In vitro studies on origin and site of action of enzyme activity responsible for conversion of human proapoprotein A-I into apoprotein A-I

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Human hepatocellular carcinoma cells (Hep G2) were shown to secrete apo A-I as a proprotein. No apo A-I synthesis could be detected with endothelial cells from human umbilical cord veins. Conversion of proapo A-I into apo A-I is a slow (of the order of hours) process, mediated by a Ca^{2+}/Mg^{2+}-dependent enzyme which is present on the surface of plasma lipoprotein particles, endothelial cells and Hep G2 cells, and is probably synthesized by Hep G2 cells.

Human proapolipoprotein A-I *Hep G2 cell* *Endothelial cell* *HDL metabolism*

1. INTRODUCTION

Apo A-I is a major protein constituent of plasma HDL and of lymph lipoproteins in man [1]. Recent studies on the biosynthesis and processing of apo A-I have shown that the primary translation product of apo A-I mRNA is a preproprotein [2] which undergoes intracellular co-translational proteolytic cleavage into proapo A-I. Proapo A-I is secreted from the cell and converted extracellularly into the mature form observed in plasma [2,3]. This conversion may be an important regulatory step in lipoprotein formation and a critical determinant of steady-state plasma apo A-I levels [2,3]. It is, therefore, of interest to characterize the enzyme activity responsible for the processing of proapo A-I and its site(s) of action. For this purpose, radiolabelled medium of a human hepatocellular carcinoma cell line (Hep G2), which contains proapo A-I [2,3], was incubated with human blood, plasma, serum or fractions thereof, or endothelial cells under various experimental conditions. The conversion of proapo A-I into the more acidic apo A-I was monitored by isoelectric focusing after purification of the apoprotein from the medium by immunoprecipitation.

Abbreviations: apo, apolipoprotein; HDL, LDL and VLDL, high density, low density and very low density lipoproteins, respectively; LPDS, lipoprotein-depleted serum; ECGS, endothelial cell growth supplement; EGF, epidermal growth factor; BSA, bovine serum albumin

2. MATERIALS AND METHODS

The established Hep G2 cell line, derived from human liver tumour, was obtained from Dr B.B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA). Cells were grown as in [4]. Endothelial cells were isolated from human umbilical cord veins and cultured as in [5]. So that synthesized proteins could be labelled, confluent monolayers grown in 25-cm^2 plastic tissue culture flasks were washed twice with methionine-free RPMI-1640 medium, and incubated with 1.5 ml of this medium supplemented with 100–200 μCi of [^{35}S]methionine/ml (300 Ci/mmol; New England Nuclear). With endothelial cells, the medium also contained 25 μg HDL and 25 μg LDL per ml. At the end of the incubation period, the medium was centrifuged for 15 min in a Beckman Microfuge

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centrifuge to remove cells and cell debris, and samples were frozen at −20°C until used. Incubations of Hep G2 cells and endothelial cells with [^{35}S]methionine were for 8 and 18 h, respectively, unless otherwise specified. To prepare serum-free conditioned medium of Hep G2 cells, confluent cultures were washed twice with DMEM, and incubated with DMEM, supplemented with 0.03% acid-treated BSA, for 48 h. Conditioned medium of endothelial cells was prepared in a similar way with M199 medium instead of DMEM and an incubation time of 30 h.

Blood was obtained from healthy donors and collected in sterile tubes containing hirudin (Pentapharm; 300 ATU/ml final conc.), heparin (Leo Pharm. Prod.; 10 units/ml final conc.) or EDTA (0.1% final conc.) as anticoagulant. Platelet poor plasma was prepared by two centrifugations for 20 min at 2000 × g and 6000 × g, respectively. Serum was prepared from freshly collected blood from the same subjects following blood clotting and using identical centrifugation methods.

Density-gradient ultracentrifugation of serum or radiolabelled medium from Hep G2 cells was performed as in [6]. Serum fractions were dialysed against RPMI-1640, and protein was measured as in [7].

For immunoprecipitation of apo A-I we used specific rabbit anti-human apo A-I antiserum (raised in our laboratory) and goat anti-rabbit IgG (Nordic Immunology). Maximum immunoprecipitation with anti-apo A-I was achieved by adding a predetermined amount of cold carrier HDL. Immunoprecipitation was also performed with a double antibody procedure by employing rabbit anti-apo A-I as primary antibody and goat anti-rabbit IgG as second antibody. Immunoprecipitates were collected by centrifugation for 5 min in a Beckman microfuge, washed 3 times with ice-cold RPMI-1640, and dissolved in the appropriate electrophoresis buffer.

SDS–PAGE was performed as in [8] with resolving gels of 10% acrylamide and stacking gels of 4% acrylamide. M_r markers (Bio-Rad) were used for calibration of the gel. For autoradiofluorography, the gel was treated with an autoradiofluorography enhancer (En^{3}hance, New England Nuclear) according to the manufacturer's instructions, dried, and placed on X-ray film (Kodak X-Omat) for 10–20 days at −90°C.

For polyacrylamide isoelectrofocusing, immunoprecipitates were mixed with 10 μg of carrier apo HDL (for reference), and dissolved in the lysis buffer used in [9]. Electrophoresis was essentially as in [10], using a 5% slab gel with a pH-gradient of 4–8 (2.5%, v/v, Ampholines, pH 4–6, and 2.5%, v/v, Ampholines, pH 6–8, LKB). Gels were prepared for autoradiofluorography as described above.

Here, apo A-I isoproteins were designated as in [3].

3. RESULTS AND DISCUSSION

Aliquots of [^{35}S]methionine-labelled culture medium from Hep G2 and endothelial cell cultures were reacted with anti-apo A-I antibodies, and the immunoprecipitates subjected to SDS-polyacrylamide gel electrophoresis (PAGE), followed by autoradiography. Fig.1 shows that reaction of anti-apo A-I with Hep G2 medium precipitated a protein of M_r 28000 that co-migrated with apo A-I from carrier HDL. No such protein could be detected with medium from endothelial cells, indicating that these cells do not secrete apo A-I or that secretion is below the detection limits of the procedure employed (0.05% of the protein synthesized). These results suggest that the presence of apo A-I in endothelial cells as observed by indirect immunofluorescence [11] is likely to be due to a (strong) interaction of exogenous apo A-I with these cells rather than to endogenous synthesis.

The immunoprecipitate obtained with Hep G2 medium was further characterized by isoelectric focusing (fig.2). The gel stained for protein (lane a) shows the positions of the carrier plasma HDL apoproteins, and the autoradiogram (lane b) shows the positions of the newly synthesized [^{35}S]methionine-labelled apo A-I. It can be seen that newly secreted apo A-I is primarily composed of isoprotein 2, which is more basic than the major plasma apo A-I isoprotein 4. This confirms the results in [3] where it was shown that isoprotein 2 is the pro-form of isoprotein 4, with a 6-amino acid NH_2-terminal extension (Arg-His-Phe-Trp-Gln-Gln). The two positive charges in the propeptide are consistent with the two-charge difference observed on isoelectrofocusing of isoprotein 2 as compared to isoprotein 4.

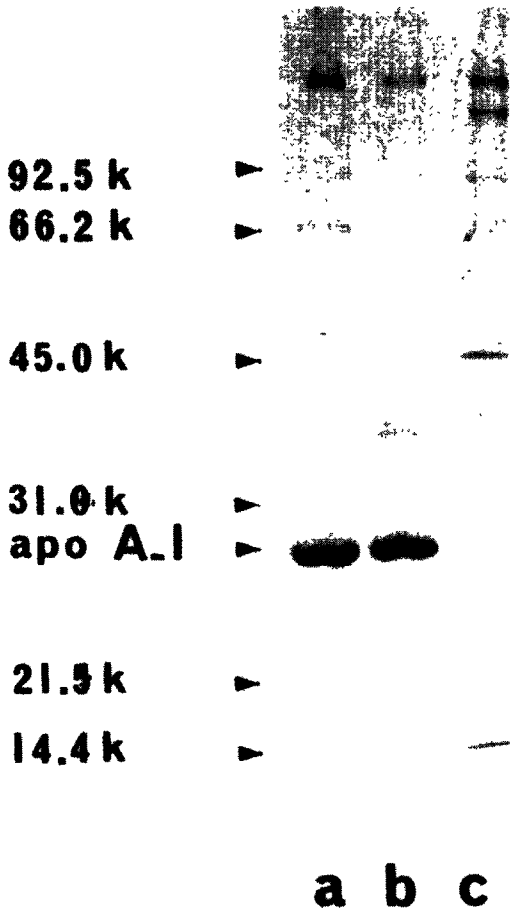

Fig.1. SDS–PAGE and autoradiography of immunoprecipitates obtained by mixing [^{35}S]methionine-labelled culture medium from Hep G2 and endothelial cells with anti-apo A-I antibodies. Lane a, immunoprecipitate of [^{35}S]methionine-labelled Hep G2 medium with rabbit anti-apo A-I as primary antibody and goat anti-rabbit IgG as second antibody; lane b, [^{35}S]methionine-labelled Hep G2 medium mixed with cold carrier HDL and immunoprecipitated with rabbit anti-apo A-I; lane c, [^{35}S]methionine-labelled endothelial cell medium, containing cold carrier HDL, immunoprecipitated with rabbit anti-apo A-I. The arrows indicate the electrophoretic mobility of the protein M_r markers and the position of human plasma apo A-I when the gel was stained for protein.

Analysis of [^{35}S]methionine-labelled Hep G2 cell-culture medium by density-gradient ultracentrifugation, followed by SDS–PAGE of the fractions, revealed that only a portion of newly synthesized apo A-I was present in fractions of density <1.21 g/ml, while the bulk of the labelled protein, including apo A-I, was in the density range >1.21 g/ml, as expected for protein not bound to lipid (not shown).

Protein synthesis by Hep G2 cells, measured by incorporation of [^{35}S]methionine into the 10% (w/v) trichloroacetic acid-precipitable fraction of the culture medium, gradually slowed down under serum-free conditions, and stopped after about 25 h (not shown). This finding permitted us to investigate whether converting activity is associated with Hep G2 cells, simply by extending the incubation period with [^{35}S]methionine from 8 h to 50 h. Fig.2, lane c, shows that prolonged incubation resulted in partial processing of isoform 2, indicating that Hep G2 cells produce converting activity.

Isoform 4 was also produced when Hep G2 medium was incubated with blood or plasma (containing hirudin or heparin), serum or endothelial cells (fig.2, lanes d–i). In all cases, processing was a slow process. Based on quantitation of radioactivity after isoelectrofocusing, the extent of conversion was about 50% after 50 h of incubation at 37°C in the presence of 10% (v/v) hirudin-plasma.

In contrast to hirudin- or heparin-containing blood or plasma, no conversion was observed with blood or plasma containing 2 mM EDTA (fig.2, lane j), which points to a metal-ion-dependent process. The lack of inhibition with 0.2 mM EDTA (fig.2, lane k) suggests that the activity is Ca^{2+}- and/or Mg^{2+}-dependent since only these cations occur in sufficient high concentrations in the media used (e.g., 0.4 mM in RPMI-1640) to escape complex formation with 0.2 mM EDTA.

Incubation of Hep G2 medium with density-gradient fractions of serum showed that the converting activity is, at least partly, associated with lipoproteins (fig.2, lanes l–p). It is not clear at this time whether the converting activity found in the LPDS fraction was artificially generated during density-gradient ultracentrifugation or can also exist free from lipoprotein under physiological conditions. Identical results were reported by authors in [12] who also provided evidence for lymph chylomicron-bound converting activity. As both plasma HDL and lymph chylomicrons bind proapo A-I as well, it was concluded by these authors that proapo A-I conversion takes place in, or on these lipoproteins. However, our data show that

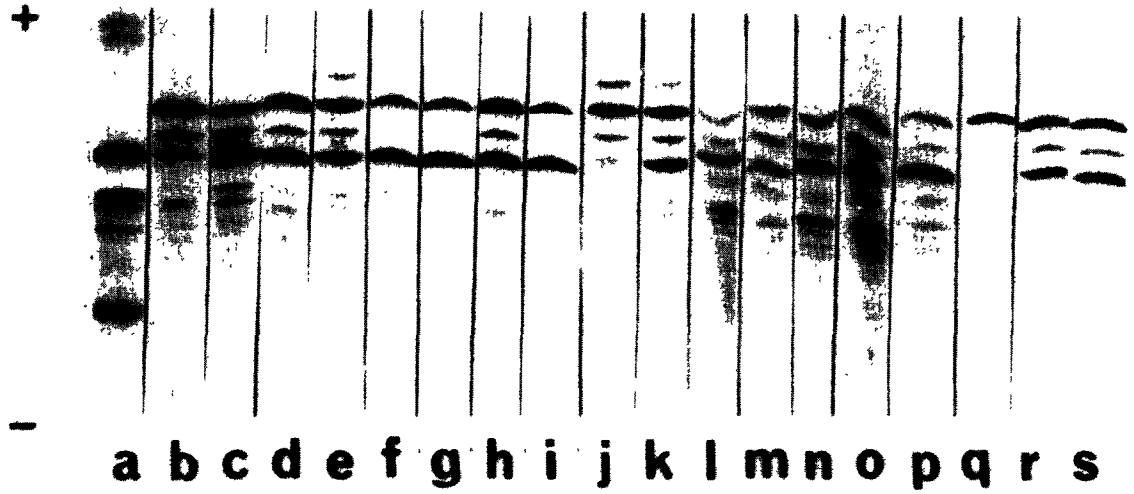

Fig.2. Isoelectrofocusing and autoradiography of immunoprecipitates of [^{35}S]methionine-labelled Hep G2 medium incubated with blood, plasma, serum or fractions thereof, or endothelial cells under various experimental conditions. Each sample was incubated with 25 μl of radiolabelled Hep G2 medium for 50 h at 37°C. Final concentrations are given in parentheses. In all cases, only the area in the vicinity of the apo A-I isoproteins is shown. Lane a, gel stained for protein; b, 8 h of incubation of Hep G2 cells with [^{35}S]methionine; c, 50 h of incubation of Hep G2 cells with [^{35}S]methionine; d, hirudin blood (20%, v/v); e, heparin blood (20%); f, hirudin plasma (10%); g, heparin plasma (10%); h, serum (10%); i, 0.32 cm^2 confluent endothelial cells; j, serum (10%) and EDTA (2 mM); k, serum (10%) and EDTA (0.2 mM); l, VLDL (67 μg protein/ml); m, LDL (533 μg protein/ml); n, HDL_2 (473 μg protein/ml); o, HDL_3 (655 μg protein/ml); p, LPDS (32 mg protein/ml); q, endothelial cells grown under serum-free conditions; r, endothelial cells grown under serum-free conditions followed by 48 h of incubation with 20% (v/v) serum; s, Hep G2 cells grown under serum-free conditions.

cleavage of the pro-segment also occurred when Hep G2 medium was incubated with Hep G2 cells or endothelial cells (fig.2, lanes c and i, respectively), even if only a fraction of the Hep G2-secreted proapo A-I molecules was part of lipoprotein particles. When the labelled Hep G2 medium was incubated with conditioned medium from Hep G2 cells or endothelial cells no conversion was observed (not shown), indicating that the converting activity expressed by these cells is cell-bound. These results suggest the possibility that apart from lipoproteins, the endothelium and hepatocytes may also provide a physiologically important surface for conversion of proapo A-I into the mature form.

In an attempt to clarify whether the endothelial cell-associated converting enzyme is synthesized by these cells, endothelial cells were washed extensively and, with daily renewal of the medium, were cultured for 50 h in M199-medium supplemented with 0.03% acid-treated BSA, 50 μg/ml LDL, 100 μg/ml ECGS, 10 ng/ml EGF, 10 μg/ml insulin, 10 μg/ml human transferrin and antibiotics, conditions under which cell growth was maintained [13]. At the end of the incubation period, the cells had lost their converting activity (fig.2, lane q), indicating that they do not synthesize the enzyme under these conditions. Subsequent incubation with 20% (v/v) serum for 48 h restored the cell-associated converting activity (fig.2, lane r), suggesting that the enzyme can be transferred from serum (lipoproteins) to the endothelial cell surface. Hep G2 cells were similarly treated and grown for 50 h in M199, supplemented with 0.03% acid-treated BSA, 50 μg/ml LDL and antibiotics, conditions under which protein synthesis was maintained. In contrast to the same experiment with endothelial cells, Hep G2 cells showed no loss of converting activity (fig.2, lane s). Unless the converting enzyme is more firmly bound to Hep G2 cells than to endothelial cells, this finding points to synthesis of the enzyme by Hep G2 cells.

In conclusion, our results show that the proapo A-I converting enzyme is Ca^{2+}/Mg^{2+}-dependent, is probably synthesized by Hep G2 cells, and occurs in association with the surfaces of lipoprotein particles, endothelial cells and Hep G2 cells. The functional significance of the proapo A-I-apo A-I system is still an open question.

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