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# The clearance of apoptotic cells in the liver is mediated by the asialoglycoprotein receptor

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Apoptosing cells are actively phagocytosed in parenchymal tissues, thus preventing the inflammatory reaction which could derive from their slow uncontrolled degradation. The molecular mechanisms by which an apoptotic cell is recognized and taken up are largely unknown. We propose that the recognition of apoptotic hepatocytes is mediated by the sugar recognition systems of the liver, particularly the asialoglycoprotein receptor (ASGP-R). The results presented here demonstrate the participation of ASGP-R in the removal of apoptotic parenchymal cells, and indicate a new perspective for the understanding of its physiological role.

Programmed cell death; Apoptosis; Liver phagocytosis; Cell turnover

## 1. INTRODUCTION

Cell loss occurs naturally in living organisms by a genetically controlled process known as *Apoptosis* [1–3]. Physiological cell death is a phylogenetically ancient process which plays a key role in development and is essential in the removal of cells during the metamorphosis of insects and amphibia [3]. Furthermore, apoptosis occurs in adult mammalian tissues regulating their cell numbers [1–3]. Typical morphological features characterize cells undergoing apoptosis [1–2]. At the onset, single cells embedded in a tissue, loose the contacts with their neighbours; in the meantime the nucleus collapses showing condensed chromatin and the cytoplasm shrinks by condensation of proteins and loss of intracellular water. The cell surface looses microvilli, the endoplasmic reticulum and Golgi dilate and fuse with the plasma membrane resulting in a bubbly appearance. In the next phase, the cell breakes up into membrane-encapsuled 'apoptotic bodies'. As final stage the apoptotic bodies are actively phagocytosed by neighbouring cells or macrophages [1–6]. These progressive morphological changes in cells undergoing apoptosis require energy as well as RNA and protein synthesis [3] suggesting that there is a specific set of biochemical events which supports the process [3].

The process leading to the recognition and selective removal of dying self-cells is in line with these general properties of the apoptotic program [4–8]. Although, the mechanisms of identification of heterologous cells and molecules have been extensively studied, remarkably little is known as to how redundant constituents of self are recognized and eliminated. Since it has been reported that the surface of apoptotic cells exposes immature glycan structures [4–6], which are normally hidden by sialic acid moieties, we decided to investigate as to whether the asialoglycoprotein receptor is involved in the phagocytosis of cell dying by apoptosis.

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## 2. MATERIALS AND METHODS

### 2.1. *Cell cultures*

Primary cultures of neonatal liver cells were prepared from newborn rats (1 to 2-day-old) as previously described [9,10]. Viability of cells was routinely checked by Trypan blue exclusion, only those preparations in which hepatocyte viability was more than 95% were used.

### 2.2. *Localization by fluorescent lectin conjugates of sugars on the surface of isolated apoptotic envelopes and in primary cultures of neonatal rat liver cells*

Cells were isolated and cultured on collagen-coated slides as described [9,10]. After extensive washing in PBS, cells were fixed in 2.5% paraformaldehyde and then incubated at room temperature with lectin–fluoresceine isothiocyanate conjugates (Sigma, USA) (Concanavalin-A (Con-A, 40 mg/ml); *Phaseolus limensis* (120 mg/ml), Ricin (RCA-120, 2 mg/ml); Ulex (40 mg/ml)) for 30 min. Cells floating in culture medium were collected by centrifugation at 800 × *g* for 5 min and apoptotic envelopes were smeared on gelatin-coated slides and incubated with the lectin–fluoresceine isothiocyanate conjugates as described above.

### 2.3. *Immunohistochemical localization of ASGP-R on neonatal rat liver cells*

The immunohistochemical staining of neonatal rat liver cells in primary cultures was performed using as primary antibody the rat asialoglycoprotein receptor antibody raised in goat (1:100). After extensive washing in PBS, cells were fixed in 2.5% paraformaldehyde and the incubation with the primary antibody was carried out overnight


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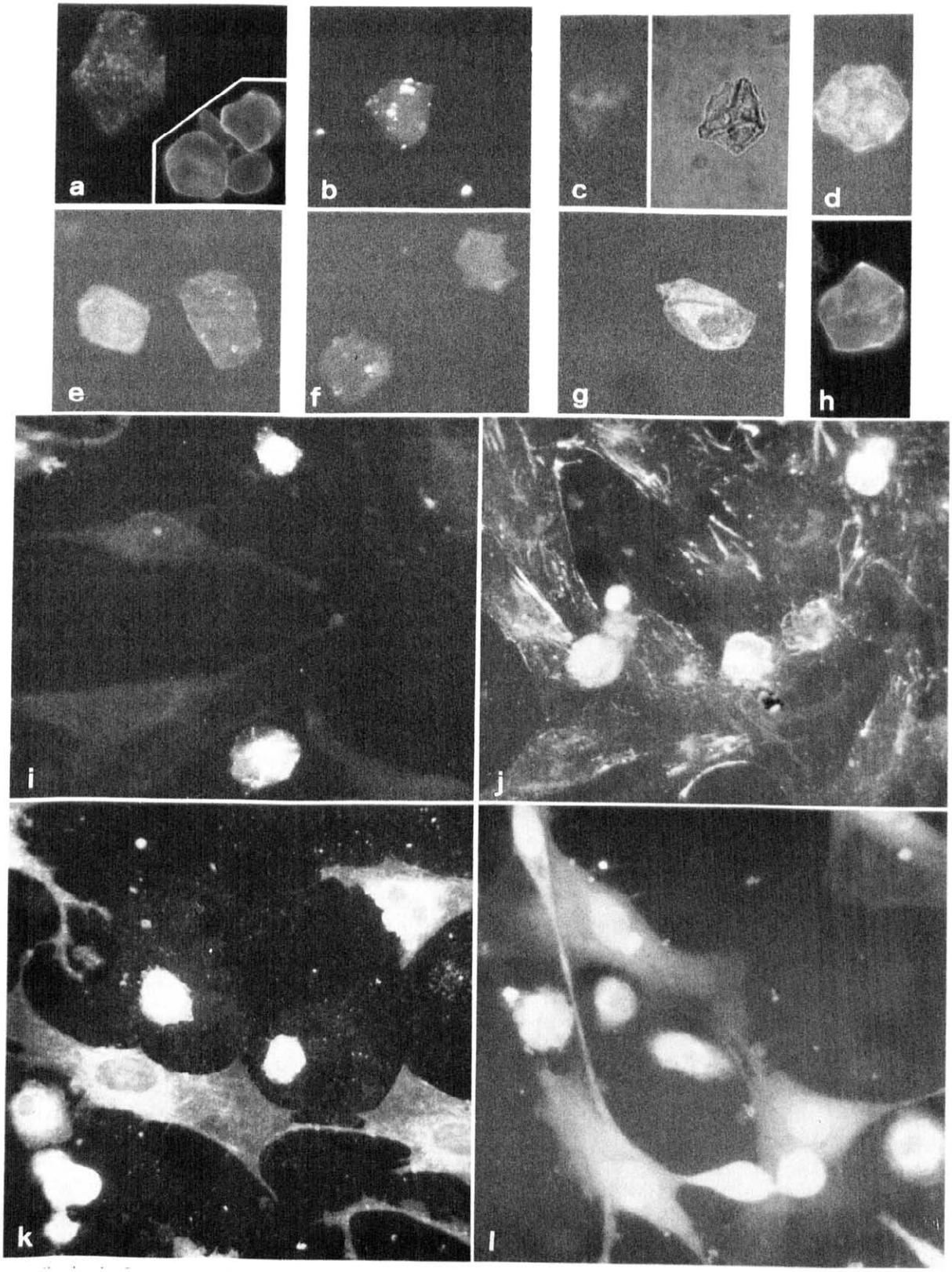


Fig. 1. Localization by fluorescent lectin conjugates of sugars on the surface of isolated apoptotic envelopes and in primary cultures of neonatal rat liver cells. Apoptotic envelopes were purified according to the procedure previously described [11]. They were smeared on gelatin coated-slides and then incubated at room temperature with lectin-fluoresceine isothiocyanate conjugates (a,e: Con-A (40 mg/ml); b,f: *Phaseolus limensis* (120 mg/ml); c,g: RCA-120 (2 mg/ml); d,h: Ulex (40 mg/ml) for 30 min. Fluorescence was analysed under a Laborlux D microscope (Leitz, Wetzlar, Germany). (a–d) Detergent-insoluble apoptotic bodies isolated from neonatal rat liver cells and (e–h) cultured 208F rat fibroblasts. Magnifications: a–h, 800×; i–l, 400×.

in a wet chamber at 4°C. The secondary antibody was a biotinylated mouse anti-goat IgG followed by a preformed avidin–biotin–horseradish peroxidase complex (ABC, Burlingame, CA). Endogenous peroxidase activity was blocked by methanol-$H_2O_2$.

2.4. *Inhibition studies*

Neonatal rat liver cells were grown at 37°C in cultures for 4 days in RPMI-1640 medium supplemented with 10% FCS. The different inhibitors were added every 20 minutes during the last culture hour at the following final concentrations: asialofetuin (ASF) 5 mg/ml; *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), galactose (Gal) and mannose, 200 mM; Arg-Gly-Asp-Ser (RGDS) and Arg-Gly-Glu-Ser (RGES), 1 mM; lactosylated bovine serum albumin (LacBSA), 5 mg/ml; anti-ASGP-R. After incubation the cells were extensively washed in PBS, fixed in 2.5% paraformaldehyde and stained with hematoxylin-eosin. The phagosomes were scored by counting their number over 4000 total cells using light microscopy. In order to avoid subjective bias, the counts were performed in duplicate by two different workers.

## 3. RESULTS AND DISCUSSION

The ASGP-R is expressed specifically on the plasma membrane of mammalian hepatocytes [12–14], thus we used primary cultures of neonatal rat liver cells as a model system for apoptosis. Neonatal rat liver cells after 4 days in culture are a mixture of hepatocytes and stromal cells [9]. The kinetic parameters of the two subpopulations are differently modulated by the growth factors present in the culture medium [9]. While the number of stromal cells increases linearly with culture time, the hepatocyte subpopulation is slightly reduced as a consequence of their intrinsic high rate of cell death by apoptosis [9,10]. Morphological analysis of the cultures reveals a large number of scattered apoptotic cells which are present as membrane-enclosed phagosomes or attached to the neighbouring cell surface during the recognition phase leading to their engulfment [9,10]. The advantage of this model is that it does not require heterologous addition of dying cells, since it already contains both the phagocytic and the target counterparts [9]. We have recently reported that mature apoptotic cells can be specifically isolated from an heterogeneous population of cells on the basis of their peculiar physico-chemical properties [11]. In fact, the specific $Ca^{2+}$-dependent activation of the cross-linking enzyme 'tissue' transglutaminase [15] leads to the formation of an intracellular highly cross-linked protein scaffold which confers to the dying cell unusual features of resistance to detergents and chaotropic agents [11].

To verify whether desialylated glycans are exposed on the surface of apoptotic cells we have tested 'apoptotic envelopes', isolated from neonatal rat liver and 208F fibroblast cultures, for their ability to react with fluorescent-conjugated lectins (Fig. 1a–h). This analysis demonstrates a cell type-specific exposition of sugar moieties on the surface of extracted apoptotic cells. While apoptotic cells isolated both from neonatal rat liver cells and 208F fibroblasts expose high levels of

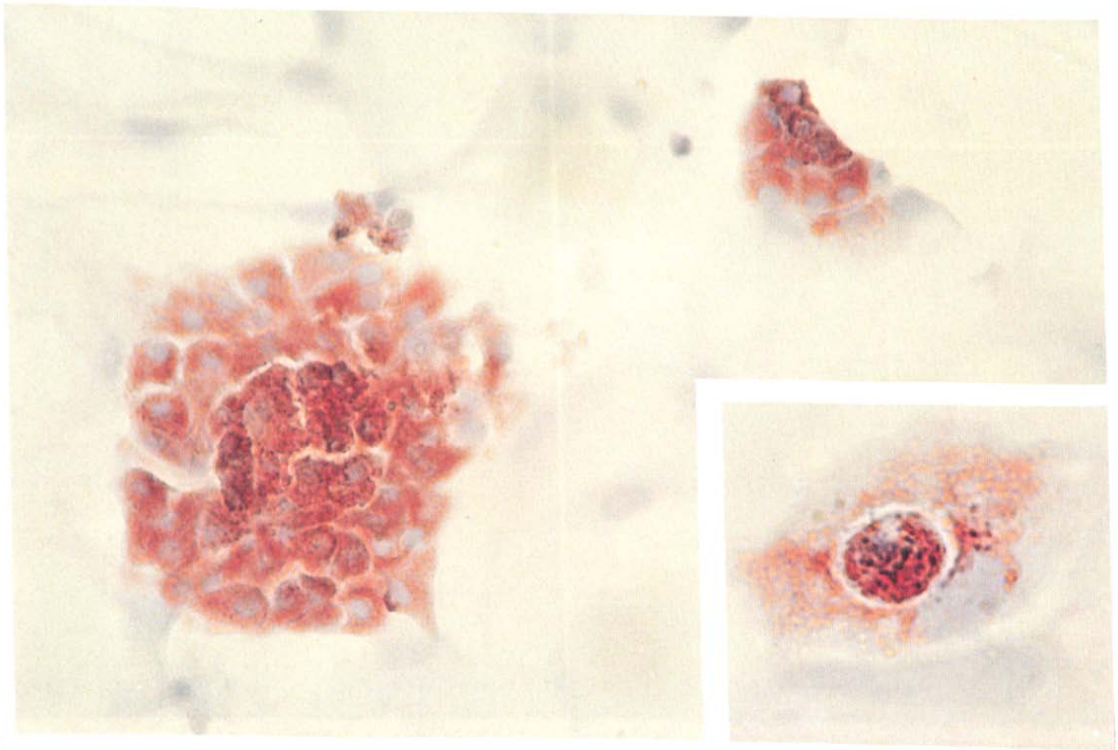

Fig. 2. Immunohistochemical localization of ASGP-R on neonatal rat liver cells. The immunohistochemical staining of neonatal rat liver cells in primary cultures was carried out as outlined in section 2. Note the intense staining in the colonies of rat hepatocytes interspersed among stromal elements on their 4th day of primary culture. In the inset a phagosome containing an apoptotic body stained with the ASGP-R antibody is shown. Negative controls in which the ASGP-R antibody was omitted did not result in any staining (data not shown) Magnifications: 300×; inset 1000×.

mannose and fucose, the GalNAc residues are specifically expressed on the surface of dying liver cells. Furthermore, apoptotic cells extracted from fibroblast cultures, show a high specific expression of galactose residues. To establish whether the extraction procedure affects the cell-surface sugar composition, we stained neonatal liver cells in primary cultures with the same lectin-conjugates. The fluorescence analysis reported in Fig. 1i–l confirms the relevant amount of desialylated glycan expressed on the surface of the shrunk apoptotic cells [7,8], indicating that the GalNAc and galactose expression takes place during the onset of the apoptotic program specifically. In contrast, the mannose and fucose residues are already expressed in the perinuclear region of the living cells. These observations suggest that the high concentration of these desialytated sugars on apoptotic body surfaces may be due to the exposition of the endoplasmic reticulum and/or Golgi content, deriving from their fusion with the plasma membrane during the apoptotic process [7,8]. These data seem to confirm that apoptotic cells undergo specific surface changes that make them susceptible to phagocytosis and that the sugar recognition systems of the liver may be involved in this process. In order to verify this hypothesis we investigated whether the asialoglycoprotein receptor protein is expressed in neonatal rat liver cells in primary cultures. The immunohistochemical localization of the ASGP-R shows that the receptor is specifically expressed on the surface of the hepatocyte islands being undetectable on the stromal cells (Fig. 2). It is noteworthy that the receptor is also localized on the surface of the apoptotic cells contained within the phagosomes (Fig. 2, inset). This confirms that the high apoptotic rate, present in the neonatal rat liver cell cultures, is due to the death of the hepatocyte subpopulation.

The involvement of the ASGP-R in the clearance of apoptotic cells is sustained by the ability of the specific antibody, GalNAc, D-Gal, Lac-BSA and ASF to block the uptake of apoptotic bodies by bystander cells (Fig. 3A). In fact, after the addition to the culture medium of the specific anti-ASGP-R, the appropriate monosaccharides and desialylated proteins reduced in all cases the clearance of dying apoptotic hepatocytes. The vitronectin receptor was recently indicated to have a major role in the recognition mechanism leading to the phagocytosis of aged neutrophils by monocyte-derived macrophages [4–6]. To verify whether in our system this receptor and/or other receptors of the integrins family were involved in the removal of apoptotic cells, the inhibitory action of the tetrapeptides Arg-Gly-Asp-Ser (RGDS) and Arg-Gly-Glu-Ser (RGES) was tested. Data (Fig. 3B) show that these receptors are not involved in the removal of dying hepatocytes by neighbouring cells, thus indicating that a cell-specific recognition system is present in liver cells, and that multiple receptors are used in a cell-specific manner in the removal of redundant cells of self.

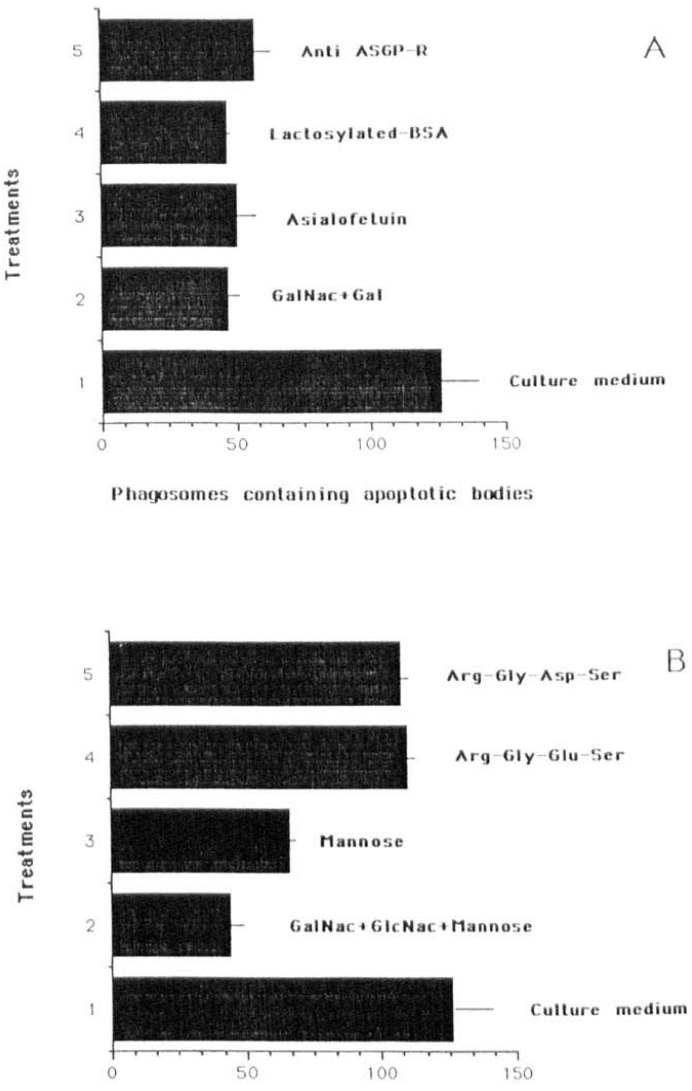


Fig. 3. Inhibition of phagocytosis of apoptotic cells in neonatal rat liver cells: (A) by anti-ASGP-R, ASF, GalNAc, LacBSA, (B) by the tetrapeptides Arg-Gly-Asp-Ser (RGDS) and Arg-Gly-Glu-Ser (RGES), mannose. Neonatal rat liver cells were grown at 37°C in cultures for 4 days in RPMI-1640 medium supplemented with 10% FCS. The different inhibitors were added every 20 minutes during the last culture hour as reported in section 2.

It is well known that liver cells express, in addition to the ASGP-R, the receptors for the recognition of GlcNAc/mannan terminating proteins [16]. Data reported in Fig. 3 indicate a cooperation among different receptors during the phagocytic process in the liver. In fact, the addition of mannan and GlcNAc to the neonatal liver cell medium is able to inhibit the clearance of apoptotic cells, also indicating the participation of mannose receptor in the uptake of dying cells. The cooperation of multiple receptors appears to be conceivable when the relative large size of an apoptotic cell is taken into account.

In this study we demonstrate that the phagocytosis of rat hepatocytes undergoing apoptosis is mediated by the

liver sugar recognition systems. The finding showing that the ASGP-R is involved in the removal of self-eliminated dead cells indicates a new perspective in the understanding of its physiological role. In fact, it must be recalled that apoptosis plays a key role in the steady-state regulation of cell numbers in tissues. In this connection it is worth mentioning that we detected a large increase in the receptor expression (data not shown) during the huge hepatocytes removal associated with the liver involuting phase occurring in rats upon injection of lead nitrate [17]. Moreover, the reduced cell renewal associated to the liver ageing process is paralleled by a significant decrease of the receptor expression [18,19], thus suggesting that the receptor expression may be modulated in a coordinate fashion with the onset of apoptosis.

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