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# Biosynthetic precursor (214 kDa) of apolipoprotein B-48 is not secreted by Caco-2 cells and normal human intestine

W. Vodek SASAK,*‡ Hans A. BULLER* and Randolph REINHOLD†

*Division of Pediatric Gastroenterology and Nutrition, Department of Pediatrics, and †Department of Surgery, New England Medical Center, Tufts University School of Medicine, Boston, MA 02111, U.S.A.

The synthesis and secretion of apolipoprotein B (apo B) was studied in a human colon carcinoma (Caco-2) cell line and in explants from normal human intestine. In Caco-2 cells, the specific activity of the intestinal disaccharidases maltase, sucrase–isomaltase and lactase was enhanced 8-, 6- and 3-fold respectively, at 19 days post-confluence as compared with 1-day-post-confluence cultures. The level of apo B secreted into the medium increased from undetectable in the cells just reaching confluency, to 115 ng/ml at 18 days post-confluence. The presence of apo B-100 and apo B-48 with mobilities on SDS/polyacrylamide-gel electrophoresis corresponding to those of human very-low-density lipoproteins and lymph chylomicrons, respectively, was detected in the media from 7-, 12- and 18-days-post-confluence cells. These two apo B proteins were also found intracellularly in 7-day-post-confluence cultures. However, more differentiated cells (12 and 18 days post-confluence) accumulated large amounts of a 214 kDa protein intracellularly. Apo B-related 214 kDa protein was also synthesized by normal human intestinal explants. A pulse–chase experiment with explants from normal human jejunum showed a slow intracellular conversion of the 214 kDa protein into the size of mature apo B-48 (264 kDa), concomitant with increasing amounts of mature apo B-48 in the medium, suggesting a precursor–product relationship. Despite large intracellular quantities, the 214 kDa protein from the normal human tissue and Caco-2 cells was absent from the medium. No apo B-100 synthesis was detected in the human explants. These findings may help in our understanding of cholesterol and lipid metabolism in health and in some disorders characterized by the inability to secrete apo B-containing lipoproteins.

## INTRODUCTION

The liver and the small intestine secrete apo B in the form of VLDL and chylomicrons, respectively. In humans, two major forms, apo B-100 (549 kDa) and apo B-48 (264 kDa), exist [1]. The failure to secrete chylomicrons leads to lipid malabsorption that results in deficiencies of lipid-soluble nutrients. In addition, in abetalipoproteinaemia and in chylomicron-retention disease (Anderson's disease) there are no detectable apo B-containing lipoprotein particles in plasma and lymph [2]. However, accumulation of apo B can be detected within the enterocyte [3,4]. The metabolic basis of these diseases is unknown; the stepwise assembly and processing of human apo B, and mechanisms that regulate secretion of lipoproteins, have not been delineated. This is, at least partially, due to the complexity of the processes involved and a lack of good experimental models to study individual steps. Recently, an established cell line (Caco-2) derived from a human colon carcinoma [5] has been shown to synthesize and secrete a variety of apolipoproteins, including apo B [6,7]. The secreted apolipoproteins are found in the medium as lipoprotein particles [6,7]. In differentiated Caco-2 cells, a significant increase in activity of the brush-border-associated enzymes is also observed [5].

In the present study, apo B synthesis and secretion were examined as a function of differentiation of Caco-2 cells. As differentiation progressed, a 214 kDa protein was the major intracellular form. The 214 kDa protein was found to be a precursor of the mature apo B-48 (264 kDa) in explants from normal human intestine. The 214 kDa form was not found in the medium and accumulated intracellularly.

## EXPERIMENTAL

### Cell maintenance and labelling

Caco-2 cells obtained from the American Type Culture Collection (Bethesda, MD, U.S.A.; ATCC no. HTB-37) were cultivated as described previously [5,6]. The cells spontaneously differentiate in confluent cultures and express properties typical of differentiated small-intestinal cells [5]. For radioisotope labelling, the cells were washed with methionine-free DMEM and incubated for 30 min in DMEM containing 10 $\mu$M-methionine (low-methionine DMEM) and 100 kallikrein-inhibitor units of Trasylol (Sigma Chemical Co.)/ml. Small amounts of methionine in the medium were used to avoid methionine starvation of cells. Then 200 $\mu$Ci of L-[$^{35}$S]methionine (691–701 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.)/ml was added and the cells were incubated for 6 h at 37 °C.

Abbreviations used: apo B, apolipoprotein B; VLDL, very-low-density lipoproteins; DMEM, Dulbecco's Modified Eagle Medium; PAGE, polyacrylamide-gel electrophoresis.

‡ To whom correspondence should be addressed, at: Tufts–New England Medical Center, 750 Washington Street, Box 200, Boston, MA 02111, U.S.A.

### Organ culture

Normal human small intestine obtained from surgical specimens was utilized within 1 h from the time of excision. The explants were prepared as described for rat jejunal material [8]. The explants (10/dish in 0.9 ml) were preincubated for 30 min in low-methionine DMEM and then labelled for 5 h with 200 $\mu$Ci of L-[$^{35}$S]methionine/dish. For pulse–chase experiments, the cells were preincubated for 30 min in low-methionine DMEM and then pulsed for 20 min with 0.5 mCi of L-[$^{35}$S]methionine/ml. The label was chased for the times indicated with DMEM containing a 200-fold excess of non-radioactive methionine in a sealed jar, gassed periodically with $O_2/CO_2$ (19:1). The studies with human material were approved by the institutional Human Studies Committee.

### Immunoprecipitation and SDS/PAGE

Media were collected and immediately supplemented with a protease inhibitor cocktail as reported elsewhere [9,10] to yield final concentrations of 20 $\mu$g of soya-bean trypsin inhibitor/ml, 20 $\mu$g of lima-bean trypsin inhibitor/ml, 2 mM-benzamidine, 25 $\mu$g of Polybrene/ml, 1 $\mu$M-D-phenylalanyl-L-prolyl-L-arginylchloromethane and 0.02% (w/v) GSH (all from Sigma). The media were then centrifuged to remove any detached cells, and adjusted to 25 mM-Tris/HCl, pH 7.5, 20 mM-NaCl and 0.5% (v/v) Triton X-100 (immunoprecipitation buffer). The cells were scraped into the immunoprecipitation buffer, protease inhibitors were added to the same concentrations as present in the media, and the suspension was homogenized on ice in a Brinkman tissue homogenizer for 3 × 20 s, followed by centrifugation at 30000 *g* for 1 h. The supernatant from the cell homogenate and the media were subjected to immunoprecipitation with a goat affinity-purified polyclonal antibody prepared against human LDL and recognizing both apo B-100 and apo B-48 [11]. The antibody was bound to CNBr-activated Sepharose beads (Pharmacia) according to the manufacturer's instructions. The immune complex was washed once with 50 mM-Tris/HCl, pH 7.5, 150 mM-NaCl, 0.05% Triton X-100, 0.1% (w/v) SDS, and twice with this buffer, except that 0.02% SDS and 0.1% Triton was present, dissolved in Laemmli sample buffer [12] by boiling for 2 min, and analysed by PAGE on a 5%-acrylamide separatory gel with 3% stacking gel. Human VLDL containing apo B-100 and apo B-48, and myosin (Bio-Rad), were used as standards. Myosin (molecular mass 200 kDa) migrates on SDS/PAGE as a protein of $M_r$ 214000.

### Other methods

The enzyme-activity assays of maltase, sucrase–isomaltase and lactase were performed as described by Dahlqvist [13]. Secreted apo B was quantified by e.l.i.s.a. [11]. At the times indicated, the regular serum-containing medium was removed, and the cells were washed with serum-free medium and incubated in this medium for 24 h before samples were taken for analysis. Incorporation of label into total protein fraction was determined by precipitation with 10% (w/v) trichloroacetic acid. The precipitates were washed three times with cold 10% (v/v) trichloroacetic acid, solubilized in 1 M-NaOH, neutralized and counted for radioactivity in a liquid-scintillation counter.

## RESULTS

As shown in Fig. 1, the activities of specific disaccharidases increased with time post-confluence of Caco-2 cells. Enzyme activities started to rise substantially at 6 days post-confluence, with maximal activities reached at 19 days post-confluence. At that time, the activity of maltase was increased 8-fold, that of sucrase–isomaltase 6-fold, and that of lactase 3-fold. Since total cell homogenates were used here for the determination of the enzyme activities, lower values (approx. 7-fold) than those reported for sucrase–isomaltase in isolated brush-border membranes of Caco-2 cells [5] were expected. The level of apo B secreted into the medium rose markedly from undetectable (day 0 post-confluence) to 15.6 ng/ml at 6 days post-confluence, and reached a maximum of 115 ng/ml at 18 days post-confluence (Fig. 2). Apo B synthesis seems to be localized to small-intestinal cells, as no apo B was found in human colon [14], and no mRNA for apo B was detected in rat colon [15].

To characterize further apo B synthesized and secreted by Caco-2 cells, the monolayers were incubated with L-[$^{35}$S]methionine at 7, 12 and 18 days post-confluence; then apo B was immunoprecipitated and analysed on SDS/PAGE (Fig. 3). Human VLDL containing apo B-100 and small amounts of apo B-48 were also run on the

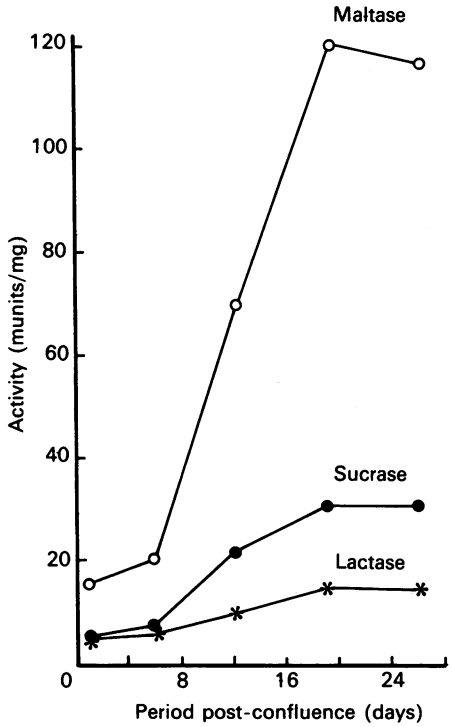


**Fig. 1. Enzyme activities of intestinal disaccharidases in Caco-2 cells**

The cells cultured as described in the Experimental section were washed extensively, scraped from the dishes and homogenized in 0.1 M-phosphate buffer, pH 6.0. The assays were carried out in duplicates as described by Dahlqvist [13]. Protein was determined by the Lowry method. ○, Maltase; ●, sucrase–isomaltase; *, lactase. Variations between separate determinations were in the range 3–12%.

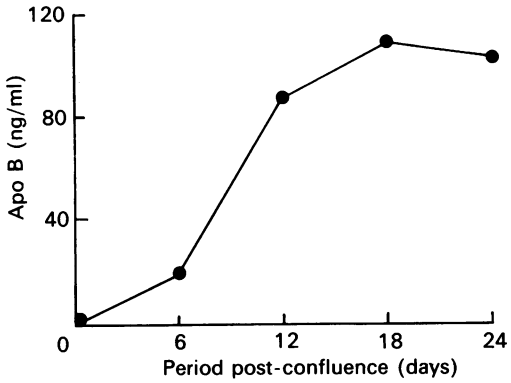


**Fig. 2. E.l.i.s.a. of total secreted apo B (apo B-100 and apo B-48) by Caco-2 cells**

At different stages of cell differentiation, the regular serum-containing medium was replaced with serum-free medium, and the cells were incubated for an additional 24 h. Apo B content in undiluted media samples was determined in triplicate as described previously [11]. Within-assay coefficient of variation was less than 2%. Between-assay coefficients of variation ranged from 3 to 5%.

gels as standards (Fig. 3, lanes 1, 4 and 7). Owing to slight differences in the electrophoresis time, the migration of apo B standards as well as labelled apo B proteins from the immunoprecipitates varied between separate runs. Apo B-48 from VLDL had the same mobility on SDS/PAGE as that from human lymph chylomicrons (results not shown). During cell differentiation there was a progressive increase in the amount of apo B-100 and apo B-48, both intracellular and that secreted into the medium (Fig. 3 and Table 1). The ratio of labelled apo B-100 to apo B-48 in the medium remained relatively constant (0.24–0.32, Table 1) throughout differentiation of the cells; however, the percentage of total apo B versus total protein fraction increased from 0.02% up to 0.36% (Table 1). In the 7-day-post-confluence cells a band of normal apo B-48 size was seen (Fig. 3, lane 3), and the ratio of labelled intracellular apo B-48 to apo B-100 (0.23) was similar to that observed for secreted apo B proteins (Table 1). However, in 12-day- and 18-day-post-confluence cultures, apo B-48 of smaller size was found, and the ratio of labelled intracellular apo B-48 to apo B-100 was enhanced to 1.16 and 10.44 respectively (Fig. 3, lanes 6 and 9, and Table 1). With the progress of differentiation, the cells synthesized markedly increasing amounts of apo B-48 as compared with apo B-100, but most of the apo B-48 accumulated intracellularly. At 7 days post-confluence, most of the total synthesized apo B-48 (84%) was found in the medium, whereas at 12 and 18 days post-confluence only 44% and 11% respectively of apo B-48 was present in the medium (Table 1). The amount of intracellular apo B versus total protein fraction increased about 140-fold between 7 and 18 days post-confluence (Table 1). The molecular mass of intracellularly accumulated apo B-48 in 12-day- and 18-day-post-confluence cells was estimated as approx. 214 kDa, since it migrated together with myosin standard. It should be noted that apo B-48 contains about

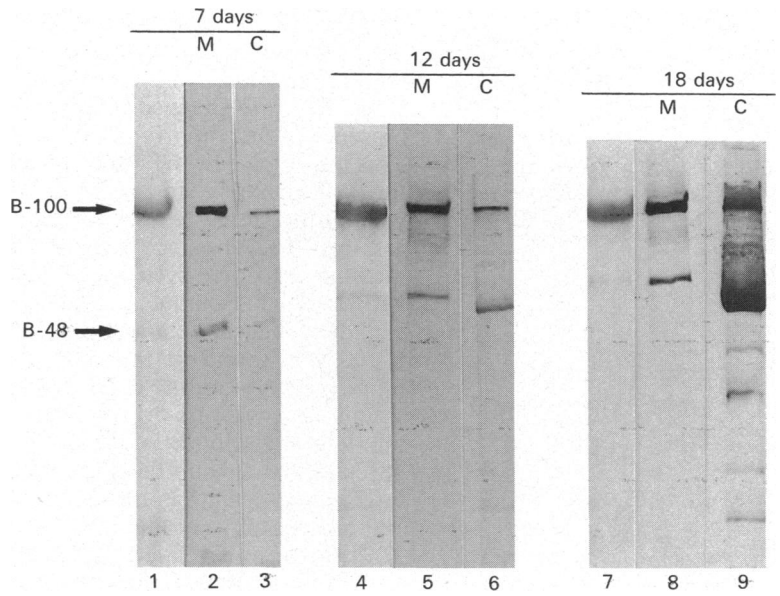


**Fig. 3. SDS/PAGE of apo B from Caco-2 cells**

The cells were plated on to 15 (60 mm-diameter) dishes at a density of $5 \times 10^4$ cells/cm$^2$ and formed a confluent monolayer in 3–4 days after plating. Then at different stages of differentiation (days 7, 12 and 18 post-confluence), the cells from five dishes per time point were preincubated with low-methionine DMEM for 30 min and labelled with L-[$^{35}$S]methionine for 6 h. The media (M) and the cells (C) samples were subjected to immunoprecipitation with anti-apo B serum. Lanes 1, 4 and 7 indicate Coomassie Blue-stained apo B-100 and apo B-48 from human VLDL. For better separation of apo B proteins, the electrophoresis was carried out for an additional 30–90 min after the tracking dye came off the gel. The gels were incubated with En$^3$Hance, dried and then exposed to Kodak X-Omat AR film at −70 °C. Because of slight differences in time of the electrophoresis between separate runs (lanes 1–3, 4–6 and 7–9), apo B-100 and apo B-48 standards of human VLDL should be compared with the immunoprecipitates analysed at the same run.

**Table 1. Apo B accumulation and secretion in Caco-2 cells**

Caco-2 cells at different stages of differentiation (days 7, 12 and 18 post-confluence) were preincubated with low-methionine DMEM and labelled with 200 $\mu$Ci of [$^{35}$S]methionine/ml for 6 h. Apo B was immunoprecipitated and analysed on SDS/PAGE (Fig. 3). After fluorography, apo B protein bands were excised from the gel, dissolved in Protosol tissue and gel solubilizer and counted for radioactivity. Incorporation of radioactivity into total protein was carried out by trichloroacetic acid precipitation as described in the Experimental section. 'Ratio' is apo B-48/apo B-100.

| | Period post-confluence (days) | B-48 (c.p.m.) | B-100 (c.p.m.) | Ratio | $10^{-3}\times$ Total protein (c.p.m.) | Total apo B (% of total protein) |
|---|---|---|---|---|---|---|
| Medium | 7 | 1497 | 4884 | 0.31 | 27896 | 0.02 |
| | 12 | 1939 | 8009 | 0.24 | 20329 | 0.05 |
| | 18 | 12883 | 39840 | 0.32 | 14754 | 0.36 |
| Cells | 7 | 281 | 1237 | 0.23 | 660156 | 0.0002 |
| | 12 | 3416 | 2933 | 1.16 | 525120 | 0.001 |
| | 18 | 102673 | 9830 | 10.44 | 406812 | 0.028 |

40% fewer methionine residues than apo B-100. Thus, when comparing the radioactivity content in apo B proteins, the amount of apo B-48 on a molar basis is about 40% higher than that of apo B-100.

In order to determine that the 214 kDa protein was not a degradation product of apo B-100, 18-day-post-confluence cells labelled with L-[$^{35}$S]methionine were subjected to immunoprecipitation with a monoclonal antibody recognizing apo B-100 but not apo B-48. Only the apo B-100 protein band was detected on a fluorogram of the gel (results not shown). This argues against a substantial degradation of apo B-100, and also suggests that the minor bands with faster mobilities on the gel (Fig. 3, lane 9) are derived from the degradation of the abundant intracellular 214 kDa protein.

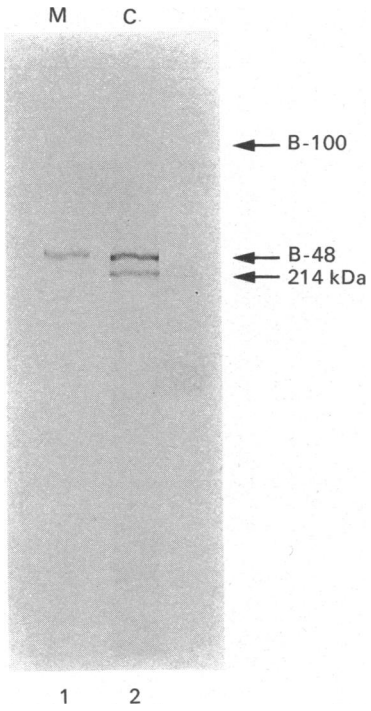


**Fig. 4. SDS/PAGE of apo B from human small intestine**

Explants from human jejunum in organ culture were preincubated with low-methionine DMEM for 30 min and then labelled continuously for 5 h with L-[$^{35}$S]methionine. Apo B from the medium (M, lane 1) and the cells (C, lane 2) was immunoprecipitated and analysed on SDS/PAGE as described in Fig. 3 legend. The migrations of myosin (214 kDa) and standard apo B-100 and apo B-48 from human VLDL are shown for comparison.

The synthesis and secretion of apo B were also examined in normal human small-intestinal specimens obtained from surgeries. Explants of jejunum in organ culture were labelled continuously for 5 h with L-[$^{35}$S]methionine, and apo B was immunoprecipitated and analysed on SDS/PAGE (Fig. 4). The intracellular fraction (Fig. 4, lane 2) consisted of two bands, the upper band of normal apo B-48 size and the lower band that migrated together with myosin standard (214 kDa). Apo B secreted into the medium by the explants revealed the presence of only apo B-48 of normal size (Fig. 4, lane 1). This indicates that the normal human intestine produces the 214 kDa protein, and that the formation of that protein in Caco-2 cells is not an artifact of the tumour-cell line. The absence of the 214 kDa protein from the medium was noted even after 18 h of continuous labelling with L-[$^{35}$S]methionine of Caco-2 cells and the human explants, suggesting a retention of that protein. No apo B-100 was found in the cells or in the medium. The immunoprecipitation carried out with non-immune serum showed no detectable protein bands. The immunoprecipitation of the 214 kDa protein and apo B-48 could be substantially inhibited by the addition of human low-density lipoprotein, which contained apo B-100 protein only (results not shown), suggesting that the 214 kDa form is related to apo B.

A pulse–chase experiment with explants from human jejunum was carried out to determine if there was a precursor–product relationship between the 214 kDa protein and the mature apo B-48 (264 kDa). After a 20 min pulse with L-[$^{35}$S]methionine, the label was chased with non-radioactive methionine for 0, 15, 30, 60 and 120 min (Fig. 5). The number of time points of the chase was limited by the small size of the surgical specimens. A maximal intracellular incorporation of the label was seen after 15 min of the chase, which was followed by a slow conversion of the 214 kDa protein into the mature apo B-48 and by steadily increasing

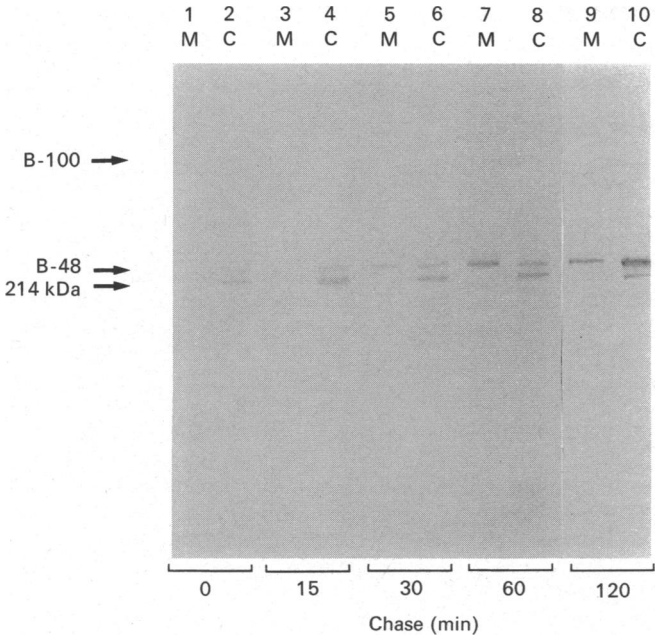


**Fig. 5. Pulse–chase labelling of apo B from human jejunal explants**

The pulse with L-[$^{35}$S]methionine (0.5 mCi/ml) was carried out for 20 min, and the label was chased with non-radioactive methionine for 0 min (lanes 1, 2), 15 min (lanes 3, 4), 30 min (lanes 5, 6), 60 mins (lanes 7, 8) and 120 min (lanes 9, 10). Apo B proteins from the cells and the media were immunoprecipitated and analysed on SDS/PAGE. The media (M) samples were applied to lanes 1, 3, 5, 7 and 9, and those of the cellular material (C) to lanes 2, 4, 6, 8 and 10. The migrations of myosin (214 kDa) and standard apo B-100 and apo B-48 from human VLDL are shown for comparison.

concentrations of apo B-48 in the medium (Fig. 5, Table 2). The amount of the 214 kDa protein after 60 min of the chase was not decreased as compared with the 30 min time point; however, the ratio of the 214 kDa protein to apo B-48 decreased (Table 2). This may be due to some differences in the size of the explant tissue, and thus varying quantities of newly synthesized apo B proteins. About 30 min was required to secrete the mature apo B-48 (Fig. 5, lane 5). The quantity of secreted apo B-48 substantially increased after 60 and 120 min of the chase (Fig. 5, lanes 7 and 9, and Table 2). When the quantities of apo B-48 in the medium are taken into account, the conversion of the 214 kDa protein into apo B-48 becomes even more evident. Altogether, these data suggest that the 214 kDa protein is a metabolic precursor of the mature apo B-48. A set of minor low-molecular-mass bands was observed in the cellular material but not in that from the medium (Figs. 4 and 5). The intensities of these minor bands were also inhibited by the presence of human low-density lipoprotein in the immunoprecipitation mixture (results not shown). This suggests that they represent either a set of incomplete apo B polypeptides and/or degradation products of the 214 kDa protein rather than those of the mature apo B-48.

## DISCUSSION

Previous studies have indicated that the adult human intestine synthesizes apo B-48 protein, whereas the fetal organ produces both apo B-48 and apo B-100 [16]. In this study, the explants of normal human intestine synthesized and secreted apo B-48, but not apo B-100. The Caco-2 cells, however, synthesized and secreted both apo B proteins, with predominantly secreted apo B-100. These results are consistent with previously published studies [6,7,17]. It is likely that the ability to synthesize both apo B proteins may be due to the recapitulation of certain fetal characteristics of the transformed cell line.

The differentiated Caco-2 cells synthesized and accumulated intracellularly a 214 kDa protein. The 214 kDa protein was also present in the normal ileum and jejunum, and was found to be apo B-related. Strong evidence that the 214 kDa protein is a metabolic precursor of the mature apo B-48 was obtained from the pulse–chase experiment with the human small-intestinal explants, which showed increasing concentrations of apo B-48 in the medium and a slow intracellular conversion of the 214 kDa into the 264 kDa protein. Slow intracellular processing of the maltase–glucoamylase precursor into the mature protein has also been shown in human intestinal explants, where the precursor form was still present even after 18 h of chase [18]. It is unlikely that the 214 kDa protein was derived from a proteolytic breakdown of the mature apo B proteins, for several reasons. First, the mixture of proteolytic inhibitors,

**Table 2. Apo B synthesis and secretion in human jejunum**

A pulse–chase experiment was performed with human jejunal explants (see Fig. 5). Densitometric scans of the bands on the fluorogram were carried out with a Quick Scan instrument (Helena Laboratories). 'Ratio' is 214 kDa/apo B-48. Abbreviation: ND, not detected.

| Chase (min) | Band intensity (arbitrary densitometric units) | | | | |
|---|---|---|---|---|---|
| | Cells | | | Media | |
| | 214 kDa | Apo B-48 | Ratio | 214 kDa | Apo B-48 |
| 0 | 7.2 | 2.0 | 3.60 | ND | ND |
| 15 | 24.5 | 8.3 | 2.95 | ND | ND |
| 30 | 13.5 | 9.0 | 1.50 | ND | 4.1 |
| 60 | 17.4 | 15.9 | 1.09 | ND | 22.4 |
| 120 | 9.8 | 43.4 | 0.23 | ND | 26.8 |

known to prevent apo B degradation, was always present during the immunoprecipitation procedure, and Trasylol was present in the medium during the labelling period. Secondly, in 7-day-post-confluence cells apo B-48 of normal size was detected; and finally, a monoclonal antibody recognizing only apo B-100 precipitated apo B-100 but not any other apo B forms, suggesting that the 214 kDa protein is not a degradation product of apo B-100.

In both the Caco-2 cells and the explants of human small intestine, no 214 kDa protein was detected in the medium. The mechanism of production of the 214 kDa apo B-48 form by differentiated Caco-2 cells and its intracellular accumulation is not clear. The supply of lipid necessary for assembly of intestinal lipoproteins seems not to be a limiting factor. The activity of acetyl-CoA:cholesterol acyltransferase in Caco-2 cells remains stable during the growth and differentiation period [19], indicating that esterified cholesterol is not in short supply. It is likely that post-translational processing and/or its rate in differentiated Caco-2 cells is different from that in undifferentiated cells. In another human colon carcinoma cell (HT-29), the processing of oligosaccharide chains differs between differentiated and undifferentiated cells [20,21]. An impairment of either glycosylation or oligosaccharide processing of apo B-48 could be responsible for the generation of the 214 kDa protein. It is noteworthy that the incorporation of mannose into the chylomicron protein fraction was decreased by 80% in patients with chylomicron-retention diseases [22]. On the other hand, the increased synthesis of apo B-48 by differentiated Caco-2 cells may not be co-ordinated with its efficient processing, and the 214 kDa form may be a pool of protein destined to be processed. In fact, a delayed processing of some microvillar hydrolases in Caco-2 cells had been reported [23]. Whichever possibility exists in Caco-2 cells, it indicates the importance of proper post-translational processing in secretion of apo B-48.

One gene is responsible for synthesis of both apo B-100 and apo B-48. A single mRNA base substitution results in the construction of a premature translational stop codon, and the formation of apo B-48 [24,25]. In our studies, no detectable changes in the mobility on SDS/PAGE was observed for apo B-100 in differentiated Caco-2 cells. It is possible that small differences in the molecular mass of apo B-100 may not be clearly detectable owing to its large size, or that a relative contribution of oligosaccharide chains, or/and other post-translationally added groups, to the mobility on SDS/PAGE differs between the two apo B proteins.

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