ISOLATION AND CHARACTERIZATION OF A CUTANEOUS LIPOPROTEIN WITH LETHAL EFFECTS PRODUCED BY THERMAL ENERGY IN MOUSE SKIN

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Received January 25, 1971

Summary:

A lipoprotein which has lethal effects when injected into recipient animals has been isolated from mouse skin which has been exposed to controlled thermal energy. The toxic activity served as a means to monitor the isolation procedure. The properties of this product were found to be identical with those of the corresponding non-toxic compound obtained from native skin according to the same isolation procedure. Both products were comprised of the same six lipid classes and six oligomeric polypeptides. The toxin differed from the native product in molecular size and density. It would appear that thermal energy induces polymerization of a normal membraneous cell wall unit.

Introduction:

The isolation and characterization of tissue lipoproteins has encountered considerable difficulties owing to numerous technical problems. Methods used for this purpose tend to dissociate lipid-lipid and lipid-protein bonds and the reassociation products which might form could differ significantly from native material (1). Moreover, the lack of any known functional property of lipoproteins has complicated both the tracing of their fate during isolation and the assessment of their native properties (2). We have now identified a lipoprotein from thermally treated mouse skin which is lethal when injected into recipient animals. This intrinsic biological activity has been used to design an isolation procedure which minimizes the formation of artifactual products of reassociation.

Materials and Methods:

The toxic lipoprotein was isolated from skin obtained from germ free inbred mice which was shaved and the subcutaneous fat removed. Equivalent products were obtained either from intact skin or from "skin residues". To prepare the latter, surface lipids were extracted with chloroform-methanol, 2:1, and skin pieces were

milled in a solution containing physiological amounts of Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}, Cl^{-}, HCO_3^{-}, and $H_2PO_4^{2-}$, pH 7.4 (Tyrode I and II). Thermal energy was applied to either starting material for 15 seconds at 250° under 500g/cm^2 pressure using an electrically heated metal stamp. A 50% weight loss occurred corresponding to the water content but there was no charring. Thermally unmodified skin was subjected to the same isolation procedure. All subsequent steps were carried out at 4° under sterile conditions and bacteriological control. The skins were suspended in 0.25M sucrose, 0.1M phosphate, pH 8.6 (1:20 w/v) and homogenized at 40,000 rpm in a Bühler apparatus equipped with 4cm diameter shearing knives. The homogenate was centrifuged at 1,200g for 20min, the solid supernatant (fat layer) and the sediment were discarded. The middle phase was dialyzed against Tyrode I and II salt solution at pH 8.6 by NaOH and solid ammonium sulfate was added. The precipitate forming between 20 and 30% salt saturation was suspended in and then dialyzed against Tyrode I and II (pH 8.6). Addition of acetone to 50% gave a precipitate which was dialyzed against water, and lyophilized. The material was purified further by ultracentrifugation in a linear density gradient of 30-60% (w/w) sucrose in water at 39,000 rpm (Spinco SW 39 rotor).

Bioassay: All fractions were tested for toxic activity by intraperitoneal injection into germ free mice (3). A 100% mortality within 48 hours served as the criterion of toxicity.

Gel filtration was performed at 20° on Sepharose 2B (2.5x100cm), equilibrated with 0.5M $MgCl_2$, 0.5M $CaCl_2$, 0.5% Triton X-100. The effluent was monitored for protein by reaction with ninhydrin after alkaline hydrolysis (4).

Disc electrophoresis was carried out according to Davis (5) at gel concentrations increasing stepwise from 4.5 to 8.5% acrylamide with a constant ratio (1:30) of BIS[1] to gel. The ionic strength and composition of gel and running buffers were kept constant. Electrophoresis in SDS[2] followed the method described by Weber and Osborn (6) while that at acid pH in the presence of urea and 2-mercaptoethanol followed the procedure of Neville (7).

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[1]BIS = N,N'-methylene-bis-acrylamide (Eastman, Kodak Ltd, USA)
[2]SDS = Sodium dodecyl sulfate 95%, recrystalized from ethanol

Results:

The physical and chemical properties and the biological activity of the products isolated from native skin were compared with those obtained from thermally modified skin. The derivative from native skin was completely inactive even when administered at 10x the dose of the toxic material. The lethal effect of the purified toxin was observed on injection of 0.25 - 0.3mg/g body weight. Lower doses were not effective. The yields after each isolation step are summarized in Table I. Both products were insoluble in aqueous buffers and physiological pH's and contained a lipid and apoprotein moiety. Solubilizing agents such as detergents and organic solvents abolished toxicity. Both products obtained by chemical isolation showed one single protein band on disc electrophoresis at low gel concentrations (4.5 - 5.5%) with decreasing mobility as the gel concentration increased. Electrophoresis of the toxic isolation product at 6.5% and higher gel concentrations revealed a second component which did not enter the gel. The first component of the toxic product had the same electrophoretic behavior as that from native skin.

The products were delipidated by extraction and analyzed by gel filtration (Fig. 1). Two components were detected in the toxic material, one appearing shortly after the void volume and a

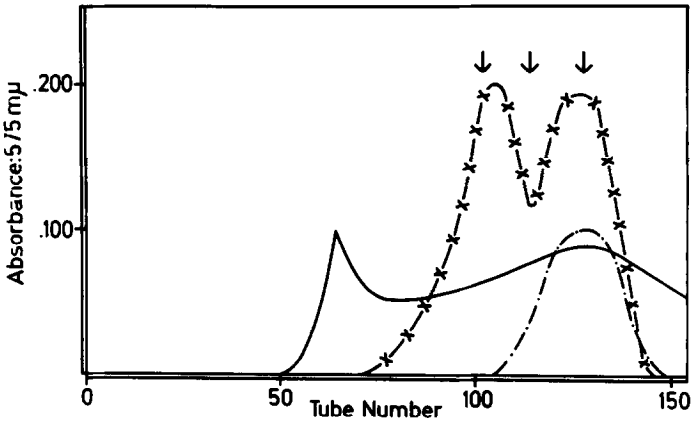

Fig. 1 gel filtration on Sepharose 2B: Apoproteins of the lipoprotein fractions obtained after chemical isolation from thermally exposed (toxic) and unexposed or native (non-toxic) mouse skin. Bed dimensions: 2.5x100cm; flow rate: 2.5ml/cm^3/hr; sample volume: 2.5ml. Blue Dextran-2000 (——); ninhydrin pattern of the toxic (-x-x-) and the non-toxic (-.-.-) product ↓ = fractions analyzed by disc electrophoresis.

TABLE I

FRACTIONS DERIVED FROM THERMALLY MODIFIED (T) AND NATIVE (N) MOUSE SKIN AT DIFFERENT ISOLATION STEPS AS % OF THE ORIGINAL WET WEIGHT

Step	% Weight			
	T			N
Original Skin	100			100
Thermal Energy	50	→	⊘	100
Chemical Isolation	5	→	→	5
Preparative Ultra-Centrifugation	↓	→	→	↓
Fraction I	0.6		Fraction I'	2.9
Fraction II	2.3			--

second emerging at the same position as the single peak found in the native apoprotein.

Ultracentrifugation of the toxic material gave two bands, different in size and density. Fraction I appeared to be identical to the single band, fraction I', present in the native product; fraction II had a higher density and was larger in size. Approximately 40% (w/w) of both products were separated as a "floating pellet" containing loosely bound lipids. All of the toxic activity was in fraction II.

All three fractions, I, II and I', contained additionally 40% (w/w) lipid, extractable by organic solvents. Analysis of the lipid moieties by thin layer chromatography with different solvent systems showed six lipid classes, identical in each of the purified compounds. The residual apoprotein from fraction II still exhibited 40% toxic activity. Reconstitution of this apoprotein with the lipid moieties extracted from fraction I, II or I' restored the original toxicity.

Electrophoresis of the apoproteins obtained by ultracentrifugation or gel filtration (Fig. 2) indicated that all three apoproteins had the same mobility in the 5.5% gel. However, in the 6.5% gel, the apoprotein from fraction II and the first peak from Sepharose 2B were excluded by the pore size limita-

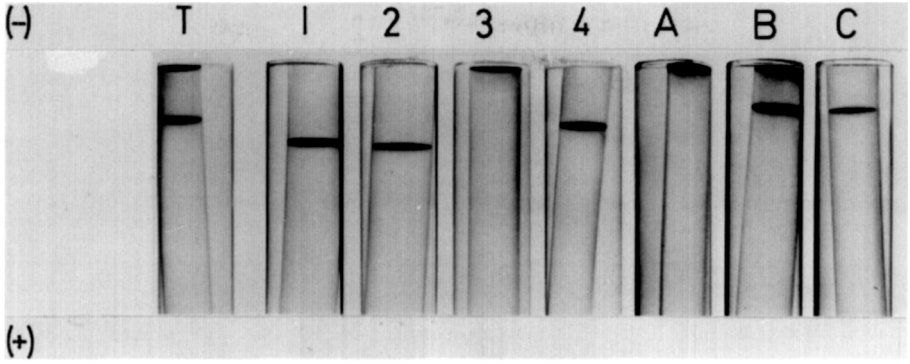

Fig. 2 Electrophoresis at different acrylamide concentrations (a.c.) of the apoproteins purified by either ultracentrifugation or gel filtration. Running buffer: 0.81M TRIS-glycine; gel buffer: 0.37M TRIS-chloride, pH 8.4 (5,8); T = toxic product before final purification (a.c. 6.5%). Purified by ultracentrifugation: 3 = fraction II, 4 = fraction I + I' (a.c. 6.5%). Obtained by gel filtration: A = peak one, B = cross over point, C = peak two (a.c. 6.5%).

tion (8) while that from fraction I still migrated with the same mobility as fraction I'.

Electrophoresis in urea and 2-mercaptoethanol at acid pH, revealed the presence of six differently charged oligomeric polypeptide subunits identical in all three apoproteins (Fig.3).

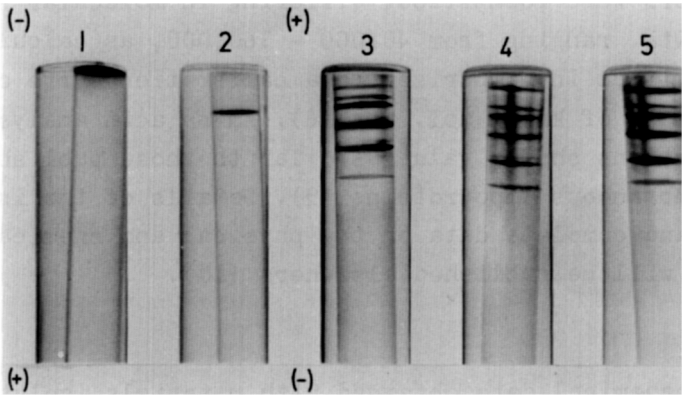

Fig. 3 Separation by charge in a discontinous electrophoresis system in the presence of urea and 2-mercaptoethanol at acid pH (7) of the oligomeric "peptide-subunits" of the toxic and the non-toxic endproducts purified by ultracentrifugation. Normal electrophoretogram for comparison. 1 = fraction II; 2 = fraction I + I' at pH 8.4 (6.5% a.c., normal); 3 = fraction II; 4 = fraction I; 5 = fraction I' at acid pH.

This microheterogeneity was confirmed after abolishing charge differences of the oligomeric polypeptides by adding SDS to the apoproteins and the electrophoretic systems. Again six separa-

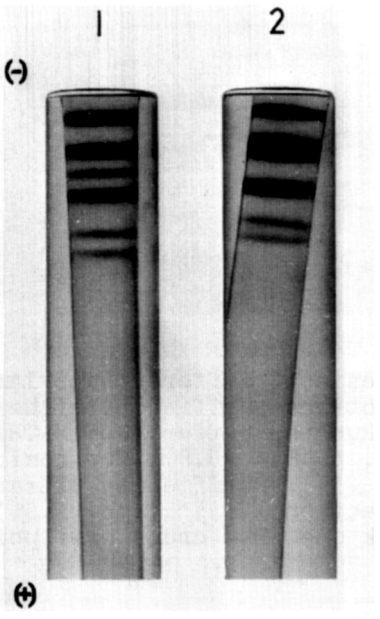

Fig. 4 Electrophoretic separation by size at neutral pH in the presence of SDS, urea and 2-mercaptoethanol of oligomeric "polypeptide-subunits" of the toxic and the inactive endproducts. 10% acrylamide and 37:1 ratio in respect to BIS (9); 1 = toxic, 2 = inactive compound.

ted oligomers were identified, differing in molecular size (Fig. 4), with mol. wts. ranging from 40,000 - 160,000, as calculated by their mobilities in comparison to a calibration curve obtained with oligomers of known mol. wts (6). Amino acid analysis of both apoproteins showed values similar to those published for insoluble membraneous lipoproteins (9). Details of the isolation procedure and complete data on the physical and chemical characterization will be published elsewhere (10).

Discussion:

The physicochemical data obtained with materials isolated from native skin on one hand and thermally altered skin as well as skin "residues" on the other indicate that they are lipoproteins of very similar chemical composition and physical characteristics. The two products differ primarily in their toxicity but also in size and density. This would suggest that the controlled application of thermal energy to skin converts a naturally occurring lipoprotein into a higher molecular weight derivative. The extractable lipids from both sources are identical by thin layer chromatography as are the "substructural" protein oligomers. Hence it

would seem that the toxin is a heat induced polymer of the product isolated from native skin.

Infrared and circular dichroic spectral analyses also indicate some changes and preliminary interpretation suggests that a conformational alteration accompanies this polymerization, though, the ultimate basis for toxicity can only be conjectured upon at the present time (11).

The same polymer could be isolated from skin "residues" as well as from intact skin. Moreover, the yield and the analytical data would limit the possible candidates for the original source of the toxin to a component of the cell wall membrane. The precursor, present in native skin, may in fact represent a basic globular repeating unit of membrane structure even though the ultimate protein subunits are not identical. The results suggest that this compound represents an equivalence of association of units corresponding to a lipoprotein entity from which membranes could be built integrally (12). This novel example for transformation of a native protein into a new product with lethal effect must be visualized in context with the unexplained "late death" phenomenon observed in human burn injury (13).

Acknowledgements:

The authors wish to thank Prof. K. Bernhard, Department of Biochemistry, University of Basel, for his encouragement and advice, Prof. P. Zahler, Theodor Kocher Institut, Berne, for the CD spectral analyses, Dr. R. Studer and co-workers, Chemical Research Division, Hoffmann-La Roche Inc., Basel, for the amino acid analyses and Mrs. Maya Kunz for her technical assistance. We are in debt to Dr.J. F. Riordan, Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, Boston, for helpful discussions and assistance in preparing the manuscript.

This research was in part supported by "Schweizerischer Nationalfonds", grant number 5314.3.

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