Detection of Apolipoprotein C in Human and Rat Enterocytes

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ABSTRACT

Apoproteins have important physiologic functions in lipoprotein metabolism. Several apoproteins are produced in the intestine including ApoA-I, ApoA-IV, and ApoB. Each appears to participate in intestinal lipid transport. The liver also produces several apoproteins, including ApoC-II and ApoC-III, but the data demonstrating the ability of the intestine to produce ApoC is incomplete. Our aim was to ascertain whether ApoC-II and ApoC-III were present in human and rat jejunum, and if so, whether their presence was altered by fat feeding. The technique of immunolocalization and a newly developed double antibody radioimmunoassay for rat ApoC-II and III were used. ApoC-II and III was found in the supranuclear regions of enterocytes along the entire lengths of villi in the jejunum of 12-h-fasted rats. 1 hour after the gastric ingestion of corn oil, ApoC-II and III was found primarily in between cells and in the lamina propria. Similar results were obtained in human jejunal biopsies with ApoC-II and III. ApoC-II and III was also detected by radioimmunoassay in enterocytes isolated from jejunum of neonatal and adult rats. Thus, ApoC-II and ApoC-III are clearly present in the intestine as well as in the liver. In addition, because their localization is altered after fat feeding, they are also likely to be produced in the enterocyte.

The production of apoproteins by enterocytes and the important roles of apoproteins in intestinal fat absorption are well established. ApoA-I and ApoB have been localized in enterocytes immunologically and their intercellular localization changes during fat absorption (1–3). Simultaneously, there are alterations in levels of these apoproteins in intestinal cells (3, 4). In addition, ApoA-I, ApoA-IV, and ApoB have been found in chylomicrons isolated from intestinal lymph (5–8). In tracer experiments, lymph chylomicrons also incorporate substantial amounts of labeled amino acids into ApoA-I, ApoA-IV, and ApoB (9, 10). It seems clear, therefore, that these apoproteins somehow participate in intestinal lipid transport.

By contrast, ApoE, although it is found in small intestinal crypt cells, does not alter its intercellular localization in the same way as do the other apoproteins during fat absorption (3). Also, chylomicrons contain only small amounts of ApoE (5, 8), and in tracer experiments, ApoE isolated from lymph chylomicrons contains very little, if any, labeled amino acid (9, 10). Thus, ApoE, which probably has an important role in hepatic lipid transport (3, 11, 12), has an unknown or perhaps no important role in the transport of intestinal lipids.

ApoC-II and ApoC-III, which are important as modulators of the activity of lipoprotein lipase (13, 14), are found in both intestinal lymph chylomicrons (9, 10) and in very low density lipoprotein (VLDL) isolated from hepatic perfusions (15–17). However, while VLDL-ApoC incorporates labeled amino acids during hepatic perfusion (10, 18), chylomicron-ApoC isolated from intestinal lymph contains relatively little label (9, 10). To assess the role of the intestine in the production of ApoC, we have adapted immunologic techniques for the localization (19, 20) and the quantitation (3) of proteins in cells, and applied these techniques to studies of ApoC in human and rat intestine.

MATERIALS AND METHODS

Human intestinal biopsies were obtained from fasting normal volunteers with a biopsy tube (Quinton Instruments, Seattle, Wash.), or at surgery using protocols and consent procedures approved by the Washington University Human Studies Committee. Tissue from overnight-fasted Sprague-Dawley or Wistar rats were obtained under ether anesthesia. For indirect immunofluorescence localization studies (3), tissues were rinsed in ice-cold 0.16 M NaCl, quickly blotted, frozen in liquid nitrogen-cooled Freon at −158°C and stored in screw-cap vials at −70°C until used. 4-μm-thick frozen sections were cut from tissue blocks in a microtome cryostat. Fixing and staining of sections was carried out as previously described using rabbit anti-rat apoprotein or rabbit anti-human apoprotein antisera (see below) at 1:200-1:400 dilutions freshly prepared in phosphate-buffered saline, and goat anti-rabbit IgG (fluorescein-conjugated IgG fraction, Meloy Laboratories Inc., Springfield, Va.). Controls for the immunofluorescence
studies consisted of tissue substrate incubated on glass microscope slides as follows: (a) with buffer alone, i.e., without either rabbit anti-rat apoprotein antiseraum or florescein conjugated goat anti-rabbit IgG conjugate, (b) with conjugate alone, (c) with nonimmune rabbit serum (NIRS) at the same dilution as immune sera and conjugate, (d) with immune sera directed against irrelevant antigens and conjugate, and (e) with specific anti-rat or human apoprotein antisera absorbed with the appropriate apoprotein antigen.

For immunolocalizations, tissues were fixed in picric acid-2% formaldehyde-phosphate buffer, pH 7.4 (21), for 10 min, embedded, paraffin sectioned (4 µm), and mounted on microscope slides. Mounted sections were deparaffinized by two washes in xylene and three washes in isopropanol and then incubated sequentially with nonimmune goat serum (NGS), rabbit anti-rat (1:100-1:200 dilutions) or anti-human apoprotein serum (1:200-1:400 dilutions), NIRS goat anti-rabbit IgG, and goat peroxidase-anti-peroxidase complex. Peroxidase reaction was developed with 0.0125% diaminobenzidine-tetrahydrochloride (DAB), 0.0025% H2O2 (22). Slides were then stained with 4% osmium, dehydrated, and covered with a coverslip (Pernott, Fisher Scientific Co., Pittsburgh, Pa.). Controls for immunoperoxidase localizations consisted of tissue sections incubated with DAB and H2O2 but (a) with buffer alone, (b) with PAP but in the absence of antiapoprotein antisera or nonimmune serum, (c) with NIRS, and (d) with antiapoprotein antisera which had been absorbed with the appropriate antigen.

For both fluorescence and immunoperoxidase staining, adjacent sections were stained with hematoxylin and eosin, and in the identification of tissue structures.

Apoprotein contents were determined in homogenates of mucosal scrapings and intact isolated cells of the rat. Mucosa was scraped by the use of glass slides from nonfixed intestine kept chilled on a glass plate. Scraping removed the mucosa, leaving only underlying muscularis. Isolated intestinal cells were prepared by the method of Merchant and Heller (23) using buffer B (0.137 M NaCl, 2.69 mM KCl, 8.10 mM Na2HPO4, 1.5 mM EDTA, and 0.5 mM dihydrothreitol at pH 7.4). Segments of intestine were distilled with the buffer and incubated at 37°C for 20, 40, and 60 min. The released cells were harvested by centrifugation and washed twice by centrifugation in 15 mM Tris, pH 7.4. On histologic examination of the treated intestinal segments, these incubation times yielded cells enriched from the outer, middle, and inner villi (and crypt) areas, respectively. Phase microscopy of the washed isolated cell preparations revealed intact cells or clumps of 2 or 3 cells, with little cellular debris. Mucosal scrapings and isolated cells were homogenized in 4 vol and 2 ml of 15 mM Tris, pH 7.4, respectively, and assayed for their contents of total protein (24) and apoproteins (25, and see below) and for their activities of sucrase (26). The ApoC-III, radioimmunoassay (RIA), homogenates of mucosal scrapings were treated with urea as follows: Mix 100-µl samples with 300 µl of 8 M urea, incubate for 2 h at 37°C, dilute sample 1:10 in buffer, and assay. In separate experiments it was shown that the amounts of urea used did not interfere with the assay.

Specificities of antisera were tested in double antibody immunoprecipitations as described (3). The monospecificities of the anti-rat Apo-A-I, anti-rat ApoB, and ApoE antisera have been previously documented. The anti-rat apoprotein antisera bound <2% of the inappropriate apoprotein. The antisera used in the immunolocalization studies in human tissues were the same antisera used for RIA's of those apoproteins (27-31). The antisera against human apoproteins A-I, A-II, B, E, C-II, and C-III were able to bind >85-95% of the appropriate 125I-labeled apoproteins, but <7% of other 125I-labeled apoproteins.

Because the double antibody RIA for rat ApoC-III has not been previously published, the reagents and the assay procedure will be described here. ApoC-III was isolated from rat high density lipoprotein (HDL) (d 1.070-1.210, one spin at d 1.070, two at d 1.210, each spin for 2 × 105 g-min) by column chromatography. First, HDL was lyophilized and delipidated with ether (23, vol/vol) then the relatively small ApoC's were isolated from the larger apoproteins by chromatography on a 2.5 × 90 cm column of Sephadex G200 (0.01 M Tris, 8 M urea, pH 8.6). The ApoC-containing peak was then chromatographed on DEAE cellulose (Whatman DE 52) using a 2-liter Tris-urea linear gradient (32). ApoC-III was identified by its position in the elution profile, by isoelectric focusing (33, 34) and by amino acid analysis (performed in the laboratory of Dr. Ralph Bradshaw, Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo.). ApoC-III, which yielded a single band and had the appropriate amino acid composition (32), was used as an immunizing antigen, as 125I-labeled tracer, and as assay standard. Anti-ApoC-III antisera were produced in rabbits. Anti-rabbit IgG was prepared in goats. Iodination was carried by lactoperoxidase and H2O2 as described for human Apo-A-II (28). The resulting 125I-labeled antisera were isolated from unreacted 125I-Na by chromatography on Sephadex G-25. Label was stored frozen at -20°C in 0.05 M barbital, pH 8.6, 15% bovine serum albumin (fraction V. BSA, Sigma Chemical Co., St. Louis, Mo.) and purified before each assay by chromatography on Sephadex G50 (0.05 M barbital, pH 8.6, 0.1% Triton X-100, assay buffer). The calculated specific radioactivity of 125I was ~15 µCi/µg and iodinations were carried out every 4-5 wk. Greater than 95% of the label was precipitable by 10% TCA and ~90% was precipitable by excess anti-rat-ApoC-III antisera (R-211). The anti-ApoC-III antisera bound <2% of rat 125I-ApoC-II, 125I-ApoA-I, 125I-ApoB, and 125I-ApoE. Thus, all of the antisera used in this report were monospecific for their respective antigens.

The RIA tubes contained 100 µl of antisera (final dilution 1:4,400), 100 µl of 125I-ApoC-III (~15,000 cpm, 12 mg), 50 µl of NIRS (final dilution 1:500), 10-200 µl of samples or standards (diluted as needed in assay buffer), and sufficient assay buffer to bring the volume to 550 µl. (All dilutions were carried out using buffer.) After mixing of the tubes, they were incubated for 42 h at 4°C. 50 µl of goat anti-rabbit IgG was then added (final dilution 1:2,200), and incubation was continued at 4°C for another 16 h. Tubes were then centrifuged at 3,000 g for 30 min. precipitates were "washed" in ice-cold saline and then counted in a Packard Autogamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Tubes that contained all reagents except for anti-ApoC-III, were included in each assay to test for nonspecific precipitation. The counts in these tubes, <3% added counts, were subtracted from each assay tube to obtain net counts. Results were calculated on a programmable calculator (Hewlett-Packard Co., Palo Alto, Calif.) using a program provided by the vendor which weights points to obtain a best-fit curve using the logit B/B0 vs. dose (ng) plot.

\[
\text{Logit } \frac{B}{B_0} = \frac{B}{B_0} = \ln \left( \frac{B}{B_0} \right)
\]

where \(B_0\) = net counts per minute in the absence of standard or sample and \(B\) = net counts per minute in the presence of standard or sample. In an attempt to shorten and simplify the assay, incubation conditions were changed as follows: Add 250 µl of sample buffer, 100 µl of NIRS or antisera, 100 µl of labeled ApoC-III. Incubate for 2 h at 37°C and for 16 h at 5°C. Add 300 µl of 30% polyethylene glycol (P-155 Carbows PEG 6000 Flake, Fisher Scientific Co.) 35 µl 17 dilution of bovine serum (nonhemolyzed, adult from Pel-Freez Biologicals Inc., Rogers, Ark.). Vortex, let stand 8 or 10 min, centrifuge at 2,400 rpm for 25 min. Count precipitates. These incubation conditions yielded plasma values comparable to the ones obtained with the double antibody assays and the latter conditions were used to obtain tissue levels of ApoC-III.

RESULTS

ApoC-III Assay

The addition of increasing amounts of ApoC-III standard to the assay tubes resulted in the production of typical competitive displacement curves (Fig. 1). The coefficient of variation of triplicate tubes in 12 assays ranged 2-6% and averaged 4%. The mean slopes, intercepts, and r values (± SD) of 12 curves run over 10 mo were -2.62 ± 0.31, 2.60 ± 0.29, and 0.995, respectively. To ascertain whether the assay detects not only ApoC-III, but also the less sialylated forms of ApoC-IIIb, ApoC-IIIc was bound to the assay. On a per unit mass basis it produced only 5% of the displacement produced by ApoC-III. VLDL, HDL, and whole plasma produced displacement curves which had slopes within ± 1 SD of the slopes of the standard curves, but rat ApoA-I, ApoC-II, and ApoE produced no displacement of counts even at doses of 1,000 mg, indicating that the assay was specific for ApoC-III. LDL (d 1.025-1.050)
appeared to contain <2% ApoC-III₃ (ApoC-III₃ by RIA × 100 + LDL-protein [by Lowry's method, reference 24]). Four rat HDL (d 1.070–1.19) and four d < 1.070 fractions contained 9–13% and 23–35% ApoC-III₃ (percent of lipoprotein protein). These percentages are compatible with those obtained on column chromatographic separation of rat HDL and VLDL + LDL apoproteins (32, 35, 36), suggesting that the assay is accurate. As a separate check on accuracy, increasing amounts of ApoC-III₃ were added to a rat plasma pool (four plasmas) and the plasma samples with and without the added ApoC-III₃ were assayed. Recoveries of added ApoC-III₃ were 95% ± 9 (n = 9). ApoC-III₃ contents of 11 female and 13 male rats fed Purina rat chow were 17 ± 4 and 18 ± 4, respectively. Delipidation of the plasmas (27) had no effect on their contents of ApoC-III₃.

**Intestinal Mucosal Contents of ApoC-III₃**

Homogenates of mucosal scrapings of adult rat jejunal mucosal scrapings contained 55 ± 6 ng of ApoC-III₃/mg of protein, and 28 ± 11 ng of ApoA-I/mg (Table I). Homogenates of jejunal and ileal mucosal scrapings of 16-h-fasted, 13-d-old suckling rats contained 30 and 25 ng of ApoC-III₃/mg homogenate protein, respectively. The analogous ApoA-I contents of these homogenates were 45 ng/mg for the jejunum and 48 ng/mg for the ileum (3).

Homogenates of isolated cells derived largely from the outer villus of adult rats contained more ApoC-III₃ than did middle villus cells (Table I). Lowest ApoC-III₃ levels were found in isolated cells derived mostly from the lower villus and crypt regions. A similar gradient from the top to the bottom of the villus was noted for sucrase activity. (The ApoC-III₃ contents of isolated cells homogenized in 150 mM Tris, pH 7.4, in the presence of 1% Triton X-100 were not significantly different from those homogenized in the absence of Triton X-100. Aliquots of cell homogenates were centrifuged at 105,000 g for 30 min. Supernatant fractions and pellets contained 78 and 22% of the total tissue levels of ApoC-III₃. Therefore, results of whole homogenates are given.)

It was important to determine that the preparation of isolated cells was not contaminated by plasma. Three experiments were performed which excluded this possibility. First, ¹²⁵I-labeled ApoA-I and ¹²⁵I-ApoC-III₃ were injected intravenously into rats at a level of radioactivity such that contamination of intestinal tissue by plasma of 0.1% would be easily detected. No ¹²⁵I counts were found in the isolated intestinal cell preparation with either ¹²⁵I-labeled apoprotein. Second, we measured ApoA-I and ApoC-III₃ levels in mucosal scrapings and also in the isolated cells derived from adjacent intestinal segments of three separate rats. ApoA-I contents in three scraped mucosal preparations which might contain plasma contaminants were 40, 26, 19 ng/mg protein and in isolated washed cells of the same intestines were 243, 207, 251 ng/mg. Values for ApoC-III₃ in mucosal scrapings were 61, 52, 51 ng/mg protein and in cells isolated from the entire villus were 65, 51, 54. As can be seen from Table I, the specific activity of cells from the outer and middle villus is greater than that from the mucosal scrapings. Finally, the presence of albumin in this cell preparation was looked for by immunoelectrophoresis and none was detected. Thus, it seems unlikely that plasma contamination contributed significantly to the apoprotein content in the isolated cell preparation.

As a further control on the specificity of our methods, ApoA-
I and ApoC-III were determined in several other organs and tissues of the rat. Because the plasma concentrations of ApoA-I and ApoC-III were 528 µg and 19 µg/ml, respectively, far in excess of intestinal tissue levels, we prepared animal carcasses free of plasma by extensively perfusing the whole animal via the aorta with Krebs-Ringer bicarbonate buffer, and removed the heart, gastrocnemius muscles, brain, and lung for analysis. No ApoA-I or ApoC-III was detectable in homogenates (10% wt/vol) of these tissues. By contrast, ApoC-III was found in 48-h primary cultures of rat hepatocytes at levels of 55 ng/mg protein (Schonfeld and Patsch, unpublished observations).

**Immunolocalization of ApoC-III in Rat Intestine**

ApoC-III was localized within the rat jejunal enterocytes by the PAP procedure (Fig. 2). Specific reaction product was found in the supranuclear regions of enterocytes throughout the length of the villus in tissues from 16-h-fasted rats (Fig. 2A and B). This region corresponds to that of the Golgi apparatus. There was little or no staining of the lamina propria.

Tissues were also sampled 1 h after the administration of 1.5 ml of corn by stomach tube (Fig. 2 C and D). (The animals were awake during intubation and until just before tissue samples were taken.) The amount of supranuclear staining for ApoC-III along the whole villus decreased but staining appeared between enterocytes at the tip of the villus. In addition, the lamina propria became heavily stained, particularly in the lacteals (see arrows). Similar results were obtained with ApoA-I (Fig. 3A and B) and ApoB (not shown). Indeed, after fat feeding the amount of A-I seen between and at the base of enterocytes was much greater than in the case of ApoC-III (compare Fig. 2C with Fig. 3B). In addition, as can be seen in both villi shown in Fig. 3B, the amount of ApoA-I staining in the Golgi region was somewhat diminished when compared with the fasted tissue (Fig. 3A).

Stain was also deposited along the brush border and in the region of the terminal web not only in tissues stained with antisera, but also in tissues stained with NIRS (Fig. 3C and D). Therefore, staining of this region was considered a nonspecific reaction. Nonspecific staining of the brush border was also seen with the immunofluorescent technique (see Fig. 4A). Erythrocytes were also nonspecifically stained (Fig. 3C and D). Staining for ApoC-III was looked for in tissue slices made from rat stomach, colon, brain, pancreas, and muscle. The only positive staining found was in blood vessels. However, slices made from rat liver demonstrated that hepatocytes stained strongly for ApoC-III and weakly for ApoA-I (not shown).

**Immunolocalization of ApoC-II and ApoC-III in Human Intestine**

ApoC-II in human jejunum behaved as it did in rat (Fig. 4C and D). (The anti-human ApoC-III antisera did not distinguish between the sialylated forms of ApoC-III [31].) Specific immunofluorescence caused by ApoC-III was present in the supranuclear (Golgi) areas of enterocytes throughout the length of the villus in 12 to 14-h-fasted subjects. On rebiopsy after the subjects drank 50 ml of a corn oil formula, most of the ApoC-III immunofluorescence was found in the basal portions of cells and in the lamina propria. ApoC-II was also found in fasting human enterocytes (Fig. 3B) and behaved similarly after corn oil feeding (not shown). Supranuclear fluorescence was not seen in tissues incubated with nonimmune serum (Fig. 4A).

**DISCUSSION**

It is now clear that several of the apoproteins found in lipoproteins of intestinal lymph are synthesized in the gut itself (1-10). Similarly, the liver synthesizes several of the apoproteins found in lipoproteins of hepatic origin (11-13, 15-18). However, the patterns of synthesis in the two organs are quantitatively not alike. Whereas the major apoprotein products of the intestine are ApoA-I and ApoA-IV, the major products of the liver are ApoE, ApoC-II, and ApoC-III. Both organs produce ApoB in abundance. These differences are interesting because the lipid moieties transported by the two organs are similar, but the mechanisms responsible for the organ specificities are unknown.

The aim of this work was to ascertain whether any of the ApoC's were present in enterocytes and whether they participated in any way in fat absorption. We investigated this question using some of the immunolocalization and apoprotein quantitation techniques we used previously for ApoA-I, ApoB, and ApoE in rat liver and intestine (3). Immunolocalization
FIGURE 4  Immunofluorescence localization of ApoC-II and ApoC-III in human jejunal enterocytes. Sections were obtained from biopsies. All sections shown are from near the tips of villi. A is a section processed in the presence of NIRS. (It represents the most intensely stained control of the five nonimmune sera used.) The nonspecific staining of the brush-border region is evident. B is a section stained for ApoC-II and is taken from an individual after 16 h of fasting; C and D are sections from the same individual stained for ApoC-III. In this preparation the nonspecific staining of the brush-border region was much less intense than in other biopsies. C is a 16-h-fasted sample; D is a rebiopsy from the same individual 1 h after the oral ingestion of 50 ml of corn oil. The corn oil induced depletion of cellular stain and the appearance of stain in the lamina propria are obvious. c, cytoplasm; n, nucleus; lp, lamina propria.

requires that the antisera be monospecific and that appropriate positive and negative controls be run. We demonstrated that the rat anti-ApoC-III antisera were indeed monospecific. Similarly, we demonstrated the monospecificity of the human anti-ApoC-II and anti-ApoC-III antisera (31). Using these antisera, we found that ApoC-III was present in rat intestine (and liver) but not in other rat tissues. The determination of tissue levels of ApoC-III required an immunoassay which was specific, precise, and accurate. The RIA's for rat ApoC-III and for human ApoC-II and ApoC-III fulfill these criteria. Therefore, tissue levels could be determined with precision. The RIA's confirmed that ApoC-II and ApoC-III were present in human intestine and that ApoC-III was present in the rat intestine (and liver, but not in other rat tissues). Furthermore, the intracellular localizations of the apoproteins were altered with fat feedings. (Rat ApoC-III levels in intestinal cells are also altered [Alpers, Grimme, and Schonfeld, unpublished observations]). In this respect, the behaviors of ApoC-II and ApoC-III resemble those of ApoA-I and ApoB (2, 3). These data suggest that ApoC-II and C-III are indeed present in the enterocyte and that these proteins are involved in fat absorption.

While we were studying intestinal apoproteins by immunologic means, Wu and Windmuller reported on the intestinal synthesis of ApoC-II and ApoC-III in rat in their elegant perfused intestinal system (9, 10). They demonstrated the incorporation of small amounts of label in the ApoC-II and ApoC-III of chylomicrons, VLDL, and HDL isolated from intestinal lymph. From other experiments they concluded that the intestine contributed ~8% of the circulating ApoC pool. Thus, two independent lines of evidence suggest that ApoC-II and ApoC-III may be produced in the intestine.

The roles, if any, of ApoC-II and ApoC-III in intestinal fat transport remain to be elucidated, but the presence of chylomicrons in the plasmas of subjects with ApoC-II deficiency after fat feeding (37) suggests that ApoC-II may not be essential for intestinal chylomicron production.

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