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Identification of the inhibitor of the plasminogen activator as the major protein secreted by endothelial rat liver cells

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Freshly isolated Kupffer and endothelial liver cells exhibit a rate of 'de novo' protein synthesis which is twice as high per mg cell protein as that of parenchymal liver cells and contribute significantly (7.5% and 5.9%, respectively) to total liver protein secretion. In parenchymal cells the main secretory protein is a 68 kDa protein (containing 19% of the secreted radioactivity, presumably albumin). In Kupffer cells a 49 kDa protein contains 8% of the secreted radioactivity, while in endothelial liver cells a 55 kDa protein is the most prominent secretory protein (containing 11% of the secreted radioactivity). By aid of a specific antibody the 55 kDa protein was identified as the inhibitor of the plasminogen activator and in the liver this protein was only secreted by the endothelial cells.

Plasminogen activator inhibitor; Liver endothelium; Liver parenchyma; Kupffer cell; (Rat liver)

1. INTRODUCTION

The liver is a heterogeneous tissue and contains besides parenchymal cells also Kupffer and endothelial cells. Endothelial cells, which possess receptors for mannose-terminated glycoproteins [1] and modified LDL [2], and Kupffer cells, which have receptors for lactate-dehydrogenase-M4 [3] and native LDL [4], play an important role in the receptor-mediated endocytosis of macromolecules by the liver.

In addition to removal of proteins from the circulation, the liver is the major organ for the production of plasma proteins. Parenchymal cells are known to produce a variety of plasma proteins, with serum albumin as the main product. The contribution of endothelial liver cells to the production of secretory proteins by the liver is unknown, while only a few secretory proteins of the Kupffer cells, like erythropoietin [5], a cytotoxic factor [6] and monokines [7–9], are identified.

The general lack of information on protein synthesis and secretion by non-parenchymal liver cell types, stimulated us to determine protein synthesis and secretion in Kupffer and endothelial liver cells. These studies were performed in freshly isolated cells and not in cultured cells because protein synthesis in parenchymal cells changes upon culture [10–13]. Also changes in enzymatic make up during culture of non-parenchymal cells have been noticed [14].

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2. MATERIALS AND METHODS

L-[^{35}S]Methionine, ^{3}H(3,4)-valine and Amplify were from Amersham (England); collagenase (type I) was from Sigma Chemical Co. (St. Louis, MO); and metrizamide came from Nyegaard and Co. A/S (Norway).

Three types of liver cells (i.e. parenchymal, endothelial and Kupffer cells) were isolated from male Wistar rats (3 months old, weighing 200–300 g) by in situ perfusion of the rat liver with collagenase as described before [2]. Cells were incubated at 37°C in plastic tubes in a concentration of 2 mg cell protein per ml RPMI 1640 and incubation was performed in a rotating lab shaker. Cells were gassed every 30 min for 15 s with 95% O_2/5% CO_2.

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The rate of protein synthesis was determined by measuring the incorporation of ^{3}H-labeled valine (50 mCi/ml RPMI 1640 (valine concentration: 5 mM)) in cellular and secreted protein at the indicated incubation times. Cells and medium were separated by centrifugation (parenchymal cells 50 × *g*, 30 s, endothelial and Kupffer cells 500 × *g*, 5 min) and cellular and secreted proteins were precipitated with 10% TCA, washed three times with 10% TCA and subsequently counted for radioactivity.

For analysis of the cellular and secreted proteins, parenchymal, endothelial and Kupffer cells were incubated for 4 h in RPMI 1640 (methionine concentration: 16.7 μM) in the presence of 25–50 μCi [^{35}S]methionine. Cellular and secreted proteins were separated by centrifugation and applied to SDS-PAA gel as described before [15]. For fluorography, gels were shaken with Amplify for 30 min, dried by heating under vacuum and placed on an X-ray film (Kodak SB-5) at −78°C. Fluorograms were scanned on a Vitatron densitometer.

PAI was identified in the conditioned medium of endothelial cells as follows: ^{35}S-labeled secreted proteins from a 4 h incubation of endothelial, Kupffer and parenchymal cells were incubated for 30 min with a monospecific polyclonal antibody (raised in rabbits against the bovine plasminogen activator inhibitor [16]), which was coupled to protein A-Sepharose beads [16]. The ^{35}S-labeled protein which was bound to the antibody was removed by centrifugation and applied to a 10% SDS-PAA gel.

3. RESULTS

In the present study a rotating incubation system was used in order to keep the cells viable at prolonged incubation times (up to 6 h) and to prevent cell aggregation or adherence of the cells to the tube wall. Viability of the cells in the present system, as judged by trypan-blue exclusion and ATP content, was more than 95% during the used incubation times.

Fig.1 shows that during the incubation time a linear incorporation of valine in cellular protein is observed for the three liver cell types, whereas the valine incorporation in secreted proteins showed a lag-phase of 3/4 h. The rate of protein synthesis

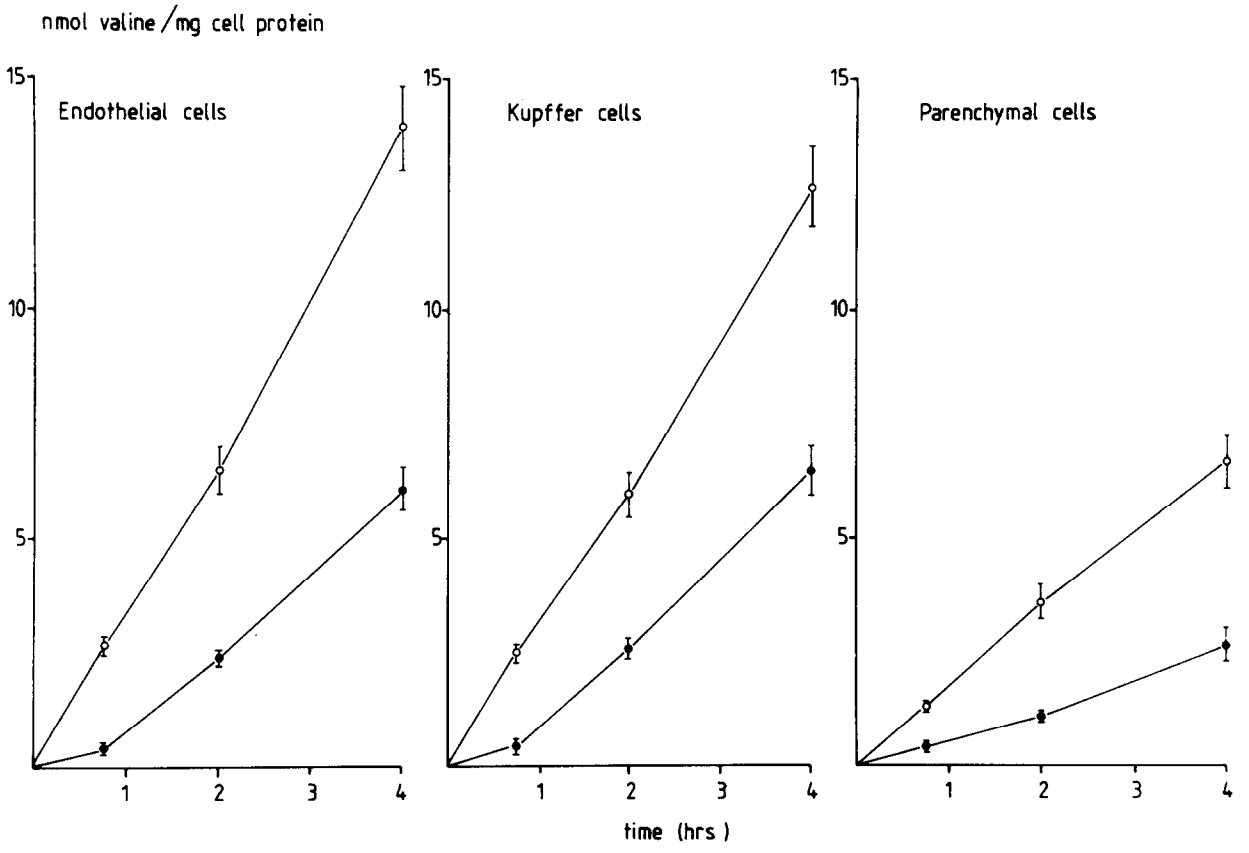

Fig.1. Incorporation of [^{3}H]valine in cellular and secreted protein by liver endothelial, Kupffer and parenchymal cells. The incorporation of [^{3}H]valine in cellular (○) and secreted proteins (●) was determined at various incubation times. Values are the mean ± SD for 4 experiments and are expressed per mg cell protein.

Table 1

The effect of inhibitors on protein synthesis and secretion of endothelial, Kupffer and parenchymal cells

	Endothelial cell		Kupffer cell		Parenchymal cell	
	Cellular	Secreted	Cellular	Secreted	Cellular	Secreted
Cycloheximide (5×10^{-6} M)	82.1 ± 3.9 (5)	80.8 ± 4.9 (5)	80.8 ± 3.9 (4)	82.3 ± 4.8 (4)	84.4 ± 3.0 (4)	86.6 ± 4.5 (4)
Colchicine (10^{-5} M)	1.3 ± 0.3 (3)	36.7 ± 3.3 (3)	0.4 ± 0.1 (3)	38.8 ± 2.9 (3)	2.8 ± 0.4 (5)	46.9 ± 3.3 (5)

The inhibitors were added to the liver cell incubations at the indicated concentrations at $t = 0$. The incorporation of [^{35}S]methionine in cellular and secreted proteins was determined after 4 h of incubation and compared to parallel control incubations (without effector). Values express the percentage inhibition (% ± SD), the number of experiments is given in parentheses

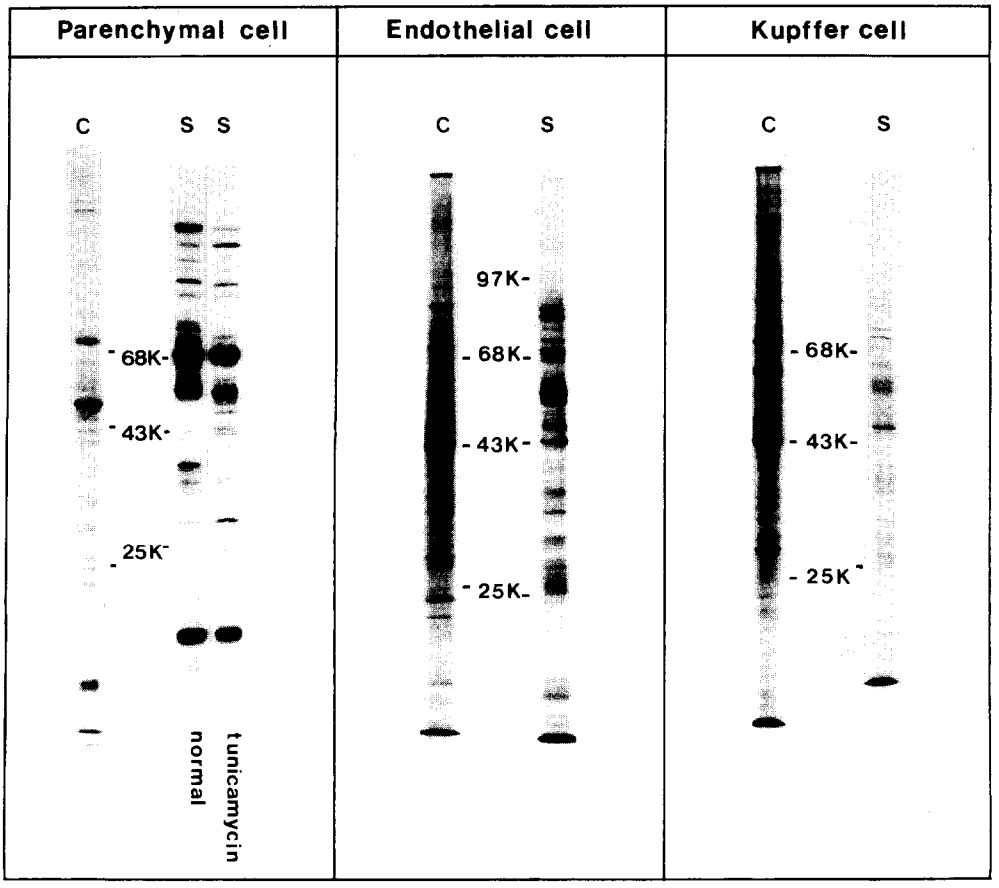

Fig.2. Analysis of the ^{35}S-labeled proteins in cellular and secreted proteins of parenchymal, endothelial and Kupffer cells from rat liver. Cellular ('lane C') and secreted ('lane S') proteins from a 4 h incubation of parenchymal, endothelial and Kupffer cells in the presence of [^{35}S]methionine (25–50 μCi) were analysed on a 10% SDS-PAA gel and visualised by fluorography. Parenchymal cells were also incubated in the presence of tunicamycin (10^{-5} M). Gels were calibrated with the following markers: phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa).

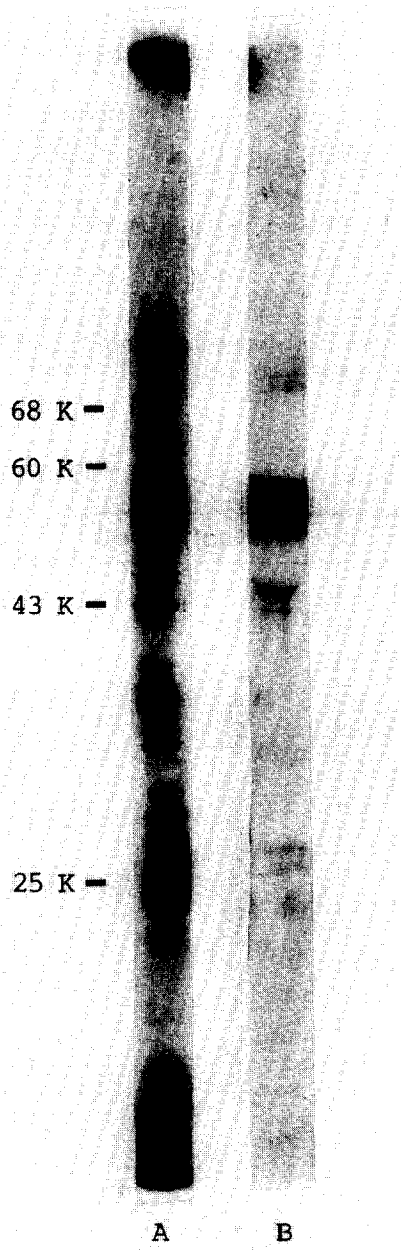

Fig.3. Identification of the inhibitor of the plasminogen activator in the conditioned medium of endothelial cells. ^{35}S-labeled secretory proteins from endothelial cells were incubated with an antibody, directed against the inhibitor of the plasminogen activator. Total (lane A) and precipitated (lane B) secretory proteins were applied to a 10% SDS-PAA gel. Gels were calibrated as described in fig.2.

(incorporation of valine in cellular plus secreted protein, expressed per mg cellular protein) was comparable in endothelial and Kupffer cells (5.68 and 5.32 nmol valine/mg cell protein per h, respectively) but twice as high as in parenchymal cells (2.32 nmol valine/mg cell protein per h). Cycloheximide and colchicine were used for further characterization of protein secretion and synthesis. Cycloheximide (5 μM) inhibited synthesis of cellular and secreted proteins in all three cell types nearly completely, whereas colchicine only reduced protein secretion (table 1).

Fig.2 shows the production of ^{35}S-labeled cellular and secreted proteins by the various liver cell types. With parenchymal cells at least 35 secreted proteins could be visualized while the major protein (M_r 67000) contained 18.7% ± 2.1% (n = 3) of the total amount of secreted ^{35}S-labeled proteins. Tunicamycin, which interferes with the glycosylation and the secretion of glycoproteins [17] influenced the secretion of several proteins, indicating a variety of glycoproteins among the secretory products in hepatocytes.

With endothelial and Kupffer cells (KCs) at least 50 different secretory proteins could be visualized. The major KC product was a 49 kDa protein, representing 8% ± 1.4% (n = 3) of the total amount of secreted material. A 55 kDa protein represented the most important endothelial cell product, containing 11.2% ± 0.9% (n = 3) of the secreted radioactivity. In the non-parenchymal liver cell types, the use of tunicamycin (10^{-5} M) had no effect on the pattern of protein secretion, while an increased dose (5–10 ×) had a lethal effect on these two cell types.

The main protein (M_r 55000) in the endothelial cell media was identified using a monospecific antibody, directed against the inhibitor of the plasminogen activator. This antibody interacted only with the 55 kDa protein (fig.3), while a control antibody did not precipitate any secretory protein of endothelial cells (not shown). The antibody directed against PAI did not precipitate any secretory protein of parenchymal or endothelial cells.

4. DISCUSSION

This paper describes that the procedure to isolate simultaneously parenchymal, Kupffer and

endothelial cells from liver by perfusion with collagenase at 37°C, delivers cells which can be used directly for measurement of protein synthesis and secretion, provided a carefully designed incubation system is used. The amino acid valine, which is neither degraded nor synthesized in the liver [19–21] was used at a high extracellular concentration (5 mM) so that the intracellular specific activity for [^{3}H]valine is identical to the extracellular specific activity for [^{3}H]valine [20,21] and a valid estimation of the rate of protein synthesis can be made.

The non-parenchymal cells appeared to be twice as active in the incorporation of [^{3}H]valine (per mg cell protein) into newly synthesized proteins as parenchymal cells. The rate of protein synthesis found in parenchymal cells (2.3 nmol valine/mg cell protein per h) is comparable with that reported before [22]. Cycloheximide and colchicine inhibition of protein secretion and synthesis (table 1) indicated that we were dealing with 'de novo' protein synthesis and secretion. The relative contribution of the various liver cells to total liver protein [23] was used to calculate that the non-parenchymal cells contribute significantly to the total secretion of proteins by the liver (together 13.4%, table 2). It may also be concluded that the sole source for some liver products will be the endothelial or the Kupffer cell.

The most prominent (19%) secreted protein in parenchymal cells, a 67 kDa protein, is presumably albumin. In agreement with Bauer et al. [24], we observed a large number (about 50) of proteins secreted by the Kupffer cells with an unidentified 49 kDa protein as major product. The number of secretory products of endothelial cells are also numerous (about 60) and quantitatively most important is a 55 kDa protein that contains 11% of the totally secreted radioactivity. For cultured bovine aortic endothelial cells PAI (M_r 52000) is described as a major secretory product which contains up to 12% of the secretory radioactivity [23]. In liver endothelial cells we were able to identify the major 55 kDa protein as PAI, by the use of a monospecific antibody. It may be speculated that the liver endothelial cells form an important source for PAI in the body.

Table 2

Relative contribution of the different liver cell types to the total liver secretion of newly synthesized proteins

Cell type	Relative contribution to total liver secretory proteins
Endothelial	7.5% ± 0.6%
Kupffer	5.9% ± 0.5%
Parenchymal	86.6% ± 7.8%

The amount of radioactivity ([^{3}H]valine) secreted by the various liver cell types was multiplied with the relative amount of protein that each cell type contributes to total liver protein

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