The galactose-specific receptor system in rat liver during development

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Summary
The number and distribution of galactose-specific binding sites were investigated in rat liver cells during perinatal development. Ligand binding to hepatocytes, macrophages and endothelial cells was followed with in vitro and in situ experiments by electron microscopy, using lactosylated bovine serum albumin adsorbed onto 5 nm colloidal gold particles as ligand.

Binding capacity, starting at a late stage of fetal development, is very low both on the hepatocyte and on the macrophage surface, which show single particles statistically distributed. By contrast, bound particles are absent from fetal endothelial cells, which also lack the typical coated regions. In vivo, experiments at 37 °C show that endocytosis occurs to some extent in prenatal life.

These results indicate that the expression of galactose-specific receptors' activity on the different liver cell types follows different developmental patterns, which are independently modulated.

Key words: galactose-specific receptors, rat liver cells, liver development, hepatocyte, macrophage.

Introduction
The removal of plasma glycoproteins from the circulation and their catabolism are major hepatic roles; the binding occurs by means of specific carbohydrate-recognition systems located on hepatocyte surface, and is followed by internalization via coated pits and vesicles and by terminal lysosomal degradation (Ashwell & Morell, 1974; Ashwell & Harford, 1982; Schwartz, 1984).

Asialoglycoproteins (ASG), e.g. galactose-terminated glycoprotein receptors are by far the most widely studied. The receptor–ligand pathway has been mapped using various electron microscope tracers, both in liver in toto and on isolated and cultured rat hepatocytes (Wall, Wilson & Hubbard, 1980). Recently, several prelysosomal compartments, collectively termed endosomes, have been identified and in many cases biochemically characterized (Wall & Hubbard, 1985; Wileman, Harding & Stahl, 1985). In these endocytic compartments the dissociation of receptor–ligand complexes occurs, thus enabling the ASG receptor to escape degradation and to recycle to the cell surface, whereas the ligand is subsequently degraded within lysosomes (Geuze, Slot, Strous, Lodish & Schwartz, 1983).

This system has been well characterized and the hepatocyte receptor molecule, an integral membrane glycoprotein composed of variable numbers of subunits and expressed at the sinusoidal surface of the cell, has been isolated from rabbit, rat and human liver. There exists, however, a large intracellular pool of ASG receptors, mainly associated with the endocytic organelles and Golgi complex, whose exact role is not yet well understood (Weigel & Oka, 1983). It should in fact be emphasized that, although the role of the hepatic galactose-binding receptor in circulating ASG clearance is well documented, this is probably not its true (or at least not its only) physiological role.

Recently it has been shown in the adult rat that the hepatocyte is not the only liver cell expressing a galactose-specific receptor activity and that receptors of similar binding specificity are found on the surface of liver macrophages and endothelial cells (Kolb-Bachofen, Schlepper-Schäfer & Vogell, 1982). The macrophage and endothelial cell receptor is, however, structurally and immunologically different from the hepatocyte receptor (Schwartz, 1984; Kempka & Kolb-Bachofen, 1985; Roos, Hartman, Schlepper-Schäfer, Kolb & Kolb-Bachofen, 1985). Binding sites, both in isolated cells and in the intact organ are,
Moreover, arranged in strikingly different patterns: single sites statistically distributed on the sinusoidal surface on the hepatocytes, clustered arrangements all over the cell membrane on liver macrophages and clustered arrangements restricted to coated pits on the endothelial cells. This receptor distribution results in different binding and uptake capacities, from molecules to large particulate ligands, as shown by experiments with gold-coated particles ranging from 5 to 50 nm (Kolb-Bachofen, Schlepper-Schäfer & Kolb, 1983; Kolb-Bachofen, Schlepper-Schäfer, Roos, Hülsmann & Kolb, 1984; Schlepper-Schäfer, Hülsmann, Djovkar, Meyer, Herbertz, Kolb & Kolb-Bachofen, 1986).

In a previous paper (Conti-DeVirgiliis, Dini & Russo-Caia, 1984) we investigated the onset of ASG-binding capacity on isolated fetal rat hepatocytes, but no qualitative data were obtained, owing to the cytochemical method employed. In the present work ligand binding of prenatal and postnatal liver cells was studied by electron microscopy of gold particles coated with galactose-exposing proteins, thus allowing a quantitative evaluation and also a topographical localization. In addition, with this technique we studied the receptor activity of the nonparenchymal cells (i.e. macrophages and endothelial cells), because it seemed interesting to know when the adult arrangement of binding sites, which is different for each cell type, is reached during the development. We also made some in vivo experiments, in order to test the internalization capacity of prenatal liver cells.

Materials and methods

Conjugate preparation
Colloidal gold solutions, with particles approximately 5 nm in diameter, were prepared by reduction of chloroauric acid (Merck) with white phosphorus. The gold particles (Au₅) were coated with lactosylated bovine serum albumin (LacBSA, Sockerbolaget) as described by Horisberger & Rosset (1977), with some variations (Schlepper-Schäfer, Kolb-Bachofen & Kolb, 1980). Saturation of coating was found with 8 μg LacBSA ml⁻¹ Au₅. One Au₅ particle bound three to four LacBSA molecules. The particle content of standard protein–gold complexes was between 4–6×10⁶ particles ml⁻¹. This ligand concentration was used in all experiments.

Cell preparation and incubation with ligand
Isolated adult liver cells were obtained from male Wistar rats anaesthetized with Farnmotil (Farmitalia) 10 mg 100 g⁻¹ b.w. and perfused with collagenase (Boehringer) according to the method of Moldeus, Högborg & Orrenius (1978). Fetal livers were obtained from pregnant females; the fetal age was calculated from the appearance of the vaginal plug and checked by weight and length. Fetal and neonatal cells were isolated according to the procedure described elsewhere (Conti-DeVirgiliis, Dini, Di Pierro, Leoni, Spagnuolo & Stefani, 1981).

Hepatocyte viability was evaluated by the Trypan blue exclusion test and further assessed by electron microscope examination. Enriched fractions of macrophage and hepatocytes were obtained from the liver cell suspension by differential centrifugation (Schlepper-Schäfer et al. 1980).

Freshly isolated cells (2×10⁶ ml⁻¹ of Eagle's medium pH 7.4, containing 20 mM CaCl₂) were incubated with 0.05 ml of standard protein–gold complex solution for 10 min at 0–4°C. Binding reaction was stopped by adding an equal volume of cold 0.2% glutaraldehyde in s-collidine buffer, pH 7.4. The cells, washed with Eagle's medium to remove the excess of ligand, were postfixed with 2% OsO₄ in s-collidine buffer, dehydrated and embedded in epoxy resin, according to Spurr (1969), for electron microscopy. Ultrathin sections were obtained with an ultramicrotome LKB Ultratome III, stained with uranyl acetate and lead citrate, and examined under a Philips EM 400T electron microscope. The number of gold particles per μm² of plasma membrane was counted on electron micrographs by a Tektronik 4051 computer equipped with a 4956 graphics tablet.

Binding specificity was tested in parallel experiments by adding 20 mM for the hepatocytes or 80 mM for the macrophages (final concentration) of N-acetyl-d-galactosamine (GalNAc) during the incubation time.

Rosette-formation assay
Binding assays were performed as described by Schlepper-Schäfer et al. (1980). Each determination was made in double; isolated hepatocytes were considered positive when binding four or more neuraminidase-treated erythrocytes. The assay specificity was tested by a pretreatment of hepatocytes with 25 mM N-acetyl-d-galactosamine (GalNAc), 25 mM d-mannose, 25 mM N-acetyl-d-glucosamine (GlcNAc).

In situ and 'in vivo' experiments
For in situ experiments, livers from day-22 fetuses, from animals 1 day after birth and from adult animals were perfused for 3–5 min in a non-recirculating system with ice-cold Eagle's medium at a flow rate of 3–4 ml min⁻¹, to remove the blood. Standard Lac BSA–Au₅ conjugate solution was injected into the perfusion medium (0.05 ml for fetal and newborn rats, 0.4 ml for adults, allowing for the different liver sizes) after a brief fixation with 1 ml of 0.1% glutaraldehyde in s-collidine buffer at 0°C; a perfusion with Eagle's medium containing 1% BSA was made for 2–5 min to saturate free aldehyde groups, followed by a further fixation for 5 min with 0.5% glutaraldehyde. For inhibition studies 80 mM GalNAc was injected into perfusion tube before ligand administration.

For in vivo experiments 0.1 ml of ligand was injected into the umbilical vein of fetuses at day 22 of intrauterine life. After 3–5 min the liver was perfused for 3–5 min with Eagle's medium at 0°C to remove the blood and then fixed with 0.5% glutaraldehyde in s-collidine. Livers, cut into small pieces, were subsequently processed for electron microscopy as described above.
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Fig. 1. Low magnification of in situ fetal liver (day 22) treated with LacBSA-Au<sub>5</sub> injected into the umbilical vein after a brief prefixation of the organ with 0.1 % glutaraldehyde in s-collidine buffer to prevent internalization of the ligand. A good tissue preservation can be seen. The presence of many haematopoietic cells (o) among hepatocytes (h) may be observed. e, endothelial cells; m, liver macrophages. Bar, 1 μm.

Results

A comprehensive evaluation of the galactose-specific binding sites expressed on the surface of the main liver cell types during perinatal life was possible with the in situ experiments, in which the ligand was injected into the circulation after a brief prefixation of the organ, in order to avoid any internalization.

Fig. 1 shows a fetal liver at low magnification, treated as described above. A good tissue preservation can be seen: the various cell types are easily recognizable and the typical features of the fetal liver are evident. The presence of many haematopoietic cells may be seen among hepatocytes, which show many lipidic droplets and are devoid of microvilli in the space of Disse; endothelial cells are less flattened and do not show the presence of coated pits.

Fig. 2 shows higher magnification of fields in which the ligand–gold complexes are present. The binding activity expressed at the cell surfaces is extremely low in prenatal life, both in hepatocytes and in macrophages, as compared with the adult level (Fig. 3); a similar condition is observed in the liver of newborn animals (Fig. 4). The protein–gold complexes are present in the fetal hepatocytes as rare, single particles distributed on the sinusoidal surface and sometimes located on the coated regions (Fig. 2). Single granules may also be seen on the surface of the fetal macrophages.

Gold particles were observed on the endothelial cell surface only after birth, with the ligand mainly restricted to the coated regions, as in the adult; in fetal life receptor sites are totally absent. It should be noted that in fetal life coated regions are extremely rare; when present, they are completely devoid of ligand. Owing to the reshaping of the endothelial cells after liver dissociation, the gold particles could be observed in these cells only with in situ experiments.
Fig. 2. In situ liver of 22-day fetus. The binding activity expressed at the cell surfaces is extremely low. Photomicrographs show higher magnification fields in which ligand particles are present on hepatocytes (A,B), liver macrophage (C,D) and endothelial cells (E,F). h, hepatocytes; e, endothelial cells; m, liver macrophages. Bars, A,C,E, 1 μm; B,D,F, 0.1 μm.
Fig. 3. *In situ* adult liver treated with LacBSA-Au₅ injected into the portal vein after a brief prefixation (see Fig. 1). A high number of bound ligand particles is present with a typically different arrangement on the three cell types. h, hepatocytes; e, endothelial cells; m, liver macrophages. Bars, A, 1 µm; B,C, 0.1 µm.
Binding on plasma membrane of LacBSA–gold particles is inhibited by GalNAc for all the cell types considered (not shown).

A crude determination of binding activity was performed on hepatocytes isolated from term fetuses, newborn and adult rats by counting the number of

Fig. 4. In situ newborn liver. A low binding activity may be seen in endothelial cells (A) and in macrophage (B). h, hepatocytes; e, endothelial cells; m, liver macrophages. Bars, 0.1 μm.
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Fig. 5. Percentage of rosette-forming hepatocytes at different ages. Low values are observed in the perinatal life (F, fetal; N, newborn), compared with the adult (A) ones. Of the different sugars added only GalNAc has an inhibitory effect.

Fig. 6. Quantitative evaluation of binding sites on the surface of hepatocytes and liver macrophages isolated from fetal (F), newborn (N), and adult (A) rats. A statistically significant ($P < 0.001$) difference in the number of gold granules/$\mu$m of plasma membrane is observed only between perinatal and adult cells.

Discussion

The purpose of this work was to study the ontogeny of galactose-specific receptors in liver cells during the prenatal and postnatal development of the rat. We have therefore investigated by electron microscopy the presence and distribution of binding sites for rosette-forming cells (Fig. 5). A similar determination was not possible for isolated liver macrophages, owing to the difficulty of recognizing these cells at the light microscopic level in the nonparenchymal fraction of perinatal liver. The results do not show an increase of rosette-forming hepatocytes immediately after birth, the values of newborn being similar to those of fetuses and far from the adult ones. Preincubation with 25mM-GalNAc inhibits rosette formation, whereas the same concentrations of D-mannose or GlcNAc do not have this effect.

The quantitative evaluation of binding sites was performed on hepatocytes and liver macrophages isolated from term fetuses, newborn and adult rats by counting the number of bound gold granules per $\mu$m of plasma membrane (Fig. 6). This evaluation is possible only with $in vitro$ experiments, because only the isolated cells may be exposed to saturating concentrations of ligand.

The results show a small increase (not statistically significant) of binding sites after birth, while the increase of binding in the adult liver cells is highly significant ($P < 0.001$).

The ligand specificity was demonstrated by the inhibition of binding activity in presence of 20 mM (for the hepatocytes) or 80 mM (for the macrophages) of GalNAc.

The low binding activity observed in fetal and neonatal macrophages was also differently distributed. The bound particles were not observed in large aggregates as in the cells from the adult liver; in fact, they were found mainly as single granules and only a few clusters composed of two or three granules were sometimes observed.

$In vivo$ experiments demonstrate that prenatal hepatocytes and macrophages can internalize modified proteins coupled to gold particles of 5 nm diameter. Fig. 7 shows such particles in endocytic vesicles found in these cells, 5 min after intravenous administration of ligand at body temperature.
Fig. 7. Liver cells from a 22-day fetus perfused in vivo with LacBSA at body temperature for 5 min and then fixed with 0.5% glutaraldehyde in s-collidine buffer. (A) Hepatocyte showing an internalized coated vesicle with protein-gold granules. (B) Protein-gold granules are detectable in endocytic vesicles also in liver macrophages. Bars, 0.1 μm.

Ligand-coated gold particles on the surface of the hepatocytes, liver macrophage and endothelial cells in the perinatal life.

The results obtained from perfused livers in situ and from isolated cells in vitro are strictly comparable, indicating that the perfusion does not affect the binding sites distribution.

Galactose-specific binding sites are already present in late fetal life, on both hepatocyte and macrophage surfaces; interestingly, endothelial cells show, on the contrary, bound particles only after birth.

However, quantitative data obtained from isolated parenchymal cells and liver macrophages demonstrate a very low binding activity in fetal life; a small increase is observed immediately after birth, the binding levels of adult liver cells being reached only after the 15th day post partum, with a gradual rise between day 2 and 15 of postnatal development (Dini & Kolb-Bachofen, 1986).

Changes of hepatocyte receptor expression have already been observed during liver development. Hickman & Ashwell (1974) incidentally reported that rabbit and human fetal livers were essentially devoid of binding activity. In our previous study (Conti-Devirgiliis et al. 1984) on isolated fetal rat hepatocytes we found that the binding capacity for asialofetuin coupled to horseradish peroxidase was lacking before the 18th day of intrauterine life; it appears at this time and increases with developmental age. In mouse fetal liver (Collins, Stockert & Morell, 1984) binding activity for asialoorosomucoid was absent until the 19th day of gestation, steadily increasing in the neonatal period and reaching adult levels by the 5th day after birth. Petell & Doyle (1985) also reported a very low asialoorosomucoid-binding activity in fetal rat liver homogenate, with a rise at the time of birth and adult levels reached at the 2nd day post partum; the increased binding capacity is due to an increased synthesis of receptor polypeptides rather than to an activation of previously existing ones.

These results are in good agreement with some data obtained from adult liver, which also indicate a relationship between ASG-receptor activity and hepatocyte growth and differentiation. A loss of ASG binding has in fact been reported during the chemical carcinogenesis of the rat liver (Stockert & Becker, 1980), in rapidly dividing human hepatoma cells (Theilmann, Teicher, Schildkraut & Stockert, 1983) and after acute and chronic phenobarbital administration (Evarts, Marsden & Thorgeirsson, 1985); partial hepatectomy has a similar effect, with a return to normal following completion of regeneration (Howard, Stockert & Morell, 1982).
As regards the topographical distribution of binding sites on the surface of the liver cells, an interesting finding of our observations is that the preclustered arrangement of receptors, characteristic of adult liver macrophages, is not present in the perinatal life. The internalization of large particulate ligands is probably due to this arrangement (Kolb-Bachofen et al. 1984). Receptor distribution on fetal and neonatal hepatocytes surface is similar to that of the adult. In situ experiments have also allowed us to compare the binding sites pattern at different ages on endothelial cells, whose shape is deeply modified when they are isolated. Ligand binding, which after birth is mainly located in coated pits, is not present in fetal life; this also due to the rarity of coated pits in this period.

Our *in vivo* experiments have shown that, in spite of their low binding activity, fetal hepatocytes and liver macrophages are able to internalize small protein-gold complexes; it is therefore possible to hypothesize that the galactose-specific receptor system concerned with the internalization of molecules (e.g. with the ASG clearance) may already function in perinatal life, even though it may not be physiologically required.

Taken together, our observations demonstrate different developmental patterns of galactose-specific receptors in the different liver cell types. Binding sites are already present in late fetal life on the hepatocyte and macrophage surface and their number increases thereafter; by contrast, they are absent from fetal endothelial cells, being detectable only after birth. In the perinatal period the macrophage-binding sites, though present, are not arranged as in the adult liver. It can therefore be concluded that the expression of galactose-specific receptor activity is independently modulated during the rat liver development.

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