Bilirubin glucuronidation by intact Gunn rat fibroblasts expressing bilirubin UDP-glucuronosyltransferase

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INTRODUCTION

Conjugation of toxic compounds with glucuronic acid is a major detoxification route in mammals. UDP-glucuronosyltransferases (UGTs) catalyse this conjugation of usually hydrophobic molecules with glucuronic acid. In this process UDP-glucuronic acid (UDPGA) serves as the glucuronic acid donor [1]. Conjugation with glucuronic acid makes the molecules more water soluble and thus facilitates their excretion. The excretion of glucuronidated compounds from the cell is mediated by organic-anion transport proteins localized in the plasma membrane [2].

The UGTs are localized in the endoplasmic reticulum of liver, kidney and a variety of other epithelial cells [3,4]. Two different UGT enzyme families are present in humans. The UGT1 family is comprised of at least five different isoenzymes with identical C-termini. These isoforms are generated via a mechanism of alternative splicing and have transferase activity towards steroid hormones and bile acids [3,4] and are encoded on separate genes. A number of UGTs of families 1 and 2 have been cloned and expressed in cells [3,4].

Bilirubin is the toxic breakdown product of the haem group of proteins such as haemoglobin and cytochrome P450sa. The major detoxification pathway of bilirubin is glucuronidation in the liver and excretion via the bile and subsequently the faeces [6]. Crigler–Najjar (CN) syndrome is an inherited unconjugated hyperbilirubinaemia, caused by a deficiency of the hepatic enzyme bilirubin UDP-glucuronosyltransferase (B-UGT) (EC 2.4.1.17) [7]. The only defect in CN patients is the absence of B-UGT; excretion of conjugated bilirubin in bile is normal [7]. The Gunn rat, which has no detectable B-UGT activity, due to a mutation in the B-UGT gene [8], is an animal model for CN syndrome.

The transplantation of hepatoma cells [9] or freshly isolated normal rat hepatocytes [10,11] can reduce serum bilirubin levels in the Gunn rat. Transplantation of genetically transformed cells which are able to glucuronidate bilirubin should therefore also correct the hyperbilirubinaemia. When autologous cells, such as fibroblasts, are transformed and re-implanted, no rejection will occur. The obvious target cell for gene therapy for CN disease is the hepatocyte. Stable transfection of cells is only possible when the cells are dividing. To induce proliferation of hepatocytes it is necessary to perform a partial hepatectomy. Stably transfected liver cells can thus be obtained by isolation, transfection and re-implantation of the hepatocytes [12]. However, the percentage of transfected cells obtained with this method is low. The advantage of using fibroblasts is that these cells are more easily obtained and more efficiently transfected than hepatocytes. Furthermore, in contrast to hepatocytes, fibroblasts can be grown in cell culture.

Bilirubin glucuronidation in liver slices was first described in 1956 by Lathe and Walker [13] and later in more detail by Campbell and Dutton [14]. Senafi et al. reported that an immortalized lung fibroblast cell line, stably transfected with B-
UGT, was able to glucuronidate bilirubin [15]. Recently, Vienneau et al. [16] showed that Gunn rat fibroblasts (GURF) are able to glucuronidate benzo[a]pyrene.

Fibroblasts synthesize UDP glucuronic acid [17], are easily transfected with a gene of interest and can be re-implanted in the donor organism [18,19]. As an initial step in the development of extrahepatic gene therapy for CN syndrome, we have investigated whether primary GURF, stably expressing B-UGT, are able to glucuronidate bilirubin efficiently.

A primary GURF cell line, which stably expressed B-UGT at a level comparable to Wistar rat hepatocytes, was obtained by transduction with a retrovirus capable of transferring B-UGT cDNA. These cells were able to glucuronidate bilirubin added to the culture medium and to excrete the resulting bilirubin glucuronides into the culture medium. This indicates that the transduced fibroblasts are capable of performing the complete sequence of reactions necessary for bilirubin glucuronidation, including bilirubin uptake and the excretion of bilirubin glucuronides.

The B-UGT activities of freshly isolated Wistar rat hepatocytes and primary GURF, stably transfected with B-UGT, were comparable at 5–10 µM bilirubin, but at bilirubin concentrations of 20–80 µM the hepatocytes were more active.

MATERIALS AND METHODS

Chemicals

Dioleoyl phosphatidylcholine (DOPC) was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.), UDP-glucuronic acid, UDP-glucose, NAD+, acrylamide and bisacrylamide from Boehringer (Mannheim, Germany) and bilirubin, saccharolactone, essentially fatty-acid-free BSA, ascorbic acid and cell-dissociation solution from Sigma (St. Louis, MO, U.S.A.). Nitroblue tetrazolium, 5-bromo,4-chloro,3-indoyl phosphate, lactone, essentially fatty-acid-free BSA, ascorbic acid and cell-dissociation solution from Sigma (St. Louis, MO, U.S.A.).

Bilirubin was added, in 0.6 ml of medium, to a near-confluent layer of fibroblasts growing in 8 cm² culture dishes. The cells were incubated for up to 30 h with bilirubin before glucuronides were measured. As a control, the same incubations were performed with untransfected cells. All incubations were performed in quadruplicate and averages ± S.D. were calculated.

Hepatocytes from Wistar rats were isolated as described previously [20]. The cells were allowed to adhere to 8 cm² primary culture dishes for 4 h at 37 °C in DMEM containing 10% fetal calf serum, 50 nM dexamethasone and 135 nM insulin. The cells were incubated with bilirubin for 1.5 h immediately following this period.

After the incubation with bilirubin, media were collected and analysed for the presence of bilirubin glucuronides, by HPLC or the diazo assay, as described below. The culture dishes were washed with PBS and 1 ml of a solution of 20 g/l Na2CO3 and 4 g/l NaOH was added to lyse the cells for protein determination.

Preparation of cell homogenates

Fibroblasts were grown in 165 cm² culture flasks, washed with PBS and harvested with cell-dissociation solution. The cells were washed with PBS, resuspended in PBS containing 2 mM EDTA and lysed by five pulses with a probe sonifier. Hepatocytes were washed immediately after isolation, resuspended in PBS containing 2 mM EDTA and lysed with five pulses from a probe sonifier. The homogenates were stored for up to two weeks at −20 °C. Cytosolic and membrane fractions were prepared by centrifuging the homogenates at 100 000 g for 1 h.

Diazob assay of bilirubin glucuronides in culture media and in incubations with cell homogenates

Liposomes composed of DOPC were prepared in 10 mM Hepes (pH 7.8)/150 mM NaCl, by sonication [21], centrifuged at 4000 g for 10 min to remove titanium particles, stored at 4 °C under argon and used within two weeks.

Rat hepatocyte or GURF homogenates (0.5–1 mg of protein) were incubated with DOPC liposomes for 30 min at 4 °C before the assay. Concentrations in the reaction mixture were 50 mM Tris/HCl, pH 7.8, 5 mM MgCl2, 3.5 mM UDPGA, 1 mM 1,4-saccharolactone, 2.5 mg/ml DOPC liposomes and 75 µM bilirubin in a total volume of 600 µl. Incubations were performed under argon in the dark at 37 °C for 3 h.
Diazo reagent [22] was prepared by dissolving 0.1 ml of ethyl anthranilate in 10 ml of 150 mM HCl. 0.3 ml of 70 mM NaNO$_3$ was added and the mixture was incubated for 5 min. To eliminate excess NO$_3$, 0.1 ml of 88 mM ammonium sulphamate was added. The bilirubin glucuronide-containing solution, 0.6 ml of the culture medium or reaction mixture from experiments with cell homogenates, was mixed with 2 ml of 0.4 M glycine/HCl, pH 2.7, and 1 ml of the diazo reagent was added. After incubation for 30 min at room temperature, the diazo reagent was inactivated with 0.5 ml of 570 mM ascorbate. The azopigments were extracted with 2 ml of methylpropyl ketone/butyl-1-acetate (17:3 v/v) and the absorbance of the organic solvent layer was measured at 530 nm. The amount of bilirubin glucuronides was calculated assuming a molar absorption coefficient of 44400 M$^{-1}$ cm$^{-1}$.

**HPLC analysis of bilirubin glucuronides**

Media from cells incubated with bilirubin or incubation mixtures from the B-UGT assays with cell homogenates were extracted by a modification of an existing procedure [23]. A dash of ascorbic acid was added to 0.6 ml of enzyme assay mixture or culture medium; subsequently 1.4 ml of chloroform/ethanol (1:1, v/v) and 1 ml of 0.4 M HCl, adjusted to pH 1.8 with glycerol and saturated with NaCl, was added and the mixture was vortexed vigorously. The tubes were centrifuged at 2000 g for 5 min. The lower, coloured phase was aspirated and transferred to a glass tube. The chloroform was immediately evaporated under a stream of nitrogen. Samples were stored under argon in the dark at $-20^\circ$C for up to 2 days. The time needed from extraction to storage was less than 30 min.

The extract was dissolved in 100 $\mu$l of chloroform/ethanol (1:1, v/v) and 20 $\mu$l was injected immediately onto a HPLC system equipped with a chromsyp C$_{18}$, column. Bilirubin and bilirubin conjugates were separated using the system of Spivak and Carey [24]. The eluate was monitored at 450 nm using a diode-array detector. The identity of the peaks was confirmed by examining the spectra. For the detection of biliverdin, elution was monitored at 380 nm. The percentage of bilirubin conjugated was calculated by determining the integrated peak areas.

**Preparation of recombinant Moloney murine leukaemia virus**

The retroviral vector, MFG-S [25], developed by R.C Mulligan was obtained by courtesy of Dr. V. Dwarki (Somatix Therapy Corporation, Alameda, CA, U.S.A.). The cDNA encoding B-UGT was cloned as a 2100 bp Neol–BamHI fragment into the retroviral vector MFG-S, so that the translation initiation codon of B-UGT replaces the translation initiation codon of the retroviral env gene. The transcription of B-UGT is under the control of the retroviral LTR promoter. The MFG-S/B-UGT plasmid was cotransfected, using CaPO$_4$ coprecipitation, with a plasmid containing a neomycin resistance gene into amphotropic retroviral packaging lines. Transfected cells were selected by culturing in media containing G418, and screened for B-UGT integration by PCR. To increase viral titres, the CRE and CRIP cells were cocultured. This resulted in a 10-fold increase in viral titres.

**Transfection of GURF with retrovirus and selection of a GURF cell line expressing high B-UGT levels**

A population of GURF, expressing B-UGT at different levels, was obtained by incubation of GURF with culture supernatant from the retroviral packaging lines.

To obtain a cell line with high expression, the cells were subcloned as follows: the cells were digested with trypsin, diluted and seeded in 96-well culture plates. To aid the growth of the clones, each well was supplemented with 100 $\mu$l of GURF culture supernatant. After colonies appeared, 20 $\mu$lM bilirubin in culture medium was added and the cells were incubated for 24 h. The medium (100 $\mu$l) was transferred to another 96-well plate and tested for the presence of bilirubin glucuronides by performing a direct diazo assay. To each well, 20 $\mu$l of saline and 100 $\mu$l of diazo reagent was added. Diazo reagent for the direct bilirubin glucuronide assay was prepared by dissolving 1 g of sulphamic acid in 1.5 ml of 25 % HCl and adding 100 $\mu$l of a 10 mg/ml NaNO$_3$ solution. The plates were incubated for 5 min and the absorption at 540 nm was measured with an ELISA reader (Medgenix Diagnostics, Soesterberg, The Netherlands). The wells with the highest signal were expanded and the cell line with the highest expression was selected for use in further experiments.

**Immunofluorescence and immunohistochemistry**

Cells were grown on glass coverslips until approx. 50 % confluence. The cells were washed twice with PBS and fixed for 45 min with acetone/methanol (1:4, v/v). After fixation, the cells were washed once with 70 % ethanol in PBS and three times with PBS/0.05 % Tween. The coverslips were incubated for 45 min with monoclonal antibody directed against the human UGT1 family [26] in PBS/0.05 % Tween/10 % goat serum. The cells were then washed five times with PBS/0.05 % Tween. For immunofluorescence detection, goat anti-mouse immunoglobulins conjugated with fluorescein isothiocyanate were used. The cells were washed five times with PBS/0.05 % Tween and mounted with Slowfade. A Zeiss Axiosvert 135 fluorescence microscope (Oberkochen, Germany) was used to visualize the cells. Photographs were taken using a Contax 167 MT camera (Tokyo, Japan) on Ilford HP 5 film (Mobberly, Cheshire, U.K.). For the immunohistochemical detection goat anti-mouse immunoglobulins conjugated with peroxidase were used. The conjugate was detected in 50 mM NaCOOH buffer, pH 4.9, containing 0.2 mg/ml 3-amino-9-ethyl carbazole and 0.01 % H$_2$O$_2$. The coverslips were washed with distilled water and mounted in gelatine/glycerol. An Olympus Vanax-T microscope and an Olympus C-35AD-4 camera (Olympus Optical Co., Tokyo, Japan) were used to visualize and photograph the cells. Photographs were taken on Agfapan APX 25 film (Agfa-Gevaert, Leverkusen, Germany).

**Determination of bilirubin toxicity**

Transfected and untransfected GURF, and hepatocytes were seeded in 96-well plates. The plates used for the experiments with hepatocytes were first coated with collagen. Bilirubin at different concentrations was added, the control cells were treated with the same medium except that no bilirubin was added. Cell viability was assessed after 24 h by measuring mitochondrial dehydrogenases using the XTT cell-proliferation kit from Boehringer Mannheim, (Almere, The Netherlands).

**Glutathione S-transferase and UDP-glucose dehydrogenase assays**

Glutathione S-transferase was measured with 1-chloro-2,4-dinitrobenzene as acceptor as described in [27]. The specific activity of UDP-glucose dehydrogenase was measured as described previously [28]. The UDP-glucose-dependent formation of NADH was measured spectrophotometrically at 340 nm. Reaction mixtures contained 50 mM...
glycine/HCl, pH 8.7, 1.5 mM NAD⁺, 1 mM UDP-glucose and 70–250 µg of cytosolic protein.

Protein was determined according to the method of Lowry et al. [29]. PAGE and Western blotting were carried out according to standard techniques, as described previously [30].

RESULTS AND DISCUSSION

Retroviral transfection of GURF and selection of a clone with high B-UGT expression

The transformation of GURF with a retrovirus capable of transferring B-UGT cDNA yielded a population of cells which expressed B-UGT at different levels. Using immunofluorescence, we estimated that about 1% of the cells overexpressed B-UGT.

We subcloned this mixed population to obtain a cell line (GURF-B1) which expressed B-UGT at levels comparable to rat hepatocytes. B-UGT activity in GURF-B1 homogenates was 2.9 ± 0.2 nmol/h per mg of protein. B-UGT activity in rat hepatocyte homogenates was 3.4 ± 0.3 nmol/h per mg of protein, as measured by diazo assay (Figure 1).

The presence of B-UGT polypeptide in GURF-B1 cell homogenates was confirmed by Western blotting. A monoclonal antibody directed against the human UGT1 family, WP1 [26], detected a protein of 51 kDa in GURF-B1 cell homogenates. Untransfected cells showed no immunoreactivity (Figure 1).

Immunohistochemistry of the GURF and GURF-B1 cell lines was performed with the monoclonal antibody WP1. With low magnification it could be seen that all cells in the GURF-B1 line expressed B-UGT (Figure 2, panel 1); the untransduced cells were not stained (Figure 2, panel 2). To determine the subcellular localization of B-UGT, immunofluorescence of the GURF and GURF-B1 cell lines was performed with the monoclonal antibody WP1. The fluorescence pattern in the GURF-B1 cell line (Figure 2, panel 3) is characteristic for localization in the ER; the nuclear envelope and a reticular pattern throughout the cytoplasm can be seen. In the untransduced line only a weak background signal is seen (Figure 2, panel 4).

The expression level of B-UGT in the GURF-B1 line was stable for at least 7 months of continuous culture, and after more than 25 passages all cells were still positive for B-UGT. No selection pressure was applied during this period.

Bilirubin glucuronidation by intact GURF-B1 and Wistar rat hepatocytes

Bilirubin glucuronidation by intact GURF-B1 and Wistar rat hepatocytes was assessed by adding bilirubin to the culture media and analysing the culture media for bilirubin glucuronides by HPLC or diazo assay. Bilirubin solutions with an equimolar amount of BSA were diluted to the desired concentrations with medium containing 10% fetal calf serum. A molar excess of BSA over bilirubin was therefore present in all experiments. In cultures of GURF-B1, incubated with 40 µM bilirubin, the production of bilirubin glucuronides was linear for up to 24 h (Figure 3). In assays of B-UGT activity of GURF and GURF-B1 for up to 36 h, no bilirubin breakdown products such as biliverdin were observed when the media were analysed by HPLC (results not shown).

The B-UGT activities of freshly isolated Wistar rat hepatocytes and GURF-B1 were measured with the diazo assay at concentrations of 5–80 µM bilirubin (Figure 4). At 5–10 µM bilirubin the glucuronidating activities of GURF-B1 and hepatocytes were comparable, whereas at 20–80 µM bilirubin the hepatocytes were more active.

Toxic effects of bilirubin on GURF-B1

The toxicity of bilirubin for cultured cells is a well-known phenomenon: oxidative phosphorylation [31] and DNA synthesis [32] are thought to be affected by bilirubin. The cytotoxic effect of bilirubin was determined by incubating GURF and hepatocytes with bilirubin and measuring mitochondrial dehydrogenases as a parameter for cell survival. Bilirubin was toxic to

Table 1 Specific activities of B-UGT, UDP-glucose dehydrogenase and glutathione S-transferase in GURF and hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>B-UGT, cell homogenates (nmol/h per mg of protein)</th>
<th>Glutathione S-transferase, cytosol (µmol/min per µg of protein)</th>
<th>UDP-glucose dehydrogenase, cytosol (nmol/h per µg of protein)</th>
</tr>
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<tbody>
<tr>
<td>GURF</td>
<td>ND</td>
<td>0.16 ± 0.02</td>
<td>32 ± 2.8</td>
</tr>
<tr>
<td>GURF-B1</td>
<td>2.9 ± 0.3</td>
<td>0.11 ± 0.01</td>
<td>39 ± 2.3</td>
</tr>
<tr>
<td>Rat hepatocytes</td>
<td>3.4 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>160 ± 13</td>
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Bilirubin glucuronidation by intact Gunn rat fibroblasts

Figure 2  Immunohistochemistry and immunofluorescence micrographs of GURF and GURF-B1

Immunohistochemistry and immunofluorescence were carried out as described in the Materials and methods section. Panel 1 shows immunohistochemistry of GURF-B1 and panel 2 of GURF (magnification 20 ×). All GURF-B1 cells express B-UGT; no immunoreaction is seen in the GURF. Panel 3 shows immunofluorescence of GURF-B1 and panel 4 shows GURF (magnification 100 ×). The staining of the GURF-B1 is characteristic for the ER: the nuclear envelope and a reticular pattern throughout the cytoplasm can be seen. In the GURF, only background staining is seen.

Figure 3  Time-course of production of bilirubin glucuronides by GURF-B1

A 95% confluent monolayer of GURF-B1 was incubated with 40 µM bilirubin as described in the Materials and methods section. Media were analysed with a diazo assay. GURF were used as a control. A typical experiment is shown, the values are means ± S.D. of quadruplicate incubations. Production of bilirubin glucuronides was linear for up to 24 h.

Figure 4  Bilirubin glucuronide production by GURF-B1 and hepatocytes, at different bilirubin concentrations

Hepatocytes and GURF-B1 were incubated with different bilirubin concentrations and media were analysed with a diazo assay, as described in the Materials and methods section. Incubations with hepatocytes were 1.5 h and incubations with GURF-B1 were 17 h. A typical experiment is shown, the values are means ± S.D. of quadruplicate incubations for : (●) hepatocytes; and (▲) GURF-B1. B-UGT activities are comparable at concentrations of 5–20 µM bilirubin but hepatocytes have higher B-UGT activity at concentrations of 40–80 µM bilirubin.

Glutathione S-transferase activity in GURF-B1, GURF and Wistar rat hepatocytes

Glutathione S-transferase alpha subclass, or ligandin [33], is a...
Bilirubin uptake by GURF

The fact that GURF-B1 cells are able to glucuronidate bilirubin indicates that bilirubin enters the cells. This is not surprising since it has been shown that bilirubin bound to albumin spontaneously associates with membranes [35,36]. From the immunofluorescence micrograph (see Figure 2) it can be seen that B-UGT is distributed throughout the cytoplasm. When bilirubin is associated with the outer leaflet of the GURF membrane it may flip-flop to the inner leaflet and diffuse to the ER membrane which is in close proximity. It has been shown in liver that ER membranes have a higher affinity for bilirubin than plasma membranes [37]. Thus, bilirubin could become available to the active site of B-UGT by passive diffusion. However, the presence of a bilirubin carrier protein in plasma membranes of GURF cannot be excluded on the basis of our experiments.

UDP-glucose dehydrogenase activity in GURF-B1, GURF and Wistar hepatocytes

To determine the amount of UDPGA in hepatocytes and GURF we measured the specific activity of UDP-glucose dehydrogenase, the key enzyme in the biosynthesis of UDPGA. Activities in Wistar hepatocyte, GURF and GURF-B1 cytosol were 160 ± 13, 32 ± 2.8 and 39 ± 2.3 nmol/h per µg of protein respectively (Table 1). Since the specific activity of UDP-glucose dehydrogenase in fibroblasts is only 20% of the specific activity in hepatocytes, the observed difference in bilirubin glucuronidation between hepatocytes and GURF-B1 at 20–80 µM bilirubin could be explained by lower UDPGA levels in fibroblasts.

Conclusions

Our results show that when B-UGT is expressed in fibroblasts, these cells are able to carry out the complete glucuronidation process. This includes the uptake of bilirubin, either by diffusion or carrier-mediated transport, and the excretion of bilirubin glucuronides. This last step must be mediated by carrier proteins since bilirubin glucuronides are too hydrophilic to diffuse over a membrane. Indeed the presence of an organic-anion transport protein has been reported in several cell types [2]. In a recent article Vienneau et al. [16] show that GURF express UGT activity towards benzo[a]pyrene. It seems likely therefore that if there are specific factors needed for the uptake and transport of UGT substrates, these factors are expressed in fibroblasts as well.

Although the specific B-UGT activities in cell homogenates of hepatocytes and GURF-B1 are comparable, the B-UGT activities of intact hepatocytes and GURF-B1 are only comparable at low (5–10 µM) bilirubin concentrations. At 20–80 µM bilirubin, hepatocytes are more active. Several factors can account for this difference. The specific activity of UDP-glucose dehydrogenase is lower in fibroblasts than in hepatocytes. Fibroblasts will therefore have lower concentrations of UDPGA available to act as glucuronic acid donor. The specific activity of glutathione S-transferase is also much lower in GURF than in hepatocytes. Since glutathione S-transferases of the alpha subclass can bind bilirubin and reduce the toxicity of bilirubin, it seems likely that the toxicity of bilirubin for GURF can be explained by the low concentration of glutathione S-transferases in these cells. Glutathione S-transferases could also participate in the transport of bilirubin from the plasma membrane to the active site of B-UGT. The lower concentrations of glutathione S-transferase in GURF-B1 could therefore also partially explain why the B-UGT activity in intact GURF-B1 is lower than the B-UGT activity in primary hepatocytes.

Our results also suggest that transplantation of GURF-B1 back in the Gunn rat may lead to lowering of serum bilirubin levels. This opens the possibility of extrahepatic gene therapy for CN syndrome.

Bilirubin glucuronidation by Wistar rat hepatocytes

At physiological bilirubin concentrations, B-UGT in hepatocytes operates far below saturation, as demonstrated by experiments in liver slices [13,14] and intact animals [38]. Our experiments also showed this overcapacity. All studies, ours included, have used only one substrate. In vivo, many different compounds will be glucuronidated simultaneously. The observed overcapacity with a single substrate may thus only reflect the total capacity of the liver for glucuronidation and export. In contrast to experiments with liver slices [13,14], we found that the B-UGT activity of intact hepatocytes approaches the B-UGT activity in homogenates of these cells. Because we have used monolayers of isolated hepatocytes, bilirubin is in contact with all cells. The diffusion of bilirubin to the cells to the middle of a liver slice could be a rate-limiting step.

UDP-glucose dehydrogenase activity in GURF-B1, GURF and Wistar hepatocytes

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