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EFFECT OF RESIDUAL ENZYMES ON DEGRADATION OF RADIOIODINATED VLDL BY COLLAGENASE-DISPERSED HEPATOCYTES

David M. Capuzzi, Charles E. Sparks, and Janet L. DeHoff

Philadelphia Veterans Administration Medical Center
and
Departments of Biochemistry and Medicine
Medical College of Pennsylvania
and
The Wistar Institute of Anatomy and Biology
Philadelphia, Pennsylvania

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SUMMARY

Liver cells isolated with crude collagenase rapidly catabolized radioiodinated VLDL. The hepatocytes retained proteolytically active elements of the preparative enzymes. Both collagenase in the absence of cells and hepatocytes prepared with collagenase degraded the C-apoproteins of labeled VLDL. Hepatocytes isolated with a chelating agent in place of collagenase deiodinated, but did not catabolize [^{125}I] labeled VLDL. Similarly prepared cells degraded labeled HDL, but addition of collagenase to incubations greatly enhanced this process. Therefore, data on hepatic catabolism of serum lipoproteins obtained with hepatocytes freshly prepared with collagenase must be interpreted with caution.

INTRODUCTION

The use of hepatocytes isolated with crude enzyme mixtures has increased dramatically as the simplicity and experimental advantages of the preparation have become apparent. When hepatocytes have been dissociated from liver by enzymatic methods, the isolated cells showed intact ultrastructure, excluded vital dyes, and synthesized macromolecules at rates comparable to the intact organ (1, 2). However, much available evidence indicates that the metabolic behavior of freshly isolated hepatocytes is dependent on the enzyme preparation used. Crude collagenase contains proteases (3) which, if retained by the hepatocytes, could affect their metabolism of proteins that traverse the plasma membranes.

Collagenase-dispersed hepatocytes have been reported to catabolize HDL (4-6), LDL (5-7), and VLDL (7) to smaller peptides. The present work

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was undertaken to determine whether this degradation was an inherent property of the hepatocyte or whether it represented the effect of enzymes retained from the dissociating solution. Our evidence shows that proteolytic enzymes derived from crude collagenase remained associated with the isolated hepatocytes and rapidly catabolized the C-apoproteins of labeled VLDL.

EXPERIMENTAL METHODS

Male Sprague-Dawley rats (200-250g), fed Purina Laboratory Chow ad libitum were used for isolation of hepatocytes. Materials and reagents were obtained from sources as previously described (8,9). Collagenase-dispersed hepatocytes were prepared by the method described by Capuzzi et al (9) in which various lots of Worthington type II collagenase (120 units/ml) in Ca++ and HCO_3^- - free Hanks medium were used to perfuse the liver and to disperse cells from the tissue. The cells were gently centrifuged at 50 x g, washed, and resuspended three times prior to use. Cells isolated without enzymes were prepared similarly except that 0.5 mM ethylene glycol-bis (β amino ethyl ether) N,N' -tetracetic acid (EGTA) was substituted for collagenase in the preparative media. The washed cells were suspended in 20 volumes of HCO_3^--free Hanks medium containing 1.25 M $CaCl_2$, pH 7.4 (incubation medium). The range of cell densities (by hemocytometer measurements) was 1.5 - 2.3 x 10^6 cells per ml. VLDL was prepared from serum of rats that were fed a 60 % sucrose-containing diet for 5-10 days prior to exsanguination. VLDL was isolated by ultracentrifugal flotation and was radioiodinated with iodine monochloride (ICL) by the McFarlane method as we previously described (8). Aliquots of suspended cells (4 ml) were incubated with [^{125}I] labeled VLDL in 25 ml plastic Erlenmeyer flasks in a rotary water-bath shaker at 120 rpm. After incubation, the medium was separated from the cells by centrifugation. The labeled peptides in the medium generated by degradation of labeled VLDL were estimated by solubility in trichloroacetic acid (TCA) and were separated by gel electrophoresis by the method of Sparks et al (8). Labeled apoproteins and peptides were also separated by gel filtration chromatography as described under Fig. 4. All samples were counted in a Searle 1195 counter which had an efficiency of 80 % for ^{125}I.

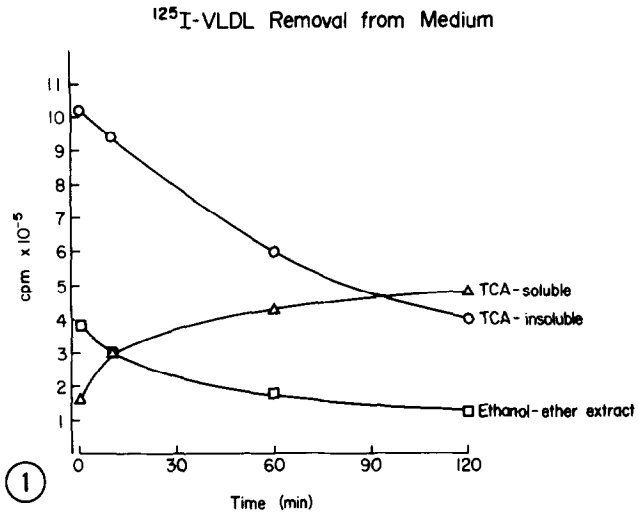
RESULTS

Preliminary Incubation

When [^{125}I] labeled VLDL was incubated with enzyme-prepared cells, there was rapid catabolism of the labeled apoproteins (Fig. 1). A progressive decline in labeled TCA-insoluble protein with generation of acid-soluble products of protein degradation consistently occurred over 120 min of incubation. These products were small peptides and inorganic iodide as shown by electrophoretic separation.

Effect of Crude Collagenase on Labeled VLDL Apoprotein

The capacity of low concentrations of crude collagenase to digest VLDL apoprotein was tested. As shown in Fig. 2, the C-apoproteins (apo C, gel mo-



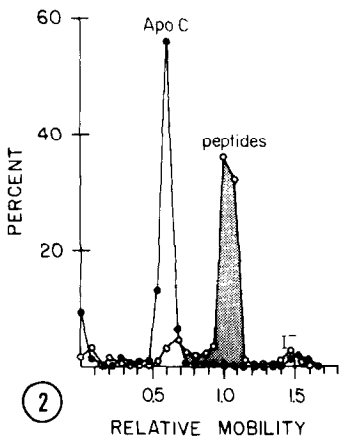


Fig. 1. Time course of catabolism of [^{125}I] labeled VLDL by enzyme cells. Liver cells isolated with collagenase (232 units/mg) were incubated with [^{125}I] labeled VLDL (16 μg protein/ml, 25 cpm/ng) for various intervals. About 79% of the ^{125}I label in this starting batch of labeled VLDL was found in VLDL apoproteins. The range of label in apoproteins in other batches was 76-95%. After removal of cells, the medium was then separated into TCA-insoluble, TCA-soluble, and ethanol-ether (1:3) extractable fractions. These fractions correspond respectively to labeled VLDL apoproteins, degradation products of the apoproteins, and labeled lipids. Each point represents the mean radioactive cpm of duplicate samples.

Fig. 2. Separation of [^{125}I] labeled VLDL apoproteins before and after incubation with collagenase. Labeled rat VLDL (10μg) was incubated with crude collagenase (8 μg, 177 units/mg) in 1 ml of incubation medium for 1 hr at 37°. The medium was analyzed before (●-●-●) and after (o-o-o) the incubation period by electrophoresis on 15% polyacrylamide gels in 0.1% sodium dodecyl sulfate (SDS-PAGE). After electrophoresis the gels were sliced into 0.4 cm sections and counted. The net radioactivity of each gel slice was calculated as a % of total cpm recovered and plotted against relative mobility with respect to Bromophenol blue. Recovery of label was greater than 90% of label applied. The unshaded peak represents the C-apoproteins of [^{125}I] labeled VLDL before incubation. The shaded area represents [^{125}I] labeled peptides derived from the degradation of apo C by crude collagenase. ^{125}I migrates with a relative mobility of approximately 1.5.

bility 0.5-0.75) accounted for most of the total labeled apoprotein of VLDL. Following incubation of labeled VLDL (10 μg/ml) with collagenase, there was loss of 70 % of labeled apo C with formation of a new radioactive peak corresponding to the peptides derived from apo C degradation. When bovine albumin (10 mg/ml) was included in the incubation medium, the catabolism of apo C was diminished by only 25%. Failure of this 1000-fold excess of

TABLE I

Effect of the Number of Cell Washes on Retention of Collagenase by Liver Cells

Quantity of Cell-associated Collagenase μg bound/10^6 cells	Number of Cell Washings
9.4	0
3.1	1 x
1.9	2 x
1.6	3 x
1.3	4 x
1.3	5 x

Crude collagenase (1 mg in 1 ml) dissolved in Ca++ and HCO_3^--free Hanks solution was radioiodinated by the ICl method. After dialysis to remove labeled iodide, labeled collagenase (0.54 mg, 19 cpm/ng) was added to 50 ml of perfusion solution just prior to its use in isolation of liver cells. The resultant cell dispersion (prior to centrifugation) was distributed equally among six centrifuge tubes and centrifuged at 50 x g for 5 min. Each of the six samples was washed sequentially a differing number of times as shown above. The packed cells were then suspended in medium and an aliquot of each sample was counted by hemocytometer. Each sample was also radioassayed.

albumin to prevent the degradation of apo C of VLDL suggests a substrate preference of the crude collagenase for apo C.

Collagenase Retention by Hepatocytes

Crude collagenase was labeled with [^{125}I] and then used to prepare hepatocytes. Surprisingly, after the usual 3 preparative washes, the liver cells retained a significant quantity of enzyme which could not be removed with even two additional washes of the packed cells (Table I).

Metabolism of Labeled VLDL by Hepatocytes Isolated with Collagenase

Since crude collagenase could cleave [^{125}I] labeled apo C into smaller peptides and was retained by hepatocytes, the products generated by incubation of labeled VLDL with these cells were examined. Just as with the crude enzyme alone, the hepatocytes degraded the apo C of labeled VLDL to smaller peptides, but the cells also deiodinated part of the apo C (Fig. 3). The addition of soybean trypsin inhibitor to the medium did not inhibit the proteolytic effect. The catabolism of labeled apo C observed varied inversely

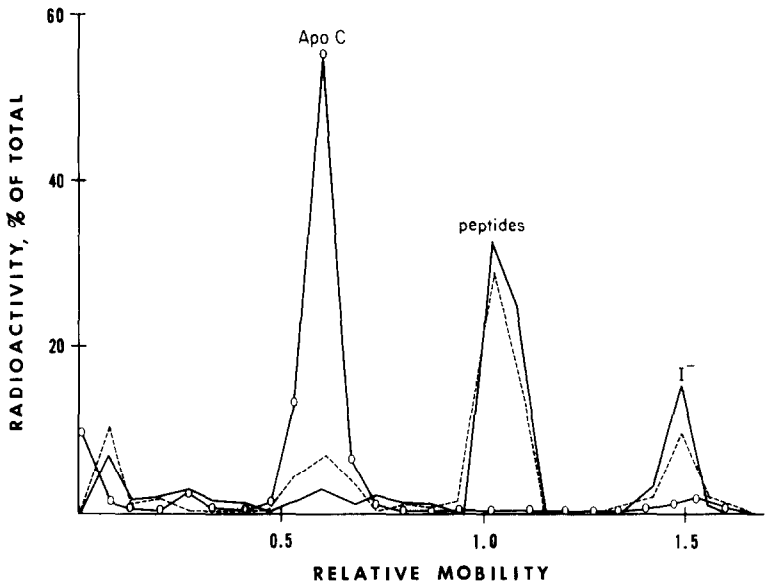


Fig. 3. Catabolism of [^{125}I] labeled VLDL by collagenase-dispersed liver cells. Labeled rat VLDL (19 μg protein/ml, 14 cpm/ng) was incubated for 1 hr at 37° in the absence or in the presence of liver cells that had been prepared with collagenase (140 units/mg). After incubation, the labeled products in the medium were separated by SDS-PAGE as under Fig. 2, and the distribution of label was plotted.
(0-0-0) Products of Labeled VLDL after incubation without cells
(———) Products of Labeled VLDL after incubation with cells prepared with one wash.
(-----) Products of Labeled VLDL after incubation with cells prepared with three washes.

with the number of cell washes used in the isolation procedure. In one experiment, the proportion of label found in catabolic products of apo C after a 1 hr incubation of labeled VLDL with cells amounted to 79 %, 75 %, and 65 % of the beginning labeled apo VLDL corresponding respectively to 1, 2 and 3 washes of the packed cells.

Metabolism of Labeled VLDL by Hepatocytes Prepared without Collagenase

Hepatocytes prepared by chelation with EGTA and then incubated for up to 3 hr with [^{125}I] labeled VLDL deiodinated, but did not catabolize apo C (Fig. 4). The label in apo C (fraction 33) declined slightly while the inorganic ^{125}I$^-$ (fraction 48) rose by 8 % after 1 hr of incubation. Thus, the proteolytic degradation of labeled VLDL by enzyme-prepared cells is probably due to residual crude collagenase, while deiodination is a property

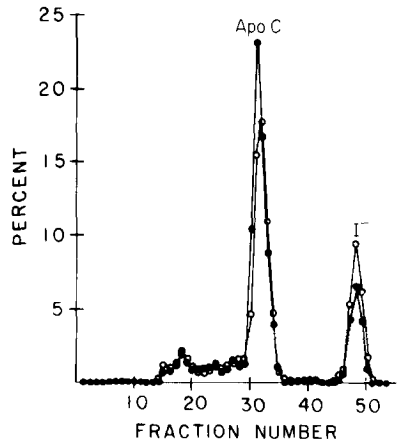


Fig. 4. Catabolism of [^{125}I] labeled VLDL by EGTA-dispersed hepatocytes. Hepatocytes isolated by perfusion with 0.5 mM EGTA as in Methods were incubated with labeled rat VLDL (25 μg protein/ml, 13 cpm/ng) for 1 hr and the incubation medium was analyzed. The medium before (●-●-●) and after (0-0-0) incubation was diluted with an equal volume of 10%, v/v, 2-mercaptoethanol/10%, v/v, glycerol/10%, w/v, SDS/0.01 M Tris-HCl, pH 8.0. The solution was boiled at 100° for 2 min and cooled to room temperature. Aliquots (2.0 ml) of medium were applied to a column (1.6 cm x 90 cm) containing Sepharose 6B CL (Pharmacia) and products were eluted at a flow rate of 6 ml/hr with buffered solution containing 0.1M Na_2HPO_4 (pH 7.4) in 1% SDS. Fractions (3.3 ml) were collected at 30 min intervals and counted. The radioactivity of each fraction was corrected for background. The % of total recovered label of each fraction was plotted against fraction number. Recoveries of greater than 95 % of the applied ^{125}I label were obtained.

of the hepatocyte itself. The absence of label from the larger peptide region of the SDS-column (fractions 36-41) reflects lack of protease activity in cells isolated with EGTA for apoproteins of labeled VLDL.

Influence of Collagenase on Metabolism of Labeled HDL by EGTA-Dispersed Cells

To determine whether labeled HDL could be degraded by liver cells and whether collagenase altered this catabolism, hepatocytes were dispersed with EGTA and incubated with radioiodinated HDL. As shown in Table II, hepatocytes prepared without collagenase catabolized a small quantity of added HDL to labeled TCA-soluble products. However, the addition of collagenase to the cell incubations produced a striking increase in the rate of catabolism of labeled HDL. In a further experiment wherein labeled HDL_3 was incubated for 1 hr with collagenase and the products separated by Sepharose column

TABLE II

Effect of Collagenase on the Catabolism of [^{125}I] labeled HDL by EGTA-Dispersed Liver Cells

Modification	Incubation Time (hr)	Quantity of added [^{125}I] labeled HDL degraded	
		TCA-soluble fraction (μg)	TCA-insoluble protein (μg)
---	0	*0.8 ± 0.1	68.5 ± 2.7
---	1	1.8 ± 0.2	57.5 ± 2.9
---	2	2.4 ± 0.2	61.4 ± 1.1
Collagenase (20 μg) added	2	22.9 ± 0.4	43.5 ± 3.0

*Mean ± S.E.M. of duplicate samples

Cells isolated nonenzymatically by perfusion with 0.5 mM EGTA as in Methods were incubated in 4 ml aliquots (1.7 x 10^6 cells/ml) with labeled HDL (22 μg protein/ml, 8 cpm/ng). HDL had been isolated from rat serum at 1.06 < d < 1.21 (10), radioiodinated as in Methods, and then incubated with the liver cells. 90% of the label in HDL was protein-bound. Where indicated, collagenase (CLS lot #47C239, 199 units/mg) was included at the start of incubation at 5 μg/ml. After incubation, mediur was removed from cells, and was separated into a delipidated TCA-insoluble fraction (undegraded labeled HDL) and an iodide-free (11) TCA-soluble fraction (split products).

chromatography, both apo A-I and apo C of HDL were degraded to smaller peptides. Thus, the potential catabolic effect of collagenase proteases is not limited to apo C of VLDL.

DISCUSSION

Perfusion of liver with collagenase-containing media was a major methodological breakthrough over previous mechanical methods which yielded damaged, "leaky" cells (12). Even with enzyme-isolated cells, metabolic variation has been observed that probably reflects subtle differences in the procedures for cell isolation (12). Several previous studies have suggested that crude collagenases affected the properties of the isolated cells. Solyom et al (13) showed that plasma membranes isolated from collagenase-dispersed hepatocytes had lower activities of some marker enzymes than those prepared from liver homogenates. Even the intracellular content of glycogen has been altered by variation of the crude enzyme mixture (14). Thomas et al (15) reported that disappearance of heparin-releaseable

lipase activity in hepatic perfusates after addition of crude collagenase suggested damage or destruction of the released lipase by the collagenase.

The present data delineate a catabolic alteration of labeled VLDL that stems from residual proteases of crude collagenase that can degrade apo C and perhaps other apoproteins. Therefore, if studies of lipoprotein catabolism are undertaken with collagenase-dispersed hepatocytes, the quantity of preparative enzyme adsorbed and its proteolytic activity against added lipoprotein substrates should be carefully assessed. Thus earlier studies (4-7) designed to investigate hepatic metabolism of serum lipoproteins with such liver cells may require re-interpretation in light of the present findings. This potential consideration probably applies also to other cell types isolated with collagenase preparations since significant adherence of the enzyme to adipocytes even after copious washings of the cells has been found (16, 17).

The present study emphasizes an important variable that is introduced when enzyme-prepared hepatocytes are used to investigate the metabolism of serum lipoproteins. The use of hepatocytes maintained in culture to overcome the initial effects of cell isolation appears preferable for such studies and may obviate some of the questions raised by this work.

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