RT-PCR and *in situ* hybridization analysis of apolipoprotein H expression in rat normal tissues

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Abstract. In this study, by using different techniques (i.e. Northern blot hybridization, RT-PCR and Southern blot hybridization) on various normal rat tissues, we were able to identify liver, kidney, heart, small intestine, brain, spleen, stomach and prostate as tissues in which the *ApoH* gene is transcribed. Moreover, for some of these tissues, by *in situ* hybridization, we found a specific localization of *apoH* transcripts. For instance epithelial cells of the bile ducts in liver and of the proximal tubules in kidney are the major sites of apoH synthesis. Our data suggest that some of the different physiological roles proposed for apoH could correlate with its direct expression, while others could correlate with its absorption from bloodstream or adjacent cells.

Introduction

Apolipoprotein H (apoH, named also ß-2-glycoprotein I) is a 50-kDa protein present in plasma both in free form and in combination with lipoprotein particles (1,2). It consists of four CCD (Complement Control Domain) modules (3) and a fifth CCD-like domain carrying a lysine rich loop, responsible for electrostatic interactions with anionic phospholipids, and a hydrophobic loop able to enter the hydrophobic core of a membrane (4). The crystal structure has revealed a fish hook-like shape (5,6).

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The physiological role of apoH remains unknown. *In vitro* data demonstrated the ability of apoH to bind to anionic surfaces, such as anionic phospholipids, heparin, DNA, mitochondria and oxidized LDL (7-10).

Considerable interest has been given to the pathological properties of apoH. One aspect is that antiphospholipid antibodies, present in plasma of patients with antiphospholipid syndrome and other autoimmune diseases, do not bind to phospholipids, while they bind to apoH when it forms a complex with the acidic phospholipids and cardiolipin or is immobilized on negatively charged surfaces (11-14). Another pathological aspect of apoH relates to the high urinary excretion of the protein in patients with Fanconi syndrome (15).

Different physiological activities have been attributed to apoH, such as a possible role in the coagulation pathway, exhibiting an inhibitory effect on ADP-mediated platelet aggregations (16,17) and on prothrombinase activity (18). Moreover, apoH may be involved in the metabolism of triglyceride-rich lipoproteins as well as in the regulation of haemostasis and in the clearance of apoptotic bodies, mediating the phagocytic uptake of phosphatidylserine containing liposomes by macrophages (19-21).

The only generally accepted property of apoH is its affinity for negatively charged phospholipids and its physiological properties could all be explained by neutralization of anionic phospholipids, regulating the exposure of negatively charged phospholipids on cellular surfaces in contact with blood. Referring to this aspect, Yan *et al* (22) have recently reported an inhibiting, instead of enhancing, effect of apoH on liposome uptake by hepatocytes, Kupffer cells and endothelial cells. Moreover, the low affinity of apoH for natural membranes exposing phosphatidylserine raises doubts about these apoH functions (23).

ApoH has been extensively characterized at amino acid and nucleotide levels from a number of species including human (24-26), rat (27), mouse (28,29), dog (30), cattle (31-33) and chimpanzee (34).

The presence of apoH protein was detected by immuno-histochemistry at the syncytiotrophoblastic layer of normal placental villous tissue, in liver, mostly in some hepatocytes

adjacent to the centrolobular veins, and in the enterocytes of jejunum and colon (35,36).

The expression of *apoH* mRNA was initially detected by Northern blotting in human, rat and mouse liver and in two liver-derived cell lines, HepG2 and Hep3B (30,37,38). More recently *apoH* mRNA was found by RT-PCR on RNA extracted from other tissues (intestine, placenta, foetal astrocytes, lymphocytes) or cultured cells (transformed cell lines) such as choriocarcinoma, hepatoma (36), colon adenocarcinoma (35), neuroblastoma, umbilical vein endothelial cells (HUVEC, but not for Alvarado-de la Barrera, 39), astrocytoma, glioblastoma cells (40) and in monocytes (41).

In previous experiments (36) some of us suggested a functional regulation of the expression of *apoH* in liver. More recently it has been demonstrated that *ApoH* gene expression is strictly related to the proliferative status of hepatic cells and that this protein could play a role in maintaining the vitality of liver cells when exposed to different stress factors such as regeneration after partial hepatectomy or growth factor depletion (42).

To shed some light on transcriptional regulation and on apoH synthesis sites we investigated the presence of *apoH* mRNA in several normal rat tissues by RT-PCR and non-radioactive *in situ* hybridization techniques.

Materials and methods

Animals. Wistar rats (Stefano Morini, San Polo d'Enza, Italy) were housed in our institutional care facility under direction of a licensed veterinary. Procedures involving animals were conducted according to the European Community Council Directory 86/609, OJL 358 1, 12 December 1987. Wistar rats were sacrificed by cervical dislocation after ether anaesthesia. Tissues were removed and frozen immediately in liquid nitrogen for RNA extraction or placed in 4% formaldehyde in PBS for *in situ* hybridization (ISH).

Probe. The probe used in Northern blotting, Southern blotting and ISH was a PCR-amplified 550-bp fragment of human *apoH* cDNA (from 60 to 610, GenBank accession no. NM_000042), cloned in pPCR-Script-Amp vector.

Northern blotting and RT-PCR. Total RNA was extracted from frozen samples using the phenol-guanidine thiocyanate method described by Chomczynski and Sacchi (43). Total RNA concentration was determined by measuring absorbance at 260 nm and the quality was detected by electrophoresis of the samples on formaldehyde agarose gel. After electrophoresis, fractionated RNA (10 μg) was transferred to nylon membranes (Hybond X; Amersham Pharmacia Biotech). The filter was hybridized to random priming labeled (α-^{32}P) deoxycytidine triphosphate (dCTP) human *apoH* specific probe, according to standard methods, with stringency washes of 0.2X SSC, 0.5% sodium dodecyl sulphate, at 65˚C. After washing, the filters were exposed to autoradiographic film (Hyperfilm; Amersham Pharmacia Biotech) with intensifying screens at -70˚C.

RT-PCR experiments were carried out with the AccessQuick RT-PCR System (Promega) using 1 μg of total RNA. The cDNA primers were ApoRat3: 5'-TTCC TCTGCCATGTTGCTATTGC-3' (from 41 to 63) and ApoRat4R: 5'-CCTTGGTACAACACGGTGGC-3' [from 693 to 674; GenBank accession no. X15551 (27)]. Although a 653-bp fragment was expected (see Results), a 797-bp product was generated by using these primers. PCR was performed on a (Eppendorf) thermal cycler under the following conditions: 94˚C (4 min); 94˚C (30 sec), 55˚C (30 sec), 72˚C (45 sec) (35 cycles); and 72˚C (5 min).

Aliquots of 10 μl of amplified products were resolved by electrophoresis in 1.5% agarose gel and stained with ethidium bromide. Following electrophoresis, samples were transferred onto nylon membranes. The filters were hybridized to random priming labeled (α-^{32}P)-(dCTP) human *apoH* cDNA probe, washed at stringent conditions (0.2xSSC, 0.5%SDS, 65˚C) and exposed for different times to X-ray film.

The RT-PCR product obtained from liver RNA was automatically sequenced (ABI PRISM 3100 system) using primers ApoRat3, apoH5R (CATCGCATGTTGTGGCAAA CATT), apoH3R (CTTCCTCAGTGCACTTGGCAG) and ApoRat4R.

In situ hybridization. Preventively formaldehyde-fixed tissues were dehydrated, cleared in xylene, infiltrated and embedded in paraffin. Sections of 7 μm thick were air dried on poly-L-lysine-coated glass slides.

Digoxigenin-(DIG)-labeled cRNA antisense and sense probes were generated by *in vitro* transcription, according to the DIG-RNA labeling procedure (Roche), using human *apoH* cDNA plasmid clone linearized with DraII or HindIII restriction enzymes and T3 or T7 RNA polymerases (Promega) respectively. Integrity and concentration of probes were assessed by agarose gel electrophoresis and by comparative dot blotting together with a DIG-labeled standard template of known concentration.

Hybridization was performed according to the protocol of Moorman *et al* (44) with the following modification: pre-hybridization and hybridization temperatures were 65˚C because of probe heterology.

Negative controls were carried out by substituting an antisense with a sense probe or by RNase A treatment (40 μg/ml at 37˚C for 30 min) prior to hybridization. The probe was detected with alkaline phosphatase-coupled anti-DIG antibody followed by an NBT/BCIP colour reaction.

Results

Since expression data on *apoH* mRNA derive mainly from RT-PCR experiments performed on transformed cell lines or immunohistochemical experiments showing the localization of the protein, we tested the presence of *apoH* transcript in normal rat tissues with RT-PCR and *in situ* hybridization assays.

To perform Northern blot analyses we extracted total RNA from several rat tissues (liver, spleen, lung, kidney, small intestine, heart, prostate, skeletal muscle, stomach). Because human and rat transcripts share 83% homology (26), we used as a probe a human *apoH* cDNA fragment, corresponding to the region coding for aa 68-191 of the human protein, cloned in pPCR Script-Amp vector. Using this method we identified the *apoH* messenger (of approximately 1200 nucleotides) only

in liver RNA (Fig. 1) as expected from previously published data (27).

For an accurate investigation of the synthesis of *apoH* transcript in other tissues we utilized the more sensitive RT-PCR technique. We evaluated the *apoH* mRNA expression in the same tissues that we used for Northern blot experiments and, as a further positive control, in human hepatoma cell line HepG2, which is known to express high *apoH* mRNA levels (35,36). The primers utilized in these experiments were constructed from highly conserved sequences in human and rat cDNA and were intentionally designed so that one hybridized to the first exon and the other to the seventh exon, in order to point out an eventual DNA genomic contamination.

The ethidium bromide staining of the gel showed the RT-PCR products obtained from rat liver and kidney RNA samples, as well as the human HepG2 RNA sample (Fig. 2A).

As assumed, the RT-PCR product obtained from human HepG2 RNA was 797 bp long, the RT-PCR products from rat RNA were the same size, although according to published data (27) 652 bp fragments were expected.

The RT-PCR product obtained from liver RNA was then purified from agarose gel and automatically sequenced using an ABI PRISM 3100 system (GenBank accession no. AM071386).

Sequence analysis, performed using the BLAST (Basic Local Alignment Search Tool) server (http://www.ncbi.nlm.nih.gov/BLAST/), confirmed that the product contained the murine *apoH* mRNA sequence, previously published (27), and also a 145-bp insert, located between nucleotide positions 71-72, as occurs in human and in other mammalian species (26,28,30,33).

Sequence analysis using rat BLAT search (http://genome.cse.ucsc.edu/cgi-bin/hgBlat) gave the maximum identity (100%) with position 97739499-97752117 of chromosome 10, band 10q32.1 corresponding to the *ApoH* gene. The sequence was also compared with human cDNA and genomic sequences, demonstrating an 81% homology and that intron positions are conserved (45,46).

Southern blot analysis of RT-PCR-amplified products allowed us to detect *apoH* cDNA also in brain, small intestine, heart, and, after a five-day exposure, in spleen, stomach and prostate samples (Fig. 2B).

Thus, the *apoH* specific transcript was detectable in a wide variety of tissues with a prevalence of expression in liver and kidney, as well as in intestine, heart and brain. Comparatively lower levels of expression were evident in spleen, stomach and prostate. Transcripts, instead, were below detectable levels in the skeletal muscle and lung.

To determine the regional distribution of *apoH* mRNA, we performed *in situ* hybridization on paraffin embedded sections of tissues expressing the *ApoH* gene at a higher level: liver, kidney, intestine and heart. A human *apoH*-specific antisense digoxigenin-labeled riboprobe was used. In line with the RT-PCR results, we found *apoH* mRNA in all tested tissues.

In liver (Fig. 3A and C), *apoH* mRNA was strongly expressed in the columnar epithelium lining the bile duct of portal canals (cholangiocytes), but not in the cells lining the intrahepatic artery, the portal vein or the lymphatic vessel. No staining was observed in the endothelial cells of central

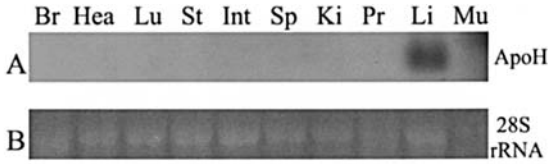

Figure 1. (A) Northern blotting of total rat RNA extracted from several normal tissues hybridized with a ^{32}P-labeled human *apoH* probe: the transcript is detected only in liver. (B) Ethidium bromide staining of the gel showing the 28S rRNA bands: the sample amounts are comparable. Br, brain; Hea, heart; Lu, lung; St, stomach; Int, small intestine; Sp, spleen; Ki, kidney; Pr, prostate; Li, liver; Mu, skeletal muscle.

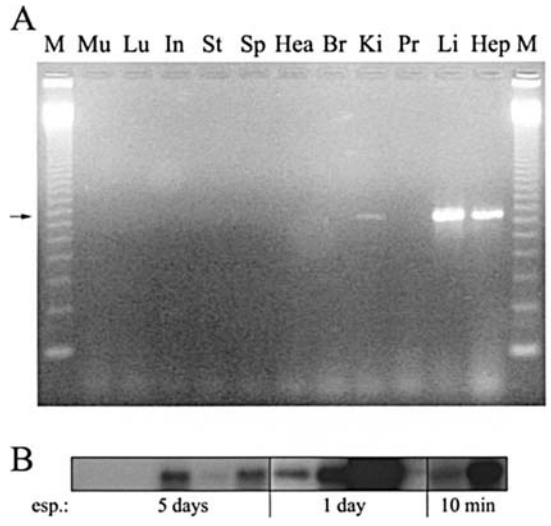

Figure 2. Detection of apoH mRNA by single-step RT-PCR followed by Southern blot analysis, performed on the same RNAs as the Northern blotting and, as a positive control, on human HepG2 cell RNA. (A) Ten microliters of amplified product was resolved by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Amplified fragments were 797 bp long, both in rat and human samples (arrow). Molecular markers (M=123 bp ladder) are shown. The *apoH* transcript is evident in kidney, liver and HepG2. (B) RT-PCR products were analyzed by Southern blot technique using the same specific *apoH* probe as for the Northern blotting. The exposure times were 10 min, 1 day and 5 days for the different samples as indicated in the figure. Note that the high stringency conditions used in this experiment favour probe hybridization with human RT-PCR product. The *apoH* transcript is detectable in liver, kidney, brain, heart, small intestine, spleen, prostate, stomach, other than HepG2 cells (Hep). For abbreviations see Fig. 1.

veins. In addition, besides a low expression detectable within the liver parenchyma, we observed higher *apoH* messenger expression in individual hepatocytes (especially in the nuclei) and also in Kupffer cells (Fig. 3D).

In kidney (Fig. 3H) the expression was evident in the cortical layer and, in particular, in the absorptive cells of proximal convoluted tubules. No staining was observed in cells of the renal corpuscles.

These results indicated that cholangiocytes and collecting duct epithelial cells were the major sites of *apoH* production in liver and kidney, respectively.

In the jejunum region of the small intestine (Fig. 3E and F) *apoH* mRNA was localized in the cells of the lamina propria, which forms the core of villi and surrounds the intestinal glands beneath the muscolaris mucosae, but not in the intestinal mucosa. Rare smooth muscle cells appeared stained.

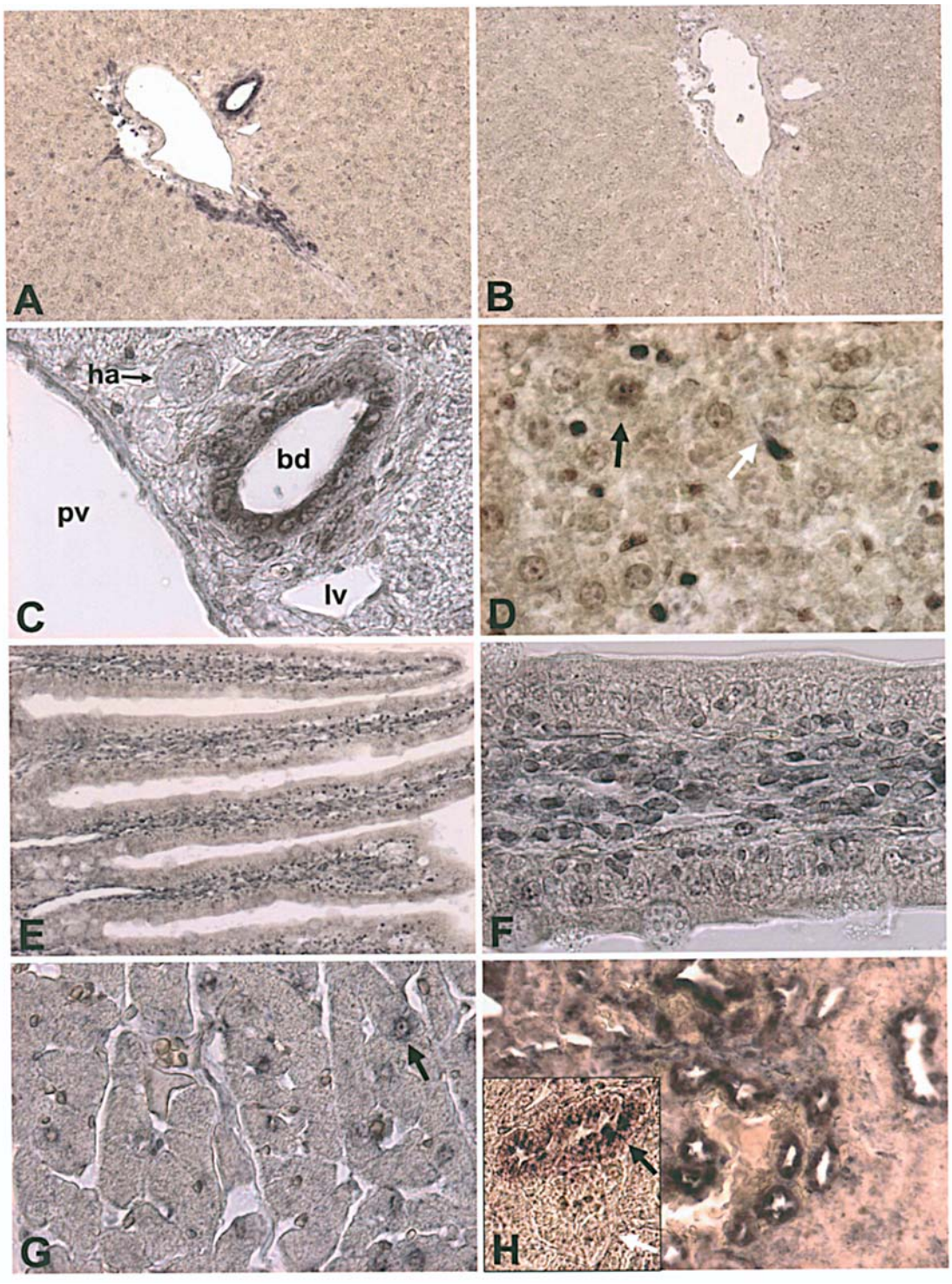

Figure 3. Distribution of *apoH* transcripts in different normal rat tissues detected by *in situ* hybridization, using digoxigenin-labeled cRNA specific probe. A dark blue to purple precipitate indicates the presence of *apoH* transcript. (A) Liver portal canal showing strong staining in bile duct epithelium. (B) Parallel section to A: no signal was detected when sense probe for *apoH* was used. (C) Higher magnification of a selected region of A, showing localization of *apoH* mRNA in cholangiocytes lining the bile duct (bd). Note the absence of reaction in both the hepatic artery (ha) and the portal vein (pv) endothelium as well as in the lymphatic vessel (lv). (D) *ApoH* transcript was also detected in some nuclei of hepatic cells (black arrow) and in Kupffer cells (white arrow). (E) In jejunum the transcript is localized in the core of villi; (F) higher magnification showing exclusive localization of *apoH* mRNA in lamina propria cells and not in epithelial cells. (G) In the heart mRNA is present in all cardiomyocytes and is localized especially in the perinuclear zone (arrow). (H) Kidney cortical region: the *ApoH* gene is expressed in proximal convoluted tubule cells (black arrow in higher-resolution inset) and no reaction product is detectable in renal corpuscles (white arrow in higher-resolution inset). Original magnification: A, B, E, x20; C, D, F, G, H, x40; H insert, x100.

In the heart (Fig. 3G), the probe appeared to hybridize within all cardiomyocytes, mostly in the cytoplasm around the nucleus (perinuclear labeling).

On all tested tissues no signal was observed in sections hybridized with the *apoH* sense strand (Fig. 3B) or in sections that had been treated with ribonuclease (not shown).

Discussion

At present, one of the most important factors in understanding the physiologic role of the apoH protein is knowing its synthesis tissues. To shed light on this issue, we analyzed several normal rat tissues by different techniques. We began our studies on *ApoH* expression by performing Northern blot analysis on total RNAs, using a human *apoH* cDNA probe: this approach confirmed that the liver is the major site of biosynthesis for apoH (37).

The RT-PCR experiments that we performed on the same total RNAs showed that, besides a strong expression in liver, the *ApoH* gene was expressed also in kidney. By subsequent Southern blot hybridization of these RT-PCR products with a human *apoH* probe, it was possible to detect the messenger in the small intestine, brain and also, at lower levels, in spleen, stomach and prostate. Moreover, the rat cDNA fragment had the same length as the human cDNA fragment. In fact, sequence analysis of the amplified RT-PCR product obtained from rat liver RNA revealed that the rat fragment which we amplified contained 145 bp more than that reported in previously published data (27) and therefore there is a higher similarity between rat and other mammalian *apoH* cDNA than previously thought.

Finally, we determined the localization of the *apoH* transcript by performing non-radioactive *in situ* hybridization experiments on tissues with a higher prevalence of *apoH* transcript, as highlighted by Northern blot and RT-PCR assays. The results obtained show a high presence of *apoH* messenger especially in the epithelial cells of the bile duct in liver and of the proximal tubules in kidney.

This specific localization of *apoH* mRNA could be consistent with a pre-eminent role of apoH in the process by which cholangiocytes and tubular cells reabsorb lipid particles from the ductal bile and the glomerular filtrate, respectively. It is in fact known that the organic and inorganic components of bile may be significantly modified by an array of absorptive mechanisms on the apical membrane of cholangiocytes. In the same way, glomerular filtrate is continuously reabsorbed by proximal tubule cells (and megalin is a good candidate as LDL-family receptor; 47).

Moreover, apoH protein was already identified in several parts of the kidney suggesting that it was filtered by the glomerulus and then reabsorbed (48). Our results indicate that apoH is produced also by the same tubular epithelial cells, thus explaining the massive urinary presence of this protein in patients with several renal tubular diseases, including adult Fanconi syndrome.

The presence of *apoH* mRNA in the lamina propria cells of jejunum (probably lymphocytes and macrophages), together with the expression in Kuppfer cells, is in agreement with the role of apoH as opsonin, mediating the phagocytic uptake of liposomes and of apoptotic membranes containing anionic phospholipids (49). Hence, phagocytes do not only recognize and take up apoptotic cell remnants showing apoH on their surface, but they also synthesize apoH. The need for producing this protein is probably due to the low affinity of apoH for natural membranes showed by Bevers *et al* (23), which could require a high concentration of the protein in the cellular microenvironment.

Although it is generally accepted that the protein is present on different endothelial cell membranes (50,51), the results on *apoH* mRNA expressed by endotheliocytes are conflicting (39,40). Moreover, we did not observe *apoH* transcripts in endothelial cells. On the other hand, apoH can adhere on the cell membrane through interaction between the cationic phospholipid binding site in the fifth domain and the anionic structures on the cell membrane or it can be recognized as a ligand for a receptor (probably annexin II-toll-like receptor, TLR-4). Furthermore, in antiphospholipid syndrome patients, many reports indicate that, when present, anti-ß2GPI antibodies may recognize the molecule on the endothelial cells and that, once bound, they might activate them inducing a proinflammatory and a procoagulant phenotype (reviewed in ref. 52).

Finally, with regard to the role of apoH in triglyceride-rich lipoprotein metabolism, our experiments showed that, at least in jejunum, enterocytes did not synthesize apoH. Thus, given that immunohistochemical studies detected the protein in these cells, we have to suppose that jejunum enterocytes take up apoH from plasma to assemble new lipid particles or, more likely, from the apoH producing cells which are present in the villi.

Our results collectively illustrate that the sites of *apoH* synthesis are often different to protein accumulation sites: for example, in liver the protein is localized mostly in the hepatocytes adjacent to the centrolobular veins (35) whereas the mRNA is expressed in few hepatocytes which are probably proliferating cells, because it is known that in hepatocytes and in HepG2 *apoH* expression depends on their proliferative rate (42).

Our results suggest also that the *ApoH* gene is expressed in cells where the protein has a direct role, for example in the recovery of liposome particles by receptor-mediated endocytosis (cholangiocytes, proximal tubule cells and also macrophages) or when the lipid need arises for metabolic (cardiomyocytes) or proliferative (hepatocytes) processes. For normal lipid metabolism and/or transport (transcytosis) instead, cells (endotheliocytes and enterocytes) could absorb and accumulate the protein (probably in combination with lipoprotein) from blood or from nearby cells.

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