Differential tissue-specific expression of human apoA-I and apoA-II[1]

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Abstract To evaluate the sources of high density lipoprotein (HDL) particles containing only apolipoprotein A-I (apoA-I), the synthesis of apoA-I and apolipoprotein A-II (apoA-II) was examined in human liver and small intestine as well as the human intestinally derived cell line, Caco-2. Human liver contained apoA-I, apoA-II as well as apolipoprotein B (apoB) mRNA. In contrast, human adult small intestine total and polyA$^+$ RNA had little or no apoA-II despite the presence of apoA-I and apoB. Intestinal biopsies from normal individuals failed to show de novo apoA-II protein synthesis in the media of organ cultures during [^{35}S]methionine pulse-chase labeling, whereas apoA-I could be readily detected. Caco-2 cells contained apoA-II mRNA and secreted apoA-II protein into the tissue culture media. These data indicate that the primary site of human apoA-II synthesis is in the liver and that the small intestine secretes apoA-I-containing high density lipoproteins. —**Eggerman, T. L., J. M. Hoeg, M. S. Meng, A. Tombragel, D. Bojanovski, and H. B. Brewer, Jr.** Differential tissue-specific expression of human apoA-I and apoA-II. *J. Lipid Res.* 1991. **32:** 821–828.

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High density lipoproteins have a significant association with coronary artery disease. High levels of HDL are associated with decreased risk (1–3) while low levels increase the risk (4–9). The proposed mechanism for this association is reverse cholesterol transport (10, 11). The two major protein components of HDL are apoA-I and apoA-II.

ApoA-I modulates the activity of lecithin:cholesterol acyl transferase (LCAT) in vitro. The physiological function of apoA-II, however, has not been elucidated. In vitro studies indicate that apoA-II can affect both hepatic lipase (12–15) and LCAT (16–19) as well as decrease the binding of HDL_3 to adipocytes (20). ApoA-II has been proposed to play a role in cardiovascular disease. Elevated apoA-II levels have been associated with reduced cardiovascular disease risk (21), but the significance of this association is questioned by a Japanese kindred having an apoA-II deficiency that is not associated with cardiovascular disease (22).

Human apoA-II is synthesized as a 100 amino acid preproprotein that is secreted primarily as the mature form containing 77 amino acids or as a proprotein having a 5 amino acid amino propeptide (23, 24). The mature protein is a dimer of 154 amino acids with a disulfide bridge at position 6 (25). Chromosome 1 contains the gene coding for apoA-II (26–28) which has been localized to the 1q21–1q23 region (29). Nucleotide sequences for the apoA-II genomic DNA (30, 31) and the mRNA have been reported (32).

The sites of synthesis for human apoA-II have been proposed to be the liver (33) and the small intestine (34). ApoA-II has been identified in the human small intestine histologically using immunochemical techniques (35) as well as by ^{14}C incorporation and polyclonal apoA-II antibody precipitation of duodeno-jejunal biopsies (36). ApoA-II mRNA has been detected in adult ileal and jejunal small intestine by in vitro translation and polyclonal antibody protein recognition (23). In addition, the presence of apoA-II in the thoracic duct lymph has been suggested to represent synthesis from the small intestine (37). However, conflicting data have been reported in

Abbreviations: apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoB, apolipoprotein B; CsCl, cesium chloride; CNBr, cyanogen bromide; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; LpA-I, lipoprotein particles containing only apoA-I; LpA-I,A-II, lipoprotein particles containing both apoA-I and apoA-II; MOPS, 3-(N-morpholino) propane sulfonic acid; PBS, phosphate-buffered saline; SSC, saline sodium citrate; $NaDodSO_4$, sodium dodecyl sulfate.

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which apoA-II mRNA was not detected in the adult human small intestine (38). Similarly, in the human fetus both the presence (39) and absence (40) of apoA-II mRNA have been reported in the small intestine. To date, no one has simultaneously evaluated both protein and mRNA production of apoA-II in human small intestine. Because of the discrepancies in the literature ascribed to the physiologic and pathophysiologic roles of HDL particles with different apoA-I/apoA-II compositions, possibly reflecting different synthetic sites, we have investigated the human liver and small intestine as sources for apoA-I and apoA-II.

METHODS

Cell culture

The HepG2 and Caco-2 cells lines were obtained from the American Type Culture Collection, Rockville, MD. The HepG2 cells were grown in a T-150 culture flask with Eagle's minimum essential media (EMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO_2, 95% air. Cells were grown to near confluency and the RNA was isolated. The Caco-2 cells were grown under the same conditions as the HepG2 cells described above except with Dulbecco's modified Eagle's medium (DMEM) instead of EMEM. After splitting the cells by trypsinization, confluence was reached in about 1 week and the cells were continued in culture up to 30 days as indicated.

Northern blot analysis

Total RNA or polyA$^+$ RNA in 5.6% (wt/vol) formaldehyde, 50% (vol/vol) formamide, as well as 2 mM ethylenediaminetetraaceticacid (EDTA), 10 mM sodium acetate, and 40 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.2, was heated to 65°C for 10 min and placed on ice prior to application to a 1% agarose gel. The samples were transferred to either GeneScreen Plus™ or nitrocellulose (41). The nitrocellulose filters were washed briefly in 2 × saline sodium citrate (SSC), 0.1% (wt/vol) sodium dodecyl sulfate ($NaDodSO_4$) at room temperature, once with 2 × SSC, 0.1% $NaDodSO_4$ at 65°C for 30 min and once with 1 × SSC, 0.1% $NaDodSO_4$ at 65°C for 30 min. The filters were sequentially stripped and rehybridized when using different probes. The nitrocellulose was stripped using two 30-min washes with a solution containing 50% formamide, 0.1 × SSC, and 0.1% $NaDodSO_4$ at 65°C. The GeneScreen Plus™ was stripped in a boiling solution of 1% $NaDodSO_4$ and 0.1 × SSC, for 30 min.

The Northern blots were quantitatively analyzed with a Betagen Betascope 603 Blot Analyzer™. To compare the human small intestine apoA-II mRNA quantity with that of human liver, a raw percentage apoA-II was determined by the ratio of small intestine apoA-II cpm to liver apoA-II cpm. This percentage was normalized with apoA-I and beta-actin by diving the raw percentage apoA-II by the ratio of small intestine apoA-I (or beta-actin) cpm to liver apoA-I (or beta-actin) cpm.

cDNA probes

A 0.3-kb apoA-II cDNA probe was generated by cleavage of the plasmid MDB 2049 with the restriction enzymes Pst 1 and Sst 1 (32). The 0.86-kb apoA-I probe was generated by cleavage of an apoA-I cDNA clone by Msp (42). The 0.6-kb apoB cDNA probe was prepared using BamH 1 and Bgl II (43). The 0.4-kb beta actin probe was made as a BamH I and Kpn I double digest (44). All four probes were labeled using with α-[^{32}P]dCTP. ApoA-I, apo-II, and apoB were nick-translated; beta-actin was labeled by random primer extension.

RNA isolation

The total RNA from Caco-2 was isolated by removing media, washing the cells with ice cold phosphate-buffered saline (PBS), adding 4 ml of guanidine thiocyanate solution (45), and homogenizing with a Polytron as previously described (41). Tissue samples were similarly prepared except that the frozen tissues were initially ground into a powder with a mortar and pestle while on dry ice prior to the addition of guanidine thiocyanate. The RNA pellet after ultracentrification with cesium chloride (CsCl) was then brought up in sterile double-distilled water and stored at −70°C after ethanol precipitation. PolyA$^+$ RNA was prepared as described by Maniatis, Fritsch, and Sambrook (46). The concentration of the RNA was determined with a Gilford spectrophotometer model 240 using the conversion of 1 absorbance unit equaling 40 μg/ml at 260 nm.

Tissue sources

Endoscopic intestinal mucosal biopsies were made after informed consent had been obtained from fasted adults. The biopsies were frozen immediately in liquid nitrogen, transported on dry ice, and maintained at −70°C until RNA isolation.

Intact small intestine was obtained from a renal transplant donor. The intestinal mucosa was washed with an inhibitor cocktail containing soybean trypsin inhibitor (100 μg/ml), lima bean inhibitor (100 μg/ml), benzamidine (2 mM), aprotinin (100 μg/ml), leupeptin (1 mM), phenylmethylsulfonyl fluoride (1 mM), bacitracin (1 μg/ml), diazoacetyl-d, l-norleucine methylester (1 mM), EDTA (0.1 mM), ε-amino-n-caproic acid (1 mM), elastatinal (20 μg/ml), chymostatin (100 μg/ml), bestatin (1 mM), and butylated hydroxytoluene (20 μM). The samples were

frozen in liquid nitrogen and stored at −70°C until RNA isolation.

Intestinal mucosal biopsy isolation and organ culture

Organ culture specimens were obtained during endoscopic evaluation of six fasted adults aged 18–22 years. All of the patients gave informed consent. Media samples were pooled from all six individuals.

Samples were immediately placed into tissue culture wells after biopsy with the mucosal surface upward as described by Trier (47). The samples were then treated as previously described (48) except that the ^{35}S-labeled media dialysate was applied to a goat anti-human apoA-I, apoA-II antibody column (1.0 cm × 30 cm) in which the antibodies were coupled to a CNBr-activated Sepharose according to the manufacturer's (Pharmacia) procedure. The column was washed with three bed volumes of 1 × PBS and then eluted with 4 M urea in 1 × PBS. Control experiments showed that both apoA-I and apoA-II bound to and were eluted from a column that was run in parallel. The eluant was dialyzed against 0.01 M ammonium bicarbonate, lyophilized, and dissolved in 0.1 M Tris-HCl, 1% $NaDodSO_4$, and 10% sucrose (pH 6.8). The dialyzate and isolated human apoA-I and apoA-II (49) were evaluated by slab gel electrophoresis using 0.1% $NaDodSO_4$ and 15% polyacrylamide in 0.1 M Tris-HCl (pH 8.3). The 1.5-mm-thick gels were electrophoresed at 250 volts at 4°C. Subsequently the samples were transferred to nitrocellulose paper for autoradiography or sequential immunoblot analysis with polyclonal goat anti-human apoA-I and apoA-II antibodies (Boehringer Mannheim, Indianapolis, IN) using a 1:1000 dilution. A second antibody consisting of anti-sheep-IgG-horseradish peroxidase complex (Bio-Rad) was then used in a 1:3000 dilution and visualized by immunoperoxidase staining (Bio-Rad).

ApoA-II two-dimensional gel immunoblot

Two-dimensional gel electrophoresis was performed as previously described (24). Briefly, the samples were analyzed by isoelectric focusing in tubes containing 8 M urea, 7.5% acrylamide, and 7.5% ampholytes (pH 4–6 or 4–7) for the first dimension. The gel was then laid onto a 15% polyacrylamide gel containing 0.1% $NaDodSO_4$. The proteins were transferred to nitrocellulose paper and incubated with a rabbit monospecific antiserum against apoA-II. A second antibody consisting of anti-rabbit-IgG-horseradish peroxidase complex (Bio-Rad) was then used and detection was by a color reagent (Bio-Rad).

RESULTS

PolyA$^+$ RNA samples (2 μg) from human small intestine and human liver (fasting samples) were examined by Northern blot analysis for apoB, apoA-I, and apoA-II mRNA (**Fig. 1**). In addition, total RNA from the human intestinally derived cell line Caco-2 was also examined. Northern blot analysis revealed the presence of apoA-I mRNA (Fig. 1, panel A) and apoB mRNA (Fig. 1, panel B) in all of the samples analyzed. However, both polyA$^+$ human small intestine samples were devoid of apoA-II (Fig. 1, panel C, lanes 3 and 4). Both Caco-2 and human liver contained apoA-II mRNA (Fig. 1, panel C, lanes 1 and 2). By using a larger quantity of polyA$^+$ RNA (10 μg), apoA-II mRNA was detected in one of the two polyA$^+$ RNA small intestine samples (**Fig. 2,** panel B, lane 3). Again the liver and Caco-2 samples both contained apoA-II (Fig. 2, panel B, lanes 1 and 2). Abundant quantities of apoA-I mRNA were present in the liver, Caco-2 cells, and both small intestine samples (Fig. 2, panel A). When normalizing for the content of apoA-I and analyzing with a Betagen Betascope 603 Blot Analyzer™, the content of apoA-II mRNA present in the positive polyA$^+$ RNA small intestine sample was 0.24% of the liver. Normalization of apoA-I mRNA using the beta-actin probe indicated that the apoA-II mRNA content in the small intestine sample was 0.23% of the liver.

Biopsies were obtained from the proximal small intestines of two fasted normolipidemic patients. ApoA-I

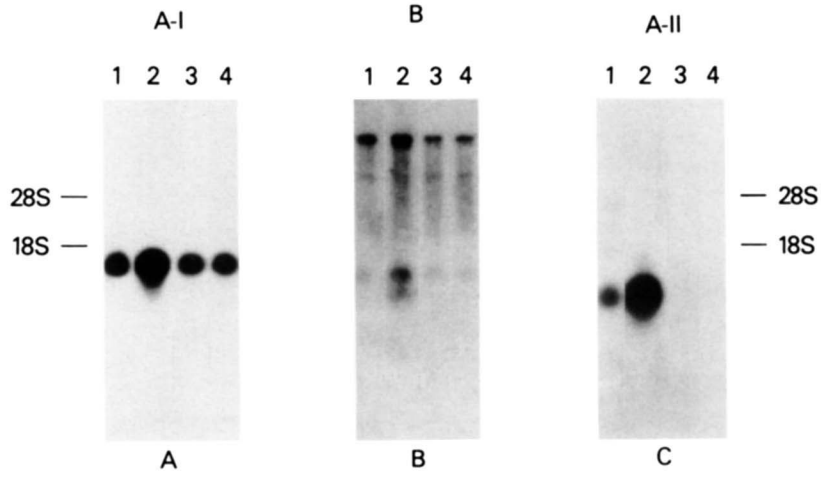

Fig. 1. The Northern blot analysis of various tissues using apoA-I, apoB, and apoA-II cDNA probes. The RNA was separated by agarose gel electrophoresis, transferred to a nitrocellulose membrane, hybridized to different probes using the same membrane, and stripped after each probe as described in Methods. Lane 1 is 10 μg of Caco-2 total RNA and lane 2 is 2 μg of human liver polyA$^+$ RNA. Lanes 3 and 4 are two different 2-μg fasting normal human small intestine mucosal polyA$^+$ RNA samples. The hybridizations were performed with apoA-I (panel A), apoB (panel B), and apoA-II (panel C) probes. In panel B, the lowest band is a residual from the apoA-I hybridization that preceded the apoB hybridization. The 18S and 28S rRNA mobilities are noted on the margins. The exposure times were 16 h for apoA-I, 24 h for apoA-II, and 12 days for apoB.

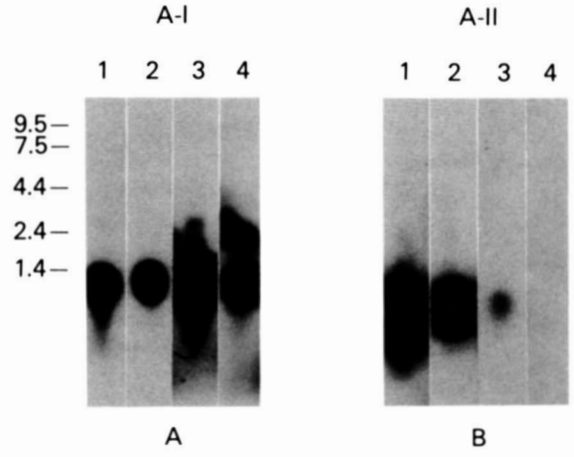

Fig. 2. The Northern blot analysis of various tissues using apoA-I and apoA-II cDNA probes. Lanes 1 and 2 have 10 μg of total RNA from human adult liver and Caco-2 cells, respectively. Lanes 3 and 4 have 10 μg of polyA$^+$ RNA from different samples of fasting adult normal total small intestine mucosa. The hybridizations were performed with apoA-I (panel A) and apoA-II (panel B) probes. The exposure times were 20 h for apoA-I and 72 h for apoA-II. The RNA ladder is on the left margin.

mRNA was seen in these biopsies as well as in the human adult liver and Caco-2 cells (**Fig. 3** panel A). ApoA-II mRNA could also be detected in these samples (Fig. 3, panel B) when a longer autoradiographic exposure time was used (7 days). When a shorter period (14 h) was used (Fig. 3, panel C), the liver and Caco-2 samples were easily observed but the small intestine samples appeared negative. The quantity of apoA-II mRNA in these biopsies relative to apoA-I and beta-actin mRNA in the liver sample, using the Betagen Betascope 603 Blot Analyzer™, was 1.1% and 0.64%, respectively.

To corroborate the RNA results, apoA-II protein biosynthesis from the small intestine was evaluated utilizing organ cultures of proximal small intestine biopsies from fasted individuals. The samples were pulse-chase labeled as is shown in **Fig. 4.** The electrophoretic positions of human apoA-I and apoA-II standards are shown on the left margin as determined by sequential immunoblotting with apoA-II and then apoA-I antibodies. The pulse-chase studies revealed a band comigrating with apoA-I, representing newly synthesized apoA-I in organ culture media. At the apoA-II location, no band was seen. Below the expected apoA-II location and above and below the expected apoA-I location were other bands; however, the bands were not recognized by the immunoblotting technique using a polyclonal apoA-II antibody (data not shown). Thus, organ cultures of fasted human small intestine can synthesize apoA-I but not apoA-II.

The human intestinal cell line Caco-2, which has been used as a model for enterocyte studies, was examined at various culture periods (**Fig. 5**). ApoA-II mRNA was present by Northern blot analysis in cells grown from 13 to 28 days. ApoA-I mRNA was also found (data not shown) as has been previously reported by Hughes et al. (50). Evidence of apoA-II protein production from Caco-2 is illustrated in **Fig. 6** which contains a two-dimensional immunoblot of Caco-2 culture media (panel A) and HepG2 culture media (panel B). Three major isoforms were detected in Caco-2 media. HepG2 media also has these isoforms as well as several additional isoforms.

DISCUSSION

Although apoA-I and apoA-II are the major apolipoproteins in HDL, evidence suggesting physical as well as physiologic heterogeneity is accumulating. The HDL_2 subfraction, which contains a higher proportion of particles containing only apoA-I, is associated with a greater protection against premature cardiovascular disease. In the one case of secondary hyperalphalipoproteinemia where both apoA-I and apoA-II levels have been reported, apoA-I was significantly increased, however, apoA-II was

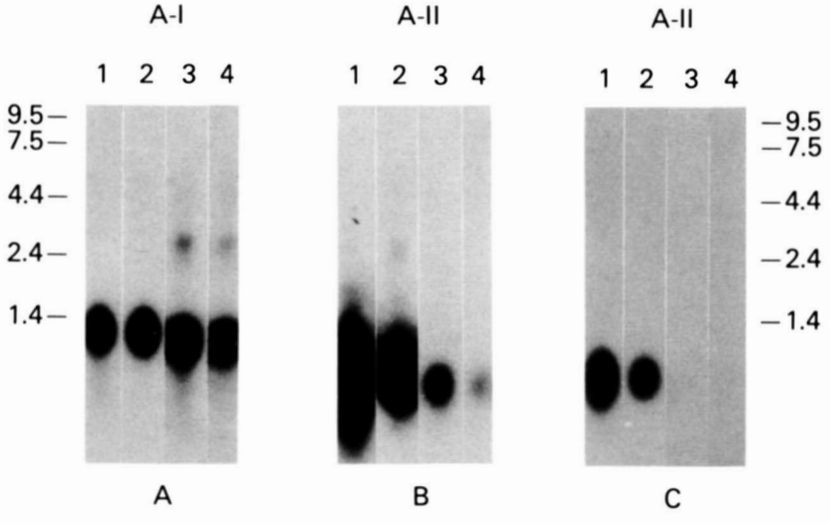

Fig. 3. The Northern blot analysis of various tissues using apoA-I and apoA-II cDNA probes. Lanes 1 and 2 have 10 μg of total RNA from human adult liver and Caco-2 cells, respectively. Lanes 3 and 4 have 10 μg of total RNA from fasting human adult upper intestinal biopsies. The hybridizations were performed with apoA-I (panel A) and apoA-II (panels B and C) probes. The exposure times were 7 h (panel A), 7 days (panel B), and 14 h (panel C). The RNA ladder is on both the left and right margins.

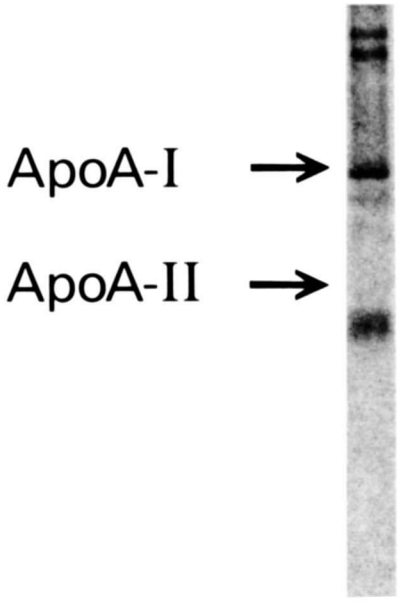

Fig. 4. The human proximal small intestine biopsy organ culture media content of apoA-I and apoA-II after $NaDodSO_4$-polyacrylamide gel electrophoresis. On the left margin are the locations of isolated human apoA-I and apoA-II standards detected by Western blot using polyclonal antibodies identification after nitrocellulose transfer as described in methods. The lane contains the [^{35}S]methionine pulse-chase labeled medium autoradiograph after 0.1% $NaDodSO_4$, 15% polyacrylamide electrophoresis.

normal (51). The present study provides an additional difference between tissue specific expression of apoA-I and apoA-II.

The results of this study established that virtually undetectable quantities of apoA-II mRNA and no de novo apoA-II protein synthesis were present in the fasting adult human small intestine. The apparent discrepancies reported in the literature regarding the intestinal synthesis of apoA-II may be due to differences in methods or in patient selection. In our studies we used Northern blot analysis of total small intestinal mucosa or upper small intestine biopsies from normal patients. It is possible that regional variations within the small intestine may exist where specific areas can be found with relatively larger or smaller quantities of apoA-II mRNA; however, when combining all of the small intestine regions together as reported in Figs. 1 and 2, only a small amount of apoA-II mRNA could be detected, less than 1% of the quantity found in the liver.

Supporting these results is the absence of apoA-II mRNA in adult upper small intestine observed by Knott et al. (38) and the finding of minimal to absent apoA-II mRNA in fetal small intestine by Hussain and Zannis (39) and Hopkins et al. (40). Inasmuch as these fetal results reflect different ages, developmental or temporal changes may be causing undetectable levels at 6–12 weeks (40) and low levels at 16 to 22 weeks (39). In other mammals, including the rat (52, 53), mouse (54), rabbit (55), rhesus monkey (38), and cynomolgus monkey (39), apoA-II mRNA has been absent in the small intestine but present in the liver. Significant amounts of apoA-I mRNA or protein are found in the liver and small intestine in the rat (56, 57), mouse (58), rhesus monkey (38), and cynomolgus monkey (59), as has been found in the human. In contrast, rabbit apoA-I mRNA is mainly in the small intestine with only trace amounts in the liver (60).

The human small intestine organ culture protein synthesis experiments were consistent with absent or low levels of apoA-II mRNA, showing no significant apoA-II protein production. These findings contrast with those of Rachmilewitz et al. (36) who reported apoA-II protein production from duodeno-jejunal mucosa when using [^{14}C]leucine labeling and antibody precipitation. Their apoA-II antibody-precipitated ultracentrifugation fractions had radioactive counts that were 60% that of apoA-I. However, when the labeled mucosal extract was applied to a $NaDodSO_4$-polyacrylamide gel, a prominent apoA-I band could be seen but no apoA-II band was visualized. The explanation for these results is as yet unclear.

Both the liver and small intestine have lymphatics that contribute to thoracic duct contents. The presence of

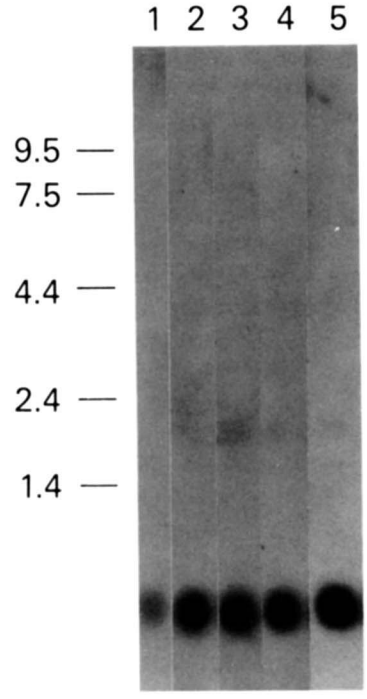

Fig. 5. The Northern blot analysis of Caco-2 at various time intervals using an apoA-II cDNA probe. Total RNA (10 μg) from Caco-2 cultures was separated by agarose gel electrophoresis, transferred to a GeneScreen Plus™ membrane and hybridized with an apoA-II cDNA probe. The RNA ladder is on the left margin. The lanes represent the following culture durations (in days): 13 (lane 1), 16 (lane 2), 20 (lane 3), 23 (lane 4), and 28 (lane 5).

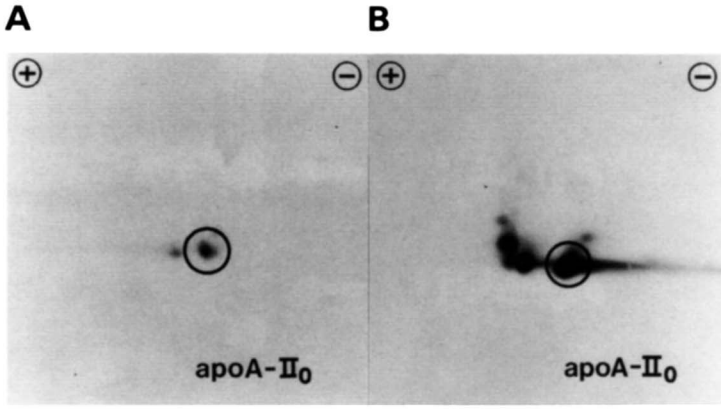

Fig. 6. The two-dimensional apoA-II immunoblot from the media of Caco-2 and HepG2 cells. Media from Caco-2 (panel A) and HepG2 (panel B) were analyzed by isoelectric focusing (positive to negative from left to right). The immunoblot was performed, following transfer to nitrocellulose after two-dimensional polyacrylamide electrophoresis, by incubation initially with a rabbit apoA-II antibody followed by rabbit antibody-IgG-horseradish peroxidase complex.

apoA-II in the thoracic duct could, therefore, represent synthesis from either the small intestine or liver or both. When Anderson et al. (37) determined that amount of apoA-II from the small intestine by analyzing thoracic duct contents, they did not include the liver lymphatic contribution in their model. Consequently, they greatly overestimated the potential small intestine contribution of apoA-II into the thoracic duct.

The Caco-2 cells, unlike normal small intestine, synthesize significant amounts of apoA-II mRNA and secrete easily detectable apoA-II protein. Previous studies have shown that Caco-2 cells produce other apolipoproteins including apoA-I, apoB, and apoE (50). HepG2 cells secrete apoA-II and other apolipoproteins like human liver and may be a better model system for their normal counterpart than Caco-2 cells. Because of the difference from normal small intestine, Caco-2 cells may be useful in delineating the *cis*- and *trans*-acting factors that affect the expression of the apoA-II gene.

Significant differences exist between human particles containing only apoA-I (LpA-I) and those having both apoA-I and apoA-II (LpA-I,A-II). With LpA-I both age and sex variability are noted in cholesterol, cholesteryl ester, phospholipids, and apoA-I content (61). In contrast, with LpA-I,A-II particles only the apoA-I content varies (61). Human LpA-I particles cause cholesterol efflux from the mouse adipocyte cell line, OB 1771, whereas LpA-I,A-II particles containing both apolipoproteins induce no cholesterol efflux (62). Additionally, liposomes containing increasing amounts of human apoA-II relative to apoA-I showed a dose-response decrease in cholesterol efflux (62). These combined results clearly establish the different functional importance of LpA-I and LpA-I,A-II particles within HDL. The results presented here revealed a significant differential tissue expression between apoA-I and apoA-II with the liver producing both apoA-I and apoA-II but the small intestine producing only apoA-I. These differences between apoA-I and apoA-II, including tissue-specific expression, may have important implications in HDL metabolism as well as to the predisposition of arteriosclerosis.

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