to be absent from preparations of VLDL by the absence of a visible supernatant layer after refrigeration for 24 h at 4 °C.

HDL were isolated according to the method of Kostner (1981). The density of serum obtained from another donor (N.I.H. Blood Bank) was adjusted to 1.070 g/ml with solid NaCl and ultracentrifugation performed at 100 000 *g* for 20 h at 16 °C. The upper third of the ultracentrifuge tubes was removed by slicing the tube, and solid NaBr was added to the material of the lower two-thirds of the tubes up to a density of 1.22 g/ml. Ultracentrifugation was then performed at 145 000 *g* for 25 h at 16 °C. After one wash under identical conditions, the floating HDL were removed by tube slicing, dialysed against a NaBr solution of density 1.110 g/ml and spun at 140 000 *g* for 40 h at 16 °C. The tubes were then cut approximately one-third of the distance from the top, between the resulting two visible concentration maxima, to obtain the separated HDL₂ (in the top fraction) and HDL₃ (in the bottom fraction).

All lipoprotein fractions were subsequently dialysed extensively against 0.9 % NaCl, pH 7.3. Apolipoprotein was measured by a modification of the Lowry method (Kruski & Narayan, 1972). Before isolation of the lipoproteins, PCMB (*p*-chloro-mercuribenzoate), (Sigma, St. Louis, MO, U.S.A.) was added to the serum to 10 mM to block lecithin:cholesterol acyltransferase and lipases, which may attack the lipoproteins. In addition, all salt solutions and buffers used throughout the preparation procedures were made to contain 0.5 mg of Na₂EDTA/ml and 1 mg of NaN₃/ml to prevent oxidative degradation of lipoproteins and to minimize microbial growth respectively.

Isolation of apolipoprotein B

Isolation of LDL from fresh normal human serum or plasma (containing 1 mM-EDTA) obtained from the N.I.H. Blood Bank was carried out by sequential flotation, as mentioned above, between densities of 1.025 and 1.050 g/ml. The LDL were dialysed exhaustively against saline (pH 7.3) and lyophilized. The lipid-free LDL fraction was prepared under N₂ with the use of ethanol/diethyl ether (3:1, v/v) and gentle rotation at 4 °C for 30 min. The organic solvent was removed by aspiration following low-speed centrifugation. This extraction procedure was repeated 4 times, after which the residue was washed with three portions of diethyl ether. To this apolipoprotein was added 5 ml of water, the protein cake was squeezed with a spatula to remove most of the trapped ether, and the remaining ether was removed by aspiration. Finally, the residue was lyophilized.

The apolipoprotein residue was solubilized, reduced and alkylated according to the following method. Treatment of the residue with 10 mM-dithiothreitol in a 0.05 M-Tris/HCl buffer (pH 8.0) containing 8.6 M-guanidine/HCl (Eastman Kodak Co., Rochester, NY, U.S.A.), followed by treatment with a 10-fold molar excess of iodoacetamide (Eastman Kodak Co.) for 1 h at room temperature. Before the enzymic digestion of this apolipoprotein B, the guanidine/HCl concentration was

brought down to under 2 M by dilution with water to precipitate the apolipoprotein. This precipitate was then exhaustively washed with water.

Identification of γ -glutamyl polyamine derivatives

A modification of the method of Folk *et al.* (1980), was employed for the detection of γ -glutamyl polyamine derivatives in apolipoprotein B. The apolipoprotein B residue (24 mg) was resuspended in 0.2 M-*N*-ethylmorpholine acetate buffer (pH 8.1). Proteolytic digestions were carried out at 37 °C in the presence of a few crystals of thymol. Proteinase concentrations used per mg of lipoprotein and times of digestion were as follows: 0.6 mg of Pronase (Calbiochem, San Diego, CA, U.S.A.), 18 h; a second 0.6 mg portion of Pronase, 8 h; 0.06 mg of prolidase containing glutathione and magnesium as cofactor, 12 h; 0.08 mg of aminopeptidase M (Sigma), 65 h; 0.08 mg of carboxypeptidase A and 0.03 mg of carboxypeptidase B (Boehringer Mannheim, Indianapolis, IN, U.S.A.), 3 h. Digestion was stopped by addition of trichloroacetic acid to 10 %, and the precipitate was removed after centrifugation. The supernatant was extracted 5 times with anhydrous ethyl ether to remove trichloroacetic acid, the aqueous phase was taken to dryness and the residue was dissolved in water. An aliquot of this sample was directly analysed for γ -glutamyl polyamines using an automated ion-exchange chromatographic procedure carried out on a Dionex D-400 amino acid analyser (Folk *et al.*, 1980). Another aliquot of the enzymic digest was chromatographed and effluent was collected directly from the column without mixing with detection reagent. Fractions were collected every 1.5 min and those corresponding to the γ -glutamyl polyamine-containing peak were pooled, concentrated, and desalted using small column of Dowex 50W-X12 (H⁺). To verify the identity of these compounds, aliquots of the chromatographic fraction were either hydrolysed in 6 M-HCl for 16 h at 110 °C or incubated with γ -glutamylamine cyclotransferase in 0.1 M-sodium phosphate buffer (pH 8.0) overnight at room temperature. This enzyme catalyses the conversion of L- γ -glutamylamines to free amines and 5-oxo-L-proline (Fink *et al.*, 1980). Free polyamines were analysed using a previously reported procedure (Folk *et al.*, 1980).

Identification of ϵ -(γ -glutamyl)lysine

A modification of the h.p.l.c. method of Griffin *et al.* (1982) was employed for the detection of ϵ -(γ -glutamyl)lysine cross-links in apolipoprotein B. Briefly, aliquots of the proteolytic digest (50–100 μ l) were incubated at room temperature for 5 min with 300–600 μ l of an assay mixture consisting of 200 mM-borate buffer (pH 10.4), 100 mM-*o*-phthalaldehyde, 50 % (v/v) methanol and 2 % (v/v) β -mercaptoethanol. Separation of *o*-phthalaldehyde derivatives was achieved on an ALTEX Ultrasphere ODS 5 μ m [4.6 mm (internal diameter) $\times$ 250 mm] column (Beckman, Berkeley, CA, U.S.A.). The elution was carried out with a linear gradient of 20 to 95 % acetonitrile in 20 mM-sodium acetate buffer, pH 6.0, over a period of 20 min at a flow rate of 1.0 ml/min. A Waters h.p.l.c. instrument equipped with two model M-6000 A pumps, a model 600 solvent programmer and a model 420-C fluorescence detector was used. The fluorescence detector excitation wavelength was set at 340 nm and the intrinsic fluorescence was monitored at 440 nm.

Monodansylcadaverine incorporation into apolipoproteins

An aliquot of lipoprotein solution (90 μ l) containing 5 mM-dithiothreitol was equilibrated at 37 °C with 10 μ l of 20 mM-monodansylcadaverine (Sigma and 5 μ l of 0.1 M-CaCl₂ (or EDTA), treated with 10 μ l of transglutaminase (either human Factor XIII, activated to Factor XIIIa with 0.5 units of human thrombin or guinea pig liver transglutaminase) at an enzyme-to-apolipoprotein concentration ratio of 1:100, and allowed to incubate for 6 h. The reaction was stopped by the addition of 20 μ l of 0.5 M-EDTA and placing the reaction mixture on ice. Human Factor XIII and guinea pig liver transglutaminase were isolated by the methods of Folk & Chung (1985). Human thrombin was a generous gift from Dr. G. Murano (Food and Drug Administration, Bethesda, MD, U.S.A.).

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis of VLDL, IDL and LDL (treated with monodansylcadaverine to identify labelled proteins) was performed using 3.5% polyacrylamide gels by the method of Weber & Osborne (1969). The apolipoproteins of HDL (treated with dansylcadaverine) were analysed using 10% polyacrylamide gels containing 8 M-urea by the method of Kane (1973), which utilizes tetramethylurea to delipidate and liberate the apolipoproteins of HDL in soluble form. The gels were analysed for fluorescent apolipoproteins by exposure to an illumination at 366 nm, and were stained for protein with either Coomassie Blue or Amidoblack.

Putrescine incorporation into apolipoproteins

The covalent incorporation of [1,4-¹⁴C]putrescine into apolipoproteins was carried out in the presence of Factor XIIIa or guinea pig liver transglutaminase. The 0.5 ml standard reaction mixtures contained 1% apolipoprotein (in the form of lipoprotein), 0.1 M-Tris/acetate (pH 7.5), 1 mM-EDTA, 10 mM-CaCl₂, 4 mM-dithiothreitol, 0.5 μ Ci of [1,4-¹⁴C]putrescine (107.1 mCi/mmol, New England Nuclear, Boston, MA, U.S.A.) and 10 μ l of enzyme (21 μ g of human Factor XIII, activated to Factor XIIIa with 0.5 units of human thrombin or 11 μ g of guinea pig liver transglutaminase). The reactions were carried out at 30 °C for various time intervals (up to 3 h) and were terminated by the addition of 4 ml of cold 7.5% trichloroacetic acid. After 30 min in the cold, the trichloroacetic acid-insoluble precipitates were filtered on Whatman GF/A glass filters and washed with about 20 ml of cold 5% trichloroacetic acid. The filters were dried and transferred to vials containing 10 ml of scintillation counting fluid (Hydrofluor; National Diagnostics, Somerville, NJ, U.S.A.), and vigorously shaken to pulverize the filters. The incorporation of putrescine into protein is expressed as pmol/h per mg of apolipoprotein substrate. For comparison, incorporation of putrescine into lipid-free protein substrates such as casein and dimethylated casein was also examined. The incorporation of putrescine in the presence of EDTA (serving as the blank) was always subtracted from that in the presence of CaCl₂.

RESULTS

In order to determine whether apolipoproteins can act as substrates for transglutaminase, we measured

the covalent incorporation of [1,4-¹⁴C] putrescine into lipoproteins (i.e. apolipoprotein associated with lipids) as catalysed by tissue transglutaminase (guinea pig liver transglutaminase) and Factor XIIIa. The results indicated that the apolipoproteins are suitable substrates for transglutaminases, although there appeared to be greater preference for the tissue enzyme than for Factor XIIIa (Table 1). The rate of [1,4-¹⁴C]putrescine incorporation into the apolipoproteins was found to be linear for at least the first hour of the reaction under the experimental conditions used. It is noteworthy that the apolipoproteins of VLDL were found to be good substrates for tissue transglutaminase and were even better than dimethylated casein, which is one of the most utilized donor substrates for various types of transglutaminase.

When the lipoproteins were treated with guinea pig liver transglutaminase in the presence of monodansylcadaverine, fluorescent labelling of the apolipoproteins took place. As shown in Fig. 1, apolipoprotein B was labelled in VLDL (gel b in Fig. 1), IDL (gel d) and LDL (gel f). In the presence of EDTA, apolipoprotein B in the lipoproteins was not labelled (gels a, c and e in Fig. 1). Based on the relative electrophoretic mobilities, the lowest labelled band in gel b represents the apolipoprotein C of VLDL. The apolipoprotein B from LDL (gel f) contained three labelled bands corresponding (from top to bottom) to the B-100, B-74 and B-26 species of apolipoprotein B. The fluorescence seen at the tops of the gel indicates polymerization of apolipoprotein. A duplicate set of gels stained with Coomassie Blue revealed that the fluorescent bands corresponded to the appropriate apolipoprotein (results not shown). However, no significant polymerization was seen at the tops of the gels containing lipoproteins that had been incubated with transglutaminase in the presence of EDTA, indicative of a calcium-dependent cross-linking by transglutaminase. Comparable results were obtained with human Factor XIIIa, although the reaction required much longer incubation periods or greater concentrations of human plasma Factor XIIIa (results not shown).

Table 1. Incorporation of [1,4-¹⁴C]putrescine into apolipoproteins as catalysed by factor XIIIa and guinea pig liver transglutaminase

Incubations were carried out for 1 h at 30 °C in 100 mM-Tris/acetate buffer, pH 7.5, containing 10 mM-CaCl₂, 0.5 μ Ci of [1,4-¹⁴C]putrescine, 1% lipid-free protein or apolipoprotein (apo) (in the form of lipoprotein) as protein substrate and either 21 μ g of human Factor XIII (activated to Factor XIIIa with 0.5 units of human thrombin) or 11 μ g of guinea pig liver transglutaminase per 0.5 ml. The rate of putrescine incorporation is expressed as pmol/h per mg of protein.

Apolipoprotein	Putrescine incorporation	
	Factor XIIIa	Guinea pig liver
Apo VLDL	607.1	4519.0
Apo LDL	32.5	109.0
Apo HDL	123.6	166.6
Apo HDL ₂	148.9	301.3
Apo HDL ₃	78.8	94.6
Dimethylated casein	1657.0	247.3

Fig. 1. Polyacrylamide-gel electrophoresis of apolipoproteins after incubation with transglutaminase

SDS/polyacrylamide-gel electrophoresis (3.5% acrylamide) was carried out with apolipoproteins from lipoproteins that had been incubated with guinea pig liver transglutaminase in the presence of monodansylcadaverine and either EDTA or calcium. Gel a, VLDL containing 20 μ g of apolipoprotein incubated in the presence of EDTA; gel b, VLDL incubated in the presence of calcium; gel c, IDL containing 5 μ g of apolipoprotein incubated in the presence of EDTA; gel d, IDL incubated in the presence of calcium; gel e, LDL containing 27 μ g of apolipoprotein incubated in the presence of EDTA; gel f, LDL incubated in the presence of calcium. The enzyme/apolipoprotein concentration ratio in each incubation mixture was 1:100. Gels were photographed for fluorescence.

To detect whether the apolipoproteins of HDL can also incorporate monodansylcadaverine, HDL₃ was treated with guinea pig liver transglutaminase in the presence of monodansylcadaverine. As shown in Fig. 2 (gel c), five main bands were fluorescent, with each corresponding to the appropriate apolipoprotein stained with Coomassie Blue (gel a). These fluorescent bands represent the main apolipoprotein bands seen when utilizing the disc gel electrophoretic system of Kane *et al.* (1980): band 1, R-serine (C-I); band 2, R-glutamine I (A-I); bands 3, 4 and 5, apolipoprotein bands derived from thiol-sensitive R-glutamine II (A-II). Significant polymerization in the presence of calcium is seen at the interface between the stacking gel and running gel, again indicative of a calcium-dependent cross-linking by transglutaminase. Purified A-I, A-II and the apolipoproteins of HDL₂ were also fluorescent-labelled *in vitro* when

Fig. 2. HDL₃ apolipoproteins after treatment with transglutaminase

HDL₃ were incubated with guinea pig liver transglutaminase in the presence of monodansylcadaverine, and the apolipoproteins were resolved by the disc gel electrophoresis system containing reducing agents, following solubilization with tetramethylurea (4.2 M). Gel a, Coomassie Blue stain of HDL₃ (182 μ g of apolipoprotein) incubated in the presence of calcium; gel b, HDL₃ incubated in the presence of EDTA; gel c, HDL₃ incubated in the presence of calcium. Gels b and c were photographed for fluorescence. 1-5 indicate the main bands produced.

incubated with either guinea pig liver transglutaminase or human factor XIIIa in the presence of dansylcadaverine and calcium (results not shown).

In order to verify whether apolipoprotein B *in vivo* contains covalent cross-links in the form of stable γ -glutamyl derivatives, apolipoprotein B was subjected to proteolytic digestion with a series of proteinases and an aliquot of the digest was analysed by automated ion-exchange chromatography. Fig. 3 shows a comparison between the chromatographic separation of a standard mixture of di- and polyamines and their *N*-(γ -glutamyl) derivatives (Fig. 3a) and that of a proteinase digest of apolipoprotein B (Fig. 3b). The chromatogram of the digest displays peaks corresponding to *N*¹*N*¹²-bis-(γ -glutamyl)spermine, *N*¹-(γ -glutamyl)spermine, spermidine and spermine. The wide bands eluting between 5 and 45 min contain amino acids and ammonia. This region is also where *N*¹-(γ -glutamyl)putrescine elutes. However, no *N*¹-(γ -glutamyl)putrescine was detected (results not shown) by a novel h.p.l.c. method (Piacentini & Beninati, 1988). In order to obtain ad-

Fig. 3. Ion-exchange chromatographic separation of free polyamines and their γ -glutamyl derivatives

Separations were performed as outlined in the Experimental section. (a) Chromatogram of a standard solution of polyamines and their γ -glutamyl derivatives. The amount of each standard was approx. 1 nmol. Peak 1, N^1N^4 -bis-(γ -glutamyl)putrescine; 2, N^1N^8 -bis-(γ -glutamyl)spermidine; 3, N^1 -(γ -glutamyl)putrescine; 4, N^1N^{12} -bis-(γ -glutamyl)spermine; 5, N^1 -(γ -glutamyl)spermidine; 6, N^8 -(γ -glutamyl)spermidine; 7, putrescine; 8, N -(γ -glutamyl)spermine; 9, spermidine; 10, spermine. (b) Chromatogram of a proteolytic digest of apolipoprotein B. The ammonia and basic amino acids were eluted between 5 and 45 min.

ditional proof of the identity of the peak corresponding to bis-(γ -glutamyl)spermine, an aliquot of the eluted fraction corresponding to peak 4 on the chromatogram (Fig. 3b) was incubated with γ -glutamylamine cyclotransferase and another aliquot hydrolysed with 6 M-HCl. The presence of free spermine was found after either enzyme incubation or acid hydrolysis. A glutamic acid/spermine ratio of close to 2:1 was calculated by acid hydrolysis (1.42 nmol/mg, glutamic acid; 0.63–0.71 nmol/mg, spermine) (Table 2).

N^1N^{12} -Bis-(γ -glutamyl)spermine is the most abundant of the γ -glutamyl polyamine derivatives (structures shown in Fig. 4) found in apolipoprotein B (Table 2). The observations that both the N^1N^{12} -bis-(γ -glutamyl)spermine and N^1 -(γ -glutamyl)spermine peaks disappear and the spermine peak increases after acid hydrolysis of the apolipoprotein B proteolytic digest further support the identity of the polyamine derivatives (results not shown).

We failed to detect ϵ -(γ -glutamyl)lysine in apolipoprotein B isolated from serum LDL (results not shown), consistent with our earlier observations (Cocuzzi & Breckenridge, 1986).

DISCUSSION

The findings (i) that apolipoprotein B contains N^1N^{12} -bis-(γ -glutamyl)spermine and (ii) that several of the apolipoproteins (A-I, A-II, B, C-I) can be labelled with monodansylcadaverine when incubated with transglutaminases and calcium, indicate that apolipoproteins are suitable substrates for transglutaminase. Apolipoproteins are known to be post-translationally modified via glycosylation (Osborne & Brewer, 1977; Brewer, 1981), proteolytic processing (Law *et al.*, 1983; Sharpe *et al.*, 1984) and fatty acid acylation (Hoeg *et al.*, 1986). The evidence presented here indicates that the transamidating mechanism may also be involved in post-translational modification of apolipoproteins.

Although there is a potential for the biological modification of glutamine residues of apolipoproteins via the transamidating mechanism, the physiological

Table 2. Levels of γ -glutamyl derivatives in apolipoprotein B after proteolytic digestion and identification of bis-(γ -glutamyl)spermine by acid hydrolysis and γ -glutamine cyclotransferase incubation

Apolipoprotein B (24 mg) was digested with a series of proteinases, and an aliquot of digest was directly analysed using an automated ion-exchange chromatographic method as described in the Experimental section (a). Another aliquot was acid-hydrolysed for 16 h at 110 °C and analysed (b). The ion-exchange chromatographic fractions corresponding to the bis-(γ -glutamyl)spermine (peak 4 in Fig. 1, gel b) were collected, pooled and desalted. An aliquot was acid-hydrolysed (c) and another aliquot was incubated with γ -glutamylamine cyclotransferase (d). Samples were analysed as reported in the Experimental section. Values are expressed as pmol/mg of protein; n.d., not detected.

γ -Glutamyl derivative	Proteolytic digestion (a)	Acid hydrolysis		γ -Glutamylamine cyclotransferase (d)
		(b)	(c)	
N^1N^{12} -Bis-(γ -Glutamyl)spermine	840	—	—	—
N^1 -(γ -Glutamyl)spermine	80	—	—	—
Putrescine	50	—	—	—
Spermidine	70	130	—	—
Spermine	70	720	630	710
Glutamic acid	—	n.d.	1420	n.d.

Fig. 4. The chemical structures of spermine, N^1 -(γ -glutamyl)-spermine, N^1N^{12} -bis-(γ -glutamyl)spermine and ϵ -(γ -glutamyl)lysine

significance of such a modification is unknown. Apart from reports that transglutaminase might possibly participate in receptor-mediated endocytosis (Davies & Murtaugh, 1984) and exocytosis (Bungay *et al.*, 1984, 1986), at this juncture one can only speculate on the role of this transamidating mechanism in normal apolipoprotein metabolism. The discovery that γ -glutamyl derivatives of polyamines occur in apolipoprotein B suggests, however, that these cross-links might play a vital role in dictating the structure of an apolipoprotein as complex as apolipoprotein B.

The modification of apolipoprotein B-containing lipoproteins *in vitro* (i.e. VLDL, IDL, LDL) by dansylcadaverine in the presence of cellular transglutaminase indicated that apolipoprotein B has many transglutaminase reactive sites. However, these sites must be distinct from the glutamine residues already taken up by spermine. Whether the N^1N^{12} -bis-(γ -glutamyl)-spermine was introduced by transglutaminase catalysis, or by a non-enzymic reaction involving an internal activated thioester bond, such as that found to occur in α_2 -macroglobulin (Sottrup-Jensen *et al.*, 1984), remains unclear. The relative amounts of amine incorporated into apolipoprotein B by either guinea pig liver transglutaminase or human plasma Factor XIIIa (Table 2) indicate that apolipoproteins are better substrates *in vitro* for the cellular enzyme than for Factor XIIIa.

In terms of pathological significance, this apolipoprotein-transglutaminase relationship raises some interesting possibilities. Since LDL is more atherogenic when altered in surface charge (Hoff, 1979) either to an electronegative form (Goldstein *et al.*, 1979) or to an electropositive form (Basu *et al.*, 1977; Goldstein *et al.*, 1977), modification *in vivo* of apolipoprotein B via transamidation with polyamines, which appear to be present in blood (Rennert & Shukla, 1978) and all living cells (Cohen, 1971), and which are substrates for transglutaminase (Schrode & Folk, 1978; Folk *et al.*, 1980; Beninati *et al.*, 1985, 1988; Piacentini & Beninati, 1988; Piacentini *et al.*, 1988) should theoretically generate an 'electropositive' atherogenic LDL. It is known that

increased clot-stabilizing transamidase activity (Hoff *et al.*, 1975), and increased apolipoprotein B levels (Wissler, 1974; Hoff *et al.*, 1977), occur in atherosclerotic lesions. The likelihood that lipid-laden foam cells in atherosclerotic lesions (Small, 1977; Hopkins & Williams, 1981), release polyamines and intracellular transglutaminase during cell death suggests that, under the appropriate conditions, modification of apolipoprotein B *in vivo* by transamidation may indeed take place.

The evidence presented here warrants further exploration into the possible involvement of the transamidation reaction in normal lipoprotein metabolism or lipoprotein disorders.

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