Editing of apolipoprotein B messenger RNA in differentiated Caco-2 cells

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Abstract During the differentiation of human enterocytes, the secretion of apolipoprotein B is switched from the apoB-100 form seen almost exclusively in fetal cells to the apoB-48 form seen almost exclusively in adult cells. This switch is accomplished by the post-transcriptional editing of the messenger RNA for apoB-100. We report that a similar switch occurs during the differentiation of the human colonic carcinoma cell line, Caco-2, and that this is accomplished by the same mRNA editing mechanism. Caco-2 cells cultured on Millipore filters developed confluent electrically resistant monolayers, and on Western blot analysis, using a monoclonal antibody directed against the amino terminal region of human apoB-100 (Mab C1.4), secreted >50% apoB-48 (of total apoB-100) into culture media, while Caco-2 cells grown on plastic secreted >95% apoB-100. To assess whether mRNA editing was responsible for the switchover from apoB-100 to apoB-48, apoB cDNA fragments spanning nucleotides 6504–6784 of apoB mRNA were synthesized using RNA isolated from Caco-2 cells grown on filters and Caco-2 cells grown on plastic. The appropriate oligonucleotide primers and Moloney murine leukemia virus reverse transcriptase were used. The resulting cDNA fragments were amplified by the polymerase chain reaction (PCR), and PCR products were subcloned and sequenced. A single cytosine/thymine base change occurred in 8/20 clones of cDNA derived from Caco-2 cells grown on filters (corresponding to a cytosine/uridine change in mRNA) and in 1/25 clones of cDNA derived from Caco-2 cells grown on plastic. PCR products of genomic sequences from Caco-2 cells did not contain the stop codon. These findings indicate that Caco-2 cells allowed to differentiate on filters can introduce an in-frame stop codon into apoB-100 mRNA, leading to translation of apoB-48. —**Jiao, S., J. B. Moberly, and G. Schonfeld.** Editing of apolipoprotein B messenger RNA in differentiated Caco-2 cells. *J. Lipid Res.* 1990. **31:** 695–700.

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Apolipoprotein B consists of two subpopulations, apoB-100 which binds to LDL receptors and apoB-48 which does not (1). Human apoB-100 (4536 amino acids) is synthesized predominantly by the liver (and possibly by the intestine) as a major component of very low density lipoproteins and low density lipoproteins (LDL) (2, 3). ApoB-48 is synthesized in the intestine and secreted as a component of chylomicrons. The human liver does not secrete apoB-48 (2–4). Both forms of apoB are coded by a single copy of the apoB-100 gene (5). The formation of apoB-48 depends upon the post-transcriptional editing of the apoB-100 mRNA. A single C→U base change is introduced at the nucleotide position 6666 of apoB-100 mRNA, resulting in a change of the codon CAA, encoding Gln^{2153}, to the stop codon UAA. The translation of this mRNA produces the shorter apoB-48 (6–8).

The mRNA editing has been described in ex vivo studies using freshly isolated tissues, but not in cultured cells. We wished to ascertain whether this process occurred in a widely used cell culture model of human enterocytes, the Caco-2 cell line, established in 1977 by Fogh, Fogh, and Orfeo (9) from a human adenocarcinoma. This cell line expresses many characteristics of differentiated small intestinal epithelium, including production and secretion of lipoproteins (10). When cultured on plastic plates, Caco-2 cells produce only apoB-100 (11, 12), and cDNA produced from such cells did not possess the stop codon necessary for translation of apoB-48 (13). Recently, Traber, Kayden, and Rindler (14) reported that Caco-2 cells cultured on filters secreted lipoproteins in a polarized fashion predominantly into the basolateral chamber, and variable amounts of apoB-48 were identified in media from the basolateral chamber by immunoprecipitation. Recent observations in our laboratory suggest that when Caco-2 cells are cultured on filters, secretion of apoB-48 into culture media predominates over apoB-100, secretion of apoA-I and E decreases, while secretion of apoA-IV in-

Abbreviations: apoB, apolipoprotein B; LDL, low density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; M-MuLV, Moloney-murine leukemia virus; PCR, polymerase chain reaction; LB medium, Luria-Bertani medium; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; IPTG, isopropyl-1-thio-β-D-galactoside; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; MOPS, 3-[N-morpholino]-propanesulfonic acid; Kb, kilobases.

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creases (J. B. Moberly and G. Schonfeld, unpublished data), suggesting that Caco-2 cells become more differentiated when grown on filters than on plastic. Therefore, we investigated whether differentiation of Caco-2 cells on filters was accompanied by the introduction of a stop codon into apoB mRNA.

MATERIALS AND METHODS

Cell culture

Caco-2 cells (kindly provided by Dr. W. Stenson, Gastroenterology Division, The Jewish Hospital, Washington University School of Medicine, St. Louis, MO) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS, J. R. Scientific, Woodland, CA, lot no. 9600), 22.2 mM glucose, 2 mM 1-glutamine, 45.5 mM bicarbonate, 1% nonessential amino acids, and 40 μg/ml gentamicin sulfate at 37°C in 10% CO_2/90% air. Cells were plated at a density of 4–5 × 10^5/cm^2 in 75-cm^2 tissue culture flasks (Corning Glassworks, Corning, NY). The culture medium was changed every 2 days before confluence. When the cell layers reached ~80% confluence, the cells were dissociated with 0.5% trypsin and 0.2 mg/ml EDTA and passaged at a split ratio of ~1:15. Caco-2 cells (passage 43) were plated either on Millipore Millicell-HA culture-plate inserts (Millipore Products Division, Bedford, MA, cat. PIHA 03050, 3-cm diameter, pore size 0.45 μm) at a density of 3–5 × 10^5 cells/filter or on 35-mm 6-well plastic plates (Costar, Cambridge, MA, cat. 3406 Mark II) at the same density. The culture medium was changed every 2 days. Three weeks after plating, media obtained from both sets of cultures over 24-h culture were harvested for the assessment of secreted apoB peptides and the cells were washed with serum-free DMEM and scraped off by a rubber policeman for analysis of mRNA. Under these conditions of culture, lactate dehydrogenase activity was virtually undetectable in any of the spent media, suggesting the cells were not damaged and that any lipoproteins detected in media were probably due to secretion, not "leakage."

Western blot of apoB

Conditioned media were added to electrophoretic sample buffer (25 mM Tris-HCl, pH 6.8, 1% SDS, 2% (v/v) 2-mercaptoethanol, and 0.03% bromophenol blue) boiled for 1 min, and subjected to 3–6% linear gradient sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Separated proteins were electorphoretically transferred to nylon membranes (Nitroplus 2000, 0.45 μm pore size, Micron Separations Inc., Westboro, MA) at 27 V for 21 h at 4°C. The membrane was blocked with 5% nonfat dry milk (Bovine Lacto Transfer Technique Optimizer, BLOTTO), and incubated with a monoclonal antibody against human apoB (C1.4). Washing and detection of the antibody were performed as previously described (15).

Oligonucleotides

The following nucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer (Foster City, CA) and purified on 15% sequencing gels.

PCR-B1: (5'–3') CTGAATTCATTCAATTGGGAGA GACA, a 26mer with its 5' end at nucleotide 6504 (antisense).

PCR-B2: CGGATATGATAGTGCTCATCAAGAC, a 25mer with its 5' end at nucleotide 6784 (sense).

PCR-B2 was used to prime the reverse transcription reaction. Both PCR-B1 and B2 were used in the polymerase chain reaction (PCR). PCR-B2 is complimentary to apoB mRNA. The same sequences were used by Powell et al. (7).

cDNA preparation

Total cytosolic RNA was isolated in 4 M guanidine isothiocyanate through the cesium chloride cushion (16). Single-strand cDNA was synthesized from 40 μg crude RNA using Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Bethesda Research Laboratory, Gaithersburg, MD, cat. 8025SA) (17). The reaction mixture consisted of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 100 μg bovine serum albumin (BSA), 50 μg/ml actinomycin D, 0.5 mM deoxynucleotide (dATT, TTT, dGTT, dCTT), 2.4 μg primer (PCR-B2), and 200 U/50 μl M-MuLV reverse transcriptase. After incubation for 1 h at 37°C, cDNA and RNA were extracted with phenol–chloroform 1:1 (v/v).

Amplification of cDNA and genomic DNA

The polymerase chain reaction (18, 19) was carried out using *Thermophylus aquaticus (Taq)* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). The reaction was performed in 100 μl of 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 1 mM DTT, 200 μM each dATP, dGTP, TTP, dCTP, 0.8 μg antisense oligonucleotide (PCR-B1), 0.8 μg sense oligonucleotide (PCR-B2), the total amount of single-strand cDNA synthesized from 40 μg of RNA by reverse transcriptase reaction (see above), and 2.5 U *Taq* DNA polymerase. After the reaction, the PCR product was incubated with 10 μg deoxyribonuclease-free ribonuclease A (Sigma) for 30 min at 37°C, separated on a 1.5% agarose gel, and isolated by electroelution (20). Genomic DNA was isolated from Caco-2 and HepG2 cells by the proteinase K/SDS procedure (16). PCR was carried out as above for cDNA except that 0.2 μg genomic DNA was used. There is no intron in the amplified region (21).

Subcloning and sequencing of cDNA and genomic DNA

Bluescript KS M13 + (Stratagene, La Jolla, CA) was used as a vector for subcloning the PCR-generated cDNAs. Ligated DNA was inducted into competent DH5 strain *E. coli* cells (Bethesda Research Laboratory) and plasmid DNA was extracted (20). DNA (0.5 μg) was sequenced on 6% polyacrylamide gel by the method of Sanger, Nicklen, and Coulson (22) using T7 DNA polymerase (United States Biochemical Co., Cleveland, OH). PCR-B1 oligonucleotide was used as a primer.

Northern blot

Total RNA (10 μg) from Caco-2 was suspended in 20 mM 3-[N-morpholino]propanesulfonic acid (pH 7.0) containing 1 mM EDTA (MOPS-EDTA), 50% formamide, and 6.6% formaldehyde and denatured by heating at 80°C for 10 min. Total RNA was separated in an 0.8% agarose gel containing 6.6% formaldehyde and MOPS-EDTA and transferred to Genescreen membrane (New England Nuclear, Boston, MA) by capillary blotting. A cDNA for human apoB, kindly provided from Dr. J. Scott, MRC Clinical Research Centre, Harrow, UK, corresponding to nucleotides 451-2223 was labeled with [^{32}P]dCTP by the random primer procedure (23) (sp act 6.6 × 10^5 cpm/ng). ApoB mRNA was hybridized with ^{32}P-labeled pH1 (2 × 10^6 cpm/ml) in buffer containing 50 mM Tris-HCl, pH 7.5, 50% formamide, 10 × Denhart's solution, 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, and 125 μg/ml of denatured salmon sperm DNA at 42°C for 24 h. The filter was washed twice with 2 × SSC at room temperature for 5 min, with 2 × SSC and 1% SDS at 65°C for 30 min, and twice with 0.1 × SSC at room temperature for 30 min. Autoradiography was carried out at −70°C for 12 h.

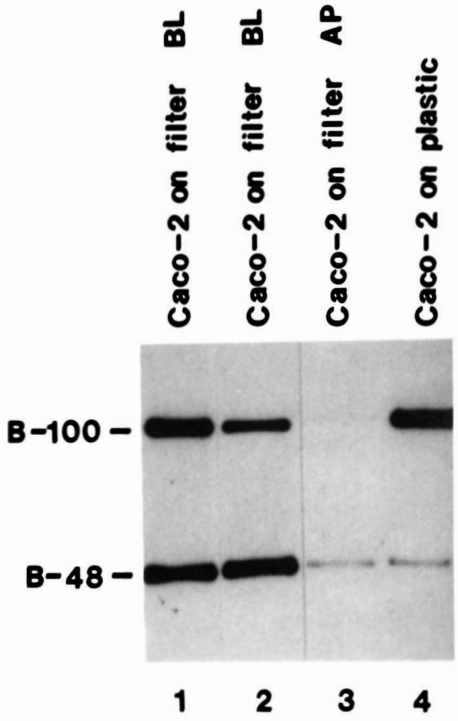

Fig. 1. Western blot of apolipoprotein B secreted by Caco-2 cells. Caco-2 cells were cultured on Millipore filters (pore size 0.45 μm) or plastic plates. Aliquots (50 μl) of 24-h conditioned media were dissolved in a sample buffer, boiled for 2 min, and electrophoresed on 3-6% gradient SDS polyacrylamide gels. Separated proteins were transferred to nylon membranes and incubated with monoclonal antibody C1.4 against the amino terminal portion of human apoB, followed by incubation with ^{125}I-labeled anti-mouse IgG. Lanes 1 and 2 are obtained from basolateral media (BL) of filter-cultured Caco-2 in a parallel experiment. Lane 3 is from apical medium (AP). Lane 4 is media obtained from Caco-2 cells grown on plastic plates.

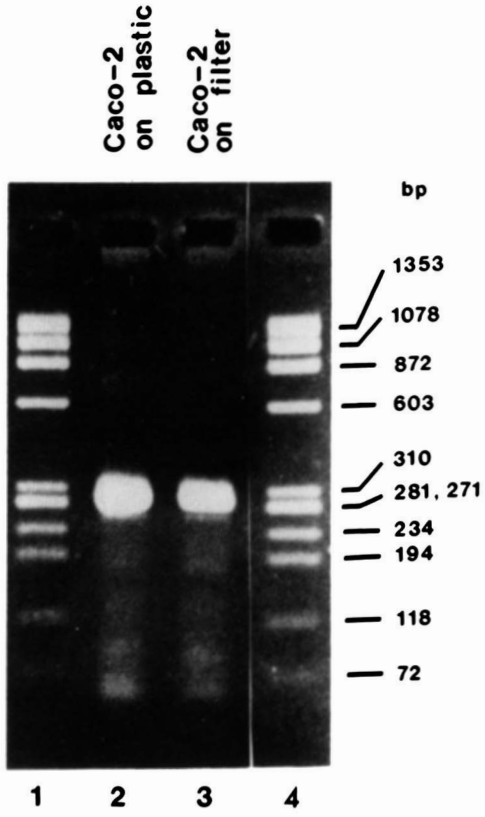

Fig. 2. Agarose gel (1.5%) electrophoresis of polymerase chain reaction (PCR) product. Apolipoprotein (apo) B cDNA was synthesized by Moloney-mouse leukemia virus reverse transcriptase from cytosol RNA obtained from Caco-2 cells grown on plastic (lane 2), and Caco-2 cells grown on filters (lane 3). Nucleotides between 6504 and 6784 of apolipoprotein B cDNA were amplified by PCR. Amplified double-stranded DNA was treated with ribonuclease A, then applied onto the agarose gel. Separated DNA was stained with ethidium bromide. Lanes 1 and 5 are *Hae*III digested-ΦX 174 RF DNA fragments used as molecular weight markers.

RESULTS

Caco-2 cells grown on plastic secreted predominantly apoB-100 as determined by immunoblotting of conditioned media (**Fig. 1**). The proportion of apoB-48 was

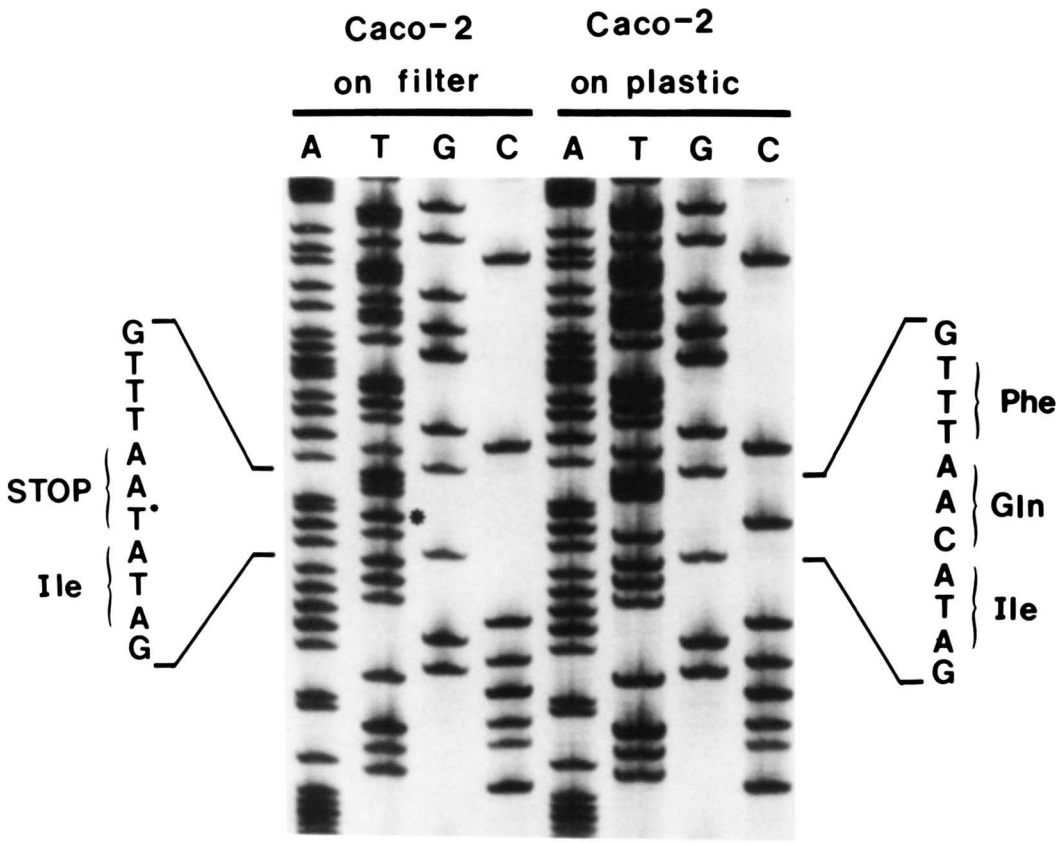

Fig. 3. Nucleotide sequences of subcloned cDNA. cDNA obtained from Caco-2 cells grown on filters (left panel) shows substitution of cytosine (C) to thymine (T) at nucleotide position 6666. The cDNA from Caco-2 cells grown on plastic did not exhibit this substitution (right panel).

much less than 5% of total apoB. However, when Caco-2 cells were grown on filters, >80% of the apoB secreted was present in the basolateral medium and apoB-48 represented >50% of apoB present both in the basolateral and apical media.

The PCR products of Caco-2 mRNA were about 280 bp in size (**Fig. 2**), consistent with the predicted sizes (281 nucleotides).

In order to quantify the relative proportions of apoB-48- and apoB-100-type mRNAs and to reveal the differences in nucleotide sequences between them, the cDNAs were amplified by PCR. The C→T changes at position 6666 were confirmed (**Fig. 3**). Eight of 20 subclones from Caco-2 cells grown on filters possessed the stop codon for apoB-48, while only 1/25 clones obtained from plastic-grown Caco-2 possessed the stop codon. PCR products of genomic DNA obtained from Caco-2 cells contained only the CAA codon, indicating that apoB-48 production results from mRNA editing.

On Northern blot analysis of apoB mRNAs isolated from cells either grown on plastic or on filters, the 14.1-Kb message was dominant, but small amounts of a 7.5 Kb mRNA also were seen.

DISCUSSION

In the present study we report that the human intestinal cell line, Caco-2, is capable of secreting both apoB-100 and apoB-48, and that the previously described mRNA editing mechanism (6–8) responsible for the switchover from apoB-100 to apoB-48 can be induced in these cells. Hughes, Ordovas, and Schaefer (12) and Lee, Dashti, and Mok (11) who grew their cells on plastic reported that apoB-48 could not be detected in media from Caco-2 cells, and Tennyson et al. (13), who grew their cells on plastic reported that Caco-2 cells did not possess the stop codon necessary for apoB production. But Traber et al. (14) who grew the Caco-2 cells on filters found both apoB-100 and apoB-48 in their culture media, and, in our hands, Caco-2 cells grown on filters secreted more apoB-48 than apoB-100 and also possessed the stop codon. In fact, even those Caco-2 cells grown on plastic produced small amounts of apoB-48 and 1 of 25 PCR subclones did possess the stop codon.

It is highly unlikely that the stop codon we found in Caco-2 is an artifact. However, during the reverse transcriptase or polymerase chain reactions nucleotides may

be misread, producing artifactual sequences (24–26). Sixty three PCR subclones derived from mRNA were sequenced and about 6300 nucleotides were read. C-T changes (corresponding to C→U changes in mRNA) were found only nine times and all were at position 6666, and the stop codons were found preferentially in Caco-2 cells grown on filters. Only two other single nucleotide substitutions were found and they were in two different positions, neither being 6666 (not shown). In the genomic DNA part of the study, an additional 23 PCR subclones and about 2300 nucleotides were sequenced and no stop codons were found.

The fact that both glutamine codon- and stop codon-containing mRNAs were found in Caco-2 cells might be explained by heterogeneous differentiation of the cell population in culture or by the fact that the RNA editing occurs post-transcriptionally rather than co-transcriptionally. The recent report by Higuchi et al. (27) that the intestine contains both forms of mRNA for apoB-100 and apoB-48 also supports that apoB mRNA editing may occur post-transcriptionally. Both 7.5 Kb and 14.1 Kb mRNAs were found in these cells. Higuchi et al. (27) reported that the 7.5 Kb mRNA contains the stop codon while the 14.1 Kb transcript contains mRNAs possessing both CAA codons and stop codons. Our data do not allow us to assign the stop codon to either mRNA subspecies.

How culturing cells on filters results in the stimulation of apoB mRNA editing and the production of apoB-48 is unknown, but culture substrates clearly can affect the signals responsible for differentiation of cells. Traber et al. (14) reported that Caco-2 cells grown on filters resemble intestinal epithelial cells morphologically. Similar findings have been reported for other epithelia (28). Davidson et al. (29) observed that thyroid hormone induced an mRNA stop codon in rat liver in vivo. Whether the thyroid hormones present in our culture medium contributed to the induction of the in-frame stop codon is unclear, but Caco-2 cells cultured on filters in serum-free medium (excluding thyroid hormones) still produce considerable amounts of apoB-48 (data not shown) suggesting that the filter matrix was sufficient for the induction of the stop codon. The resolution of the molecular basis of this phenomenon requires more intensive experiments; however, the presence of apoB mRNA editing in Caco-2 cells could provide a useful system for examining the relationship between differentiation and the mechanism of RNA editing.

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