Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 581-587

APOLIPOPROTEIN B-100 IS THE MAJOR FORM OF THIS APOLIPOPROTEIN SECRETED BY HUMAN INTESTINAL Caco-2 CELLS

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Received August 15, 1988

SUMMARY: Although the discovery of stop codon has explained the mechanism for the formation of the intestinal marker, apolipoprotein B-48, the dispute regarding the presence of apolipoprotein B-100 in the intestine is still unsettled. To further investigate the characteristics of intestinal apolipoprotein B, the newly developed human colonic adenocarcinoma Caco-2 cells which express functional properties of the differentiated enterocytes, were used. SDS-polyacrylamide gel electrophoresis analyses of the intact culture medium or its lipoproteins of d < 1.23 g/ml showed the presence of only a single protein band of apolipoprotein B-100 with no detectable apolipoprotein B-48. After immunoblotting with oligoclonal antibodies to the amino-terminal peptide of apolipoprotein B, a trace amount of apolipoprotein B-48 was observed in the isolated lipoproteins, but not in the intact culture medium. These results suggest that apolipoprotein B-100 is the major form of apolipoprotein B secreted by human intestinal cells.

Apolipoprotein B (ApoB) is the major carrier for triacylglycerol and cholesterol in the circulation. It is an essential structural component of chylomicrons, very-low-density (VLDL) and low-density lipoproteins (LDL). Human ApoB, as currently viewed by most investigators, exists in two major forms: the ApoB-100 from liver and the ApoB-48 from intestine.

Recent studies on cDNA clones of ApoB-100 have indicated that the gene for ApoB-100 is present in both liver and intestine of man, monkey and rat (1-4), suggesting the possibility of synthesis and secretion of ApoB-100 by intestine.

The abbreviations used are: ApoB, apolipoprotein B; VLDL, very low density lipoproteins (d < 1.006 g/ml); LDL, low density lipoproteins (d 1.006-1.063 g/ml); HDL, high density lipoproteins (d 1.063-1.23 g/ml); DMEM, Dulbecco's Modified Eagle Medium; N-terminal, amino-terminal; C-terminal, carboxyl-terminal; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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0006-291X/88 $1.50

This is supported by our previous observation that ApoB-100 was found in rat mesenteric chyle when analyzed immediately after collection of chyle (5). ApoB-100 was also found to be the only form of ApoB in human chylomicrons when analyzed promptly after plasma collection (6).

Most recently, an inframe stop codon was discovered in human intestinal mRNA of ApoB resulting from a C to U substitution in the codon CAA encoding Gln-2153 to UAA, which is a termination codon for ApoB-48 (7-9). This discovery of stop codon explains the mechanism of ApoB-48 formation. If the stop codon is present in all the intestinal ApoB mRNAs, one should not be able to detect ApoB-100 in the intestine. Our previous studies (5,6), as well as those by others (10), do not support this hypothesis. To further investigate the characteristics of intestinal ApoB, we have utilized the newly developed human colonic adenocarcinoma Caco-2 cell line. Caco-2 cells undergo in vitro differentiation and express structural characteristics (11) and functional properties (12-14) typical of the small intestinal enterocytes. Thus, the ApoB secreted into the medium by Caco-2 represents the intestinal form of this apolipoprotein with no contribution from plasma or the liver. Results from this study further support the hypothesis that ApoB-100 is the major ApoB form synthesized and secreted by human intestinal cells.

MATERIALS AND METHODS

Cell culture. The human colonic adenocarcinoma cell line Caco-2 were obtained from American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM glutamine, 1% nonessential amino acids, kanamycin sulfate (50 μg/ml), and 10% fetal calf serum at 37°C in a 5% CO_2, 95% air atmosphere. The culture medium was changed daily after the cells reached confluency. All experiments were conducted with 10-14-days post confluence cells at which time the differentiation-dependent expression of their functional properties typical of small intestinal enterocytes is maximal (11). At the start of each experiment, the maintenance medium was removed, monolayers were washed twice with phosphate-buffered saline and serum-free DMEM was added. After a 20 hour incubation, culture medium was removed and centrifuged at 2000 rpm for 30 min to remove cell debris.

Isolation of lipoproteins. Preservative cocktail (D) containing antibiotics/EDTA/protease inhibitor/antioxidants (15) was added to the pooled medium. The medium was divided in half and to one half, leupeptin at 1 mM final concentration was added. Lipoproteins of $d < 1.23$ g/ml were isolated by a one-step ultracentrifugation at 40,000 rpm for 36 hours at 4°C. The lipoproteins were dialyzed against 0.02 M Tris-buffered saline (pH 7.4) containing the corresponding preservatives at 4°C under N_2 and concentrated with Amicon membrane cone before analyses.

To determine the distribution of ApoB in various density classes, the medium was also fractionated into VLDL, LDL and high density lipoprotein (HDL) as previously described (16). The concentration of ApoB in density classes was measured by electroimmunoassay with antibodies to lipoprotein B (17).

Preparation of antibodies. Amino-terminal peptide of ApoB consisting of H_2N-Glu-Glu-Glu-Met-Leu-Glu-Asn-Val-Ser-Leu-Val-Cys-Pro-Lys-Asp-Ala-Thr-Arg-COOH

Table 1. Amino acid composition of the synthesized N-terminal peptide of ApoB after HPLC purification (moles/mole of peptide)

Amino Acids	Found	Expected
Asp	1.9	2
Thr	1.0	1
Ser	0.9	1
Glu	4.1	4
Pro	1.2	1
Gly	0	0
Ala	1.0	1
Cys	N.D.[a]	1
Val	2.0	2
Met	0.7	1
Ile	0	0
Leu	1.9	2
Tyr	0	0
Phe	0	0
His	0	0
Lys	1.2	1
Arg	1.1	1
Total		18

[a]N.D., not determined.

(18) was synthesized using Applied Biosystem Model 430A peptide synthesizer (Foster City, CA) according to the method described by Barany and Merrifield (19). The synthesized product was analyzed by reversed-phase HPLC. Two major peaks were found and both showed identical amino acid composition (Table 1). The difference in elution position possibly was due to dehydroserine. Edman degradation of the crude peptide yielded a single correct sequence as described above.

The N-terminal peptide of ApoB was cross-linked to rabbit albumin (Sigma) with glutaraldehyde (Sigma) (20). After extensive dialysis against phosphate buffered saline, the peptide-albumin was mixed with equal volume of Freund's complete adjuvant and inoculated into rabbit lymph nodes (21). A second intradermal injection was given on the back after a month. Blood was drawn 7 weeks later. Positive immunoreaction was shown by ELISA with the peptide only. A booster shot was given every 7-8 weeks. Positive immunoreactions with ApoB, LDL and whole plasma were shown after the third booster shot.

Monoclonal antibodies to ApoB, MB47, a generous gift of Dr. L. Curtiss (La Jolla, CA), were used as anti-C-terminal portion of ApoB. MB47 was shown to inhibit the LDL-receptor interaction and bind an epitope on thrombin fragment T_2, a carboxyl-terminal portion of ApoB-100 (22).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The lipoproteins of $d < 1.23$ g/ml or the concentrated intact culture medium were analyzed by 3.3% polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), according to the method of Fairbanks et al. (23) as modified previously (5), including recrystallization of the acrylamide and inclusion of 0.02% thiodiglycol as antioxidant in the running buffer.

Immunoblotting. Electrophoresis of lipoproteins or intact culture medium was carried out in a 3.3% polyacrylamide slab gel containing SDS using the same buffer system described above. The proteins were transferred to Immobilon transfer membrane (Millipore, Bedford, MA) by electroblotting, according to Towbin et al. (24) and immunoblotted as described previously (6). The oligoclonal antibodies to N-terminal peptide of ApoB and monoclonal anti-ApoB

(C-terminal portion) were diluted 1:1000 and 1:2000, respectively for immunoblotting.

RESULTS AND DISCUSSION

Quantitation of ApoB in lipoprotein density fractions isolated from Caco-2 cell culture medium from four experiments showed that 0-4.6% of total ApoB was recoverred in VLDL, 95-100% was in the LDL and only trace amount was found in HDL (Table 2). Since ApoB-48 was observed in HDL of Caco-2 cell culture medium (10), the analyses of ApoB were performed on the total lipoproteins of d < 1.23 g/ml or on the concentrated intact medium. As shown in Fig. 1 (gel 2), the SDS-PAGE analysis of freshly prepared lipoproteins of d < 1.23 g/ml from Caco-2 culture medium contained only a single band with mobility corresponding to ApoB-100. The ApoB-48 band was not detectable by Coomassie blue staining. After immunoblotting, a trace amount of ApoB-48 was observed with anti-N-terminal peptide of ApoB which could not easily be photographed (Fig. 1, pattern 3). Thus, ApoB-100 remained the predominant band, as illustrated by immunoblotting (Fig. 1). In comparison to a reference VLDL which contained a small amount of ApoB-48, the immunoblot showed the presence of ApoB-48 band with the oligoclonal antibodies to N-terminal peptide of ApoB (Fig. 1, pattern 5) and the absence of ApoB-48 band with anti-C-terminal portion of ApoB (MB47) (Fig. 1, pattern 6). These results validate that the anti-N-terminal peptide of ApoB is effective in detecting the authentic ApoB-48, if present, and that the anti-C-terminal portion of ApoB should react only with ApoB-100 and not with the ApoB-48, as described previously (22). To avoid the manipulation of lipoprotein isolation, concentrated intact culture medium was analyzed. Fig. 2 shows that when the concentrated medium was analyzed immediately after collection, ApoB-100 was the only form of ApoB detected by SDS-PAGE (gel 8) as well as by immunoblotting (patterns 6 and 7). When the isolated lipoproteins of d < 1.23 g/ml from the same medium, with or without leupeptin, were analyzed, a trace

Table 2. Concentration and distribution of apolipoprotein B in culture medium and lipoprotein density classes

Total medium	Fraction		
	VLDL	LDL	HDL
(μg/g cell protein/h)	(Percent distribution)[a]		
63.32 ± 8.49[b]	0-4.6	95.4-100	Trace

[a]Mean of 4 experiments.

[b]Mean ± SEM of 8 experiments.

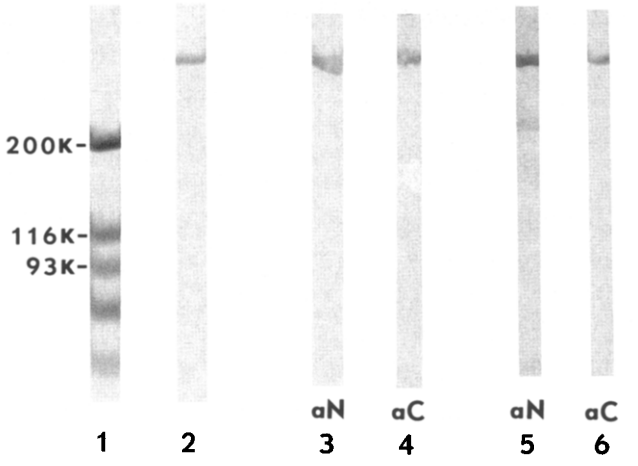

Fig. 1. Analyses of lipoproteins of d < 1.23 g/ml from Caco-2 cell culture medium by electrophoresis and immunoblotting. Gels 1 and 2, electropherograms of calibration proteins (gel 1) and lipoproteins of d < 1.23 g/ml from Caco-2 cell culture medium (gel 2) in 3.3% polyacrylamide gel slabs containing SDS. Patterns 3 and 4, immunoblots with antisera after transferring proteins from gel 2 to Immobilon transfer membrane. Patterns 5 and 6, immunoblots of a reference VLDL which contained ApoB-48. aN, oligoclonal antibodies to N-terminal peptide of ApoB; aC, monoclonal antibodies to C-terminal half of ApoB, MB47 (22).

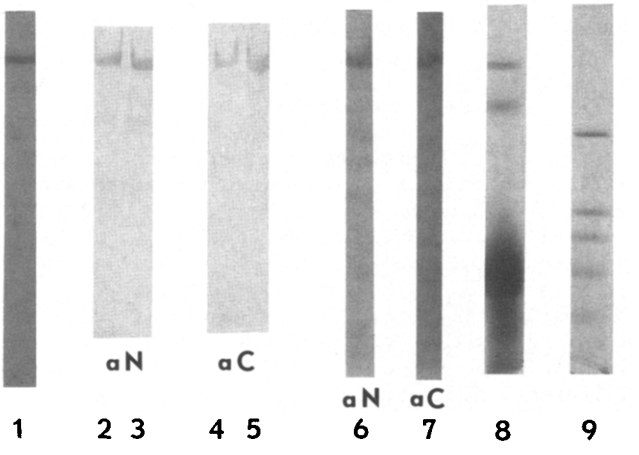

Fig. 2. Analyses of Caco-2 cell culture medium and its lipoproteins of d < 1.23 g/ml by electrophoresis and immunoblotting. Gels 1, 8 and 9, electropherograms on 3.3% polyacrylamide gel slab containing SDS. Sample in gel 1, lipoproteins of d < 1.23 g/ml from Caco-2 cell culture medium; gel 8, concentrated intact culture medium from Caco-2 cell; gel 9, calibration standards same as in Fig. 1. Patterns 2-7, immunoblots with antisera after transferring proteins from gels to Immobilon transfer membrane. Samples in patterns 2-5, lipoproteins of d < 1.23 g/ml from Caco-2 cell culture medium, with leupeptin (patterns 2 and 4) and without leupeptin (patterns 3 and 5). Samples in patterns 6 and 7, intact culture medium of Caco-2 cells with leupeptin. aN, oligoclonal antibodies to N-terminal peptide of ApoB; aC, monoclonal antibodies to C-terminal half of ApoB, MB47 (22).

amount of ApoB-48 was detected by immunoblotting for both preparations (patterns 2 and 3); thus, ApoB-100 remained the predominant band. However, by SDS-PAGE alone, again only ApoB-100 was observed, with no detectable ApoB-48 band (Fig. 2, gel 1).

Analyses of three additional preparations of lipoproteins from Caco-2 cell media confirmed the same observations. These results suggest that ApoB-100 is the major, if not the only ApoB form secreted by the intestinal Caco-2 cells under the present culture condition. Hughes et al. (10) have also shown that ApoB-100 was the only ApoB form present in VLDL and LDL secreted by Caco-2 cells. Furthermore, the authors observed that ApoB-48 was the minor and ApoB-100 the major form of ApoB in HDL of the culture medium. If the presence of stop codon in ApoB mRNA is the only mechanism for the presence of ApoB-48 in Caco-2 cell culture medium, either the number of mRNA containing stop codon is very small so that the "authentic" ApoB-48 derived from stop codon is minimal, or the majority of the ApoB-48 synthesized by Caco-2 cells is not secreted.

ACKNOWLEDGMENTS

We would like to express our appreciation to Ms. E. Carter for expert technical assistance, to Dr. K. Jackson of Saint Francis Hospital of Tulsa Medical Research Institute, Oklahoma City, OK, for synthesis of N-terminal peptide of ApoB. We are grateful to Dr. L. Curtiss of La Jolla, CA, for her generous gift of monoclonal antibody, and to Ms. J. Pilcher for typing the manuscript. This work was supported in part by grant HL-23181 from the National Heart, Lung, and Blood Institute of the National Institutes of Health, and by the resources of the Oklahoma Medical Research Foundation.

REFERENCES

1. Lusis, A.J., West, R., Mahrabian, M., Reuben, M.A., LeBouef, R.C., Kaptein, J.S., Johnson, D.F., Schumaker, V.N., Yahasz, M.P., Schotz, M.C., and Elovson, J. (1985) Proc. Natl. Acad. Sci. USA 82, 4597-4601.
2. Knott, T.J., Rall, S.C., Jr., Innerarity, T.L., Jacobson, S.F., Urdea, M.S., Levy-Wilson, B., Powell, L.M., Pease, R.J., Eddy, R., Nakai, H., Byers, M., Preistly, L.M., Robertson, E., Rall, L.B., Betsholtz, C., Shows, T.B., Mahley, R.W., and Scott, J. (1985) Science 230, 37-43.
3. Deeb, S.S., Disteche, C., Motulsky, A.G., Lebo, R.V., and Kan, Y.W. (1986) Proc. Natl. Acad. Sci. USA 83, 419-422.
4. Law, S.W., Lackner, K.J., Hospattankar, A.V., Anchors, J.M., Sakaguchi, A.Y., Naylor, S.L., and Brewer, H.R., Jr. (1985) Proc. Natl. Acad. Sci. USA 82, 8340-8344.
5. Lee, D.M., Koren, E., Singh, S., and Mok, T. (1984) Biochem. Biophys. Res. Commun. 123, 1149-1156.
6. Lee, D.M., and Singh, S. (1988) Biochim. Biophys. Acta 960, 148-156.
7. Powell, L.M., Wallis, S.C., Pease, R.J., Edwards, Y.H., Knott, T.J., and Scott, J. (1987) Cell 50, 831-840.
8. Chen, S.-H., Habib, G., Yang, C.-Y., Gu, Z.-W., Lee, B.R., Weng, S.-A., Silberman, S.R., Cai, S.-J., Deslypere, J.P., Rosseneu, M., Gotto, A.M., Jr., Li, W.-H., and Chan, L. (1987) Science 238, 363-366.
9. Hospattankar, A.V., Higuchi, K., Law, S.W., Meglin, N., and Brewer, H.B., Jr. (1987) Biochem. Biophys. Res. Commun. 148, 279-285.
10. Hughes, T.E., Sasak, W.V., Ordovas, J.M., Forte, T.M., Lamon-Fava, S., and Schaefer, E.J. (1987) J. Biol. Chem. 262, 3762-3767.

11. Pinto, M., Robine-Leon, S., Appay, M.D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J., and Zweibaum, A. (1983) Biol. Cell 47, 323-330.
12. Hauri, H.P., Sterchi, E.E., Bienz, D., Franzen, J.A.M., and Marxer, A. (1985) J. Cell Biol. 101, 838-851.
13. Grasset, E., Bernabeu, J., and Pinto, M. (1985) Am. J. Physiol. 248, C410-C418.
14. Vincent, M.L., Russell, R.M., and Sasak, V. (1985) Hum. Nutr. Clin. Nutr. 39C, 355-360.
15. Lee, D.M., Valente, A.J., Kuo, W.H., and Maeda, H. (1981) Biochim. Biophys. Acta 666, 133-146.
16. Alaupovic, P., Lee, D.M., and McConathy, W.J. (1972) Biochim. Biophys. Acta 260, 689-707.
17. Curry, M.D., Gustafson, A., Alaupovic, P., and McConathy, W.J. (1978) Clin. Chem. 24, 280-286.
18. Knott, T.J., Wallis, S.C., Powell, L.M., Pease, R.J., Lusis, A.J., Blackhart, B., McCarthy, B.J., Mahley, R.W., Levy-Wilson, B., and Scott, J. (1986) Nucleic Acids Res. 14, 7501-7503.
19. Barany, G., and Merrifield, R.B. (1979) in The Peptides, Analysis, Synthesis, Biology, Gross, E., and Meienhoser, J., eds., vol. 2, pp. 1-284, Academic Press, New York.
20. Reichlin, M. (1980) Methods Enzymol. 70, 159-165.
21. Sigel, M.B., Sinha, Y.N., and VanderLaan, W.P. (1983) Methods Enzymol. 93, 3-12.
22. Young, S.G., Witztum, J.L., Casal, D.C., Curtiss, L.K., and Bernstein, S. (1986) Arteriosclerosis 6, 178-188.
23. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
24. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.