CELLULAR DISTRIBUTION AND HANDLING OF LIVER-TARGETING PREPARATIONS IN HUMAN LIVERS STUDIED BY A LIVER LOBE PERFUSION

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ABSTRACT:
We developed and tested a novel method for perfusing parts of human liver to study uptake and handling of drug-targeting preparations. These preparations, designed for the treatment of liver fibrosis in man, have been extensively studied in animals, but little is known about the uptake and handling by human livers. Human liver tissue was obtained from livers procured from multiorgan donors and from cirrhotic livers of patients. To assess tissue viability, perfuse glutamate-oxalacetate-transaminase (GOT), glutamate-pyruvate-transaminase (GPT), and lactate dehydrogenase (LDH) levels were determined. To assess tissue functionality, the uptake of taurocholic acid and phase I and II metabolism of lidocaine and 7-hydroxycoumarin were determined. Uptake of a drug-targeting preparation was studied with Dexa10-HSA, which is designed for targeting of dexamethasone to nonparenchymal cells in the liver. During a 90-min perfusion period, no elevation of either GOT, GPT, or LDH was found. Both healthy control livers and cirrhotic livers showed phase I and II drug metabolism and functional taurocholic acid uptake. Studies with Dexa10-HSA revealed that 60 min after administration, 40% of the dose had been taken up by control livers and only 5% by cirrhotic livers. In control livers, Kupffer and endothelial cells had taken up Dexa10-HSA, whereas in cirrhotic livers only Kupffer cells were responsible for the uptake. Viability parameters and liver function tests clearly showed the applicability of this method. In the perfusion set-up, we showed uptake of the drug-targeting preparation Dexa10-HSA by healthy and cirrhotic human liver tissue, although the distribution patterns differed. This demonstrates the need to study new concepts in (diseased) human tissue.

The concept of site-specific delivery of drugs has been developed to increase concentrations of a drug at a target site, thereby maximizing the therapeutic efficacy and simultaneously reducing side effects elsewhere in the body. This approach may not only increase the efficacy and safety of drugs; it can also contribute to the understanding of pathophysiological processes in the target organ by specific elimination of cellular responses. The treatment of liver fibrosis, for instance, is hampered by the low efficacy of the conventional drugs and the serious side effects of these compounds when administered chronically. Therefore, several liver-targeting preparations are currently being explored to avoid these problems (Hashida et al., 1995; Meijer, 1995; Meijer and Molema, 1995).

Liver fibrosis is characterized by the excess deposition of extracellular matrix components elicited by virus infections, chronic alcohol abuse, genetic disorders, or chronic exposure to hepatotoxic agents (Friedman et al., 1992). These stimuli may initiate a perpetuating process leading to a gradual deterioration of liver function, which may end in complete liver failure (Gressner, 1996; Gressner, 1998). The process is induced by the concerted action of many cell types and is regulated by multiple mediators. Inciting stimuli may damage hepatocytes and cause the activation of other resident hepatic cells (Kupffer, endothelial, and stellate cells) or infiltrating inflammatory cells (Alcolado et al., 1997; Friedman, 1998). Chronic activation of the inflammatory process leads to an irreversible accumulation of extracellular matrix. Since conventional treatment with hepatoprotective, anti-inflammatory, fibrinostatic, and fibrinolytic drugs has proven to be unsatisfactory, we are exploiting the concept of drug targeting. Targeting can be achieved by the coupling of drugs to (neo)glycoproteins that are specifically taken up by various target cells in the liver (Meijer and Molema, 1995). Specific uptake of lactosylated human serum albumin (HSA1) by hepatocytes, mannosylated HSA by Kupffer cells, and succinylated HSA by endothelial cells was already shown (Jansen et al., 1991; Meijer, 1995). Recently, Beljaars et al. (1999) showed specific uptake of mannose-6-phosphate-HSA by hepatic stellate cells. In addition, drugs can also be coupled directly to HSA to obtain site-specific delivery. We have previously shown cell-specific delivery of the potent anti-inflammatory drug dexamethasone to Kupffer and endothelial cells when coupled to HSA (Melgert et al., 2000). All carriers and conjugates

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However, have been tested in experimental animals, mostly rats. Very little is known about their uptake in humans or, more importantly, in cirrhotic patients. Since studying the kinetics of these preparations in man is not feasible at this stage of drug design, we aimed at the development of an in vitro method to study uptake, cellular distribution, and handling of liver-targeting preparations in human liver tissue. This article describes a new method of perfusing parts of a human liver, which we used to study distribution and handling of drug-targeting preparations in normal as well as cirrhotic liver tissue. After examining liver viability and functionality during a 90-min perfusion period, we studied the uptake and cellular binding of the drug-targeting preparation dexamethasone-HSA (Dexa10-HSA). For this preparation we found that it accumulated in endothelial and Kupffer cells of normal livers and only in Kupffer cells of cirrhotic livers.

**Experimental Procedures**

**Materials.** The following compounds were obtained from the indicated sources: bovine serum albumin from Organon Teknika (Boxtel, The Netherlands); lidocaine from Centrachemie (Etten-Leur, The Netherlands); sodium taurocholate from Fluka (Buchs, Switzerland); dexamethasone and dexamethasone disodium phosphate from Bufa (Hilversum, The Netherlands); 7-hydroxycoumarin (7-HC) from Sigma (St. Louis, MO); HSA from the Central Blood Laboratory (Amsterdam, The Netherlands); [3H(G)]taurocholic acid from PerkinElmer Life Science Products (Boston, MA); University of Wisconsin organ preservation solution (UW) from DuPont Critical Care (Waukegab, IL). Monoethyglycinexylidide (MEGX) and glycinexylidide (GX) were kind gifts from Astra (Södertälje, Sweden), and 7-HC glucuronic and sulfate conjugates were kind gifts from P. Mutch, Glaxo Wellcome Research and Development (Herts, UK).

**Liver Tissue.** Human liver tissue was obtained from parts of donor livers procured from multiorgan donors (control liver tissue (Con)) or from cirrhotic livers of patients undergoing liver transplantation (Cir). Consent from the legal authorities, from the patients (Cir livers) and from the families concerned (Con livers) was obtained. The donor livers were reduced to perform reduced-size transplantation. The remaining redundant liver tissue after bipartition, for which no recipient was available, was stored in cold UW solution until the start of the perfusion procedure, for which wedges of 10 to 60 g were used. Total cold preservation time varied from 6 to 39 h. The use of the redundant liver tissue was approved by the medical ethical committee of our institution.

In the case of the Cir livers, indications for transplantation were primary sclerosing cholangitis (3), primary biliary cirrhosis (3), Caroli syndrome (1), chronic rejection (1), autoimmune hepatitis (2), Budd-Chiari syndrome (1), and amyloidosis (1). After explantation of the cirrhotic liver, a wedge (10–60 g) was cut from the left lobe and perfused with cold UW solution. Warm ischemia time in these livers, defined as the time between explantation of the liver and perfusion of the liver wedge with cold UW solution, varied from 5 to 30 min. The perfusion was started within 6 h.

**Liver Lobe Perfusion.** Liver wedges were cannulated with one or two cannulas, depending on the size of the wedge, as was described by Groothuis et al. (1995) for isolation of hepatocytes. Perfusion flow was adapted to the size of each wedge to exclude the influence of wedge size on results of the perfusions. The cannulas were filled with ice-cold Krebs-Henseleit buffer (Olinga et al., 1998a), and the wedge was then placed in a cabinet at 37°C. In a recirculating mode, the tissue was perfused at 30 ml·min⁻¹ per canula with 220 ml of Krebs-Henseleit buffer, saturated with 95% O₂/5% CO₂ (carbogem) and containing 1% bovine serum albumin. After 30 min of perfusion, the compounds to be studied were added and 1-mL samples of perfusate were taken at the indicated time points up to 60 min. A schematic representation of the perfusion set-up is depicted in Fig. 1.

**Liver Viability during Perfusion.** To assess the viability of the tissue during the perfusion, perfusate samples were taken at the indicated times, after 30 min of perfusion, and analyzed for glutamate-oxaloacetate-transaminase (GOT), glutamate-pyruvate-transaminase (GPT), or lactate dehydrogenase (LDH). The content of these enzymes in the perfusate was determined according to routine laboratory procedures.

**Liver Function during Perfusion.** To assess the functionality of the tissue during perfusion, three types of parameters were measured: 1) the uptake of taurocholic acid as an example of active carrier-mediated uptake, 2) the conversion of lidocaine to MEGX and GX to study phase I drug metabolism, and 3) the conversion of 7-HC to 7-HC glucuronic and 7-HC sulfate to study phase II drug metabolism.

**7-Hydroxycoumarin conversion.** After 30 min of preperfusion of either Con or Cir liver wedges, taurocholic acid was injected into the perfusion system to a final concentration of 21 μM. A tracer amount of this amount consisted of [3H(G)]taurocholic acid (Sandker et al., 1994). At the indicated times the amount of radioactivity in the perfusate was counted in a liquid scintillation counter (Tricarb 4000 series, Packard, Groningen, The Netherlands) after addition of 3 ml of Hionic-Fluor (Packard) to 0.5 ml of perfusate.

**Calculation of the extraction ratio.** The extraction ratio for the whole liver (E_{(HL)}) was estimated as follows: the initial clearance (CL_{init}) in milliliters·minute⁻¹ by the liver lobes was calculated from the ratio of the initial disappearance velocity and the initial substrate concentration, multiplied by the perfusate volume, using the nonlinear curve-fitting program Multistat as described before (Melgert et al., 1998). From these data an intrinsic clearance (CL) for the liver lobes could be calculated according to the equation CL_{init} = CL_{HL}·[Cl_{HL}]/(Q_{HL} + CL_{HL}) in which Q_{HL} is the perfusion flow through the liver lobes (30 ml·min⁻¹ per canula). Assuming a liver weight of 1.5 kg, this Cl_{HL} could be extrapolated to a whole liver. The extraction ratio was then estimated according to the equation E_{(HL)} = CL/(Cl_{HL} + Q_{HL}) in which Q_{HL} is the plasma flow through the liver (750 ml·min⁻¹ in humans) (Blom et al., 1982; Sandker et al., 1994; Olinga et al., 1998b).

**Lidocaine conversion.** After 30 min of preperfusion of either Con or Cir liver lobes, lidocaine was added to the perfusion system to a final concentration of 5 mM. At the indicated times samples from the perfusate were taken. Protein present in the perfusate was precipitated by adding perchloric acid to a final concentration of 6%. After centrifugation, MEGX and GX amounts in the supernatant were determined by high-performance liquid chromatography analysis (Olinga et al., 1998a).

**7-Hydroxycoumarin conversion.** After 30 min of preperfusion of either Con or Cir liver lobes, 7-HC was added to the perfusion system to a final concentration of 500 μM. At the indicated times samples from the perfusate were taken. Protein present in the perfusate was precipitated by adding perchloric acid to a final concentration of 6%. After centrifugation, an aliquot of the supernatant was neutralized with 0.5 M K₂CO₃ to pH 7.0. The neutralized supernatant was centrifuged and analyzed by high-performance liquid chromatography for 7-HC, 7-HC glucuronide, and 7-HC sulfate (de Kanter et al., 1998).

**Precision-cut liver slices.** Precision-cut liver slices were also used to study...
lidocaine and 7-HC conversion. Liver slices (10–14 mg) were prepared in Krebs-Henseleit buffer supplemented with 25 mM d-glucose and saturated with 95% O₂/5% CO₂ at 4°C, using a Krumdieck tissue slicer as described earlier (Olinga et al., 1997b). Slices were incubated in Williams’ E medium supplemented with 25 mM d-glucose in 6-well plates at 37°C (Olinga et al., 1997a). Lidocaine and 7-HC were added in the same concentrations as used in the perfusion set-up. The metabolites were determined in the incubation medium after a 60-min incubation period as described above.

Uptake of Dexa₁₀-HSA. We have previously described the synthesis and characterization of this dexamethasone conjugate (Melgert et al., 2000). Uptake of Dexa₁₀-HSA was studied by adding a tracer amount of ¹²⁵I-labeled Dexa₁₀-HSA and by determining the amount of radioactivity left in the perfusate during perfusion. The conjugate was labeled with ¹²⁵I to a specific activity of 0.1 mCi/mg of protein via tyramine-cellobiose according to the method of Hysing and Tolleshaug (1986). After 30 min of preperfusion of either Con or Cir liver lobes, a tracer amount (1 × 10⁶ cpm) of radionated protein was added to the system. At the indicated times samples from the perfusate were taken and all proteins were precipitated by adding trichloric acid to a final concentration of 10%. After centrifugation, the amount of precipitable radioactivity in the samples was counted with a gamma counter (Riastar Gamma Counting System, Packard Instrument Company, Meriden, CT).

Statistics. Data are given as means ± S.E.M. Statistical significance of differences between results were calculated using the Student’s t test. Results were considered significant at p < 0.05.

Results

Liver Viability during Perfusion. Figure 2, A, B, and C depict the levels of LDH, GOT, and GPT, respectively, in the perfusion medium of both Con and Cir livers during a 60-min perfusion time after 30 min of preperfusion. Although the enzyme levels increased slightly in time, none of them were significantly elevated after 60 min. The levels of all enzymes in the perfusion medium of Cir livers were lower as compared with Con livers.

Liver Function during Perfusion. Taurocholic acid uptake. The membrane transport capability of the hepatocytes was tested with taurocholic acid in both Con and Cir livers and is shown in Fig. 3. In Con livers taurocholic acid was rapidly removed from the perfusion medium in the first 10 min after administration, with the level falling to about 35% of the administered dose. In the next 50 min, the level decreased at a slower rate further to about 20% of the dose. The extraction ratio of taurocholic acid in these livers in the first 5 min was calculated to be approximately 1.0. The same uptake pattern was found in Cir livers, although the uptake in the initial phase was much slower. The calculated extraction ratio for the first 5 min was 0.32 ± 0.28. After 60 min, the level of taurocholic acid had only decreased to 50% of the administered dose in these livers.

Lidocaine conversion. Phase I drug metabolism was tested with lidocaine. Functional hepatocytes convert this compound mainly to MEGX via cytochrome P450-mediated N-deethylation, which can further be metabolized to GX. Figure 4 shows the amounts of MEGX and GX produced by Con and Cir livers after a 60-min perfusion.
MEGX per gram of liver tissue as compared with perfused Con livers. Liver slices produced more MEGX and GX using liver slices generally use lidocaine biotransformation as a functional parameter (Olinga et al., 1997b). Liver slices produced significantly more MEGX and GX than slices of the same livers (836 ± 238 versus 3431 ± 1188 nmol · g⁻¹ of liver, respectively), although the difference was not significant (p = 0.05 versus Con).

The Uptake and Distribution of Dexa₁₀⁻HSA. Uptake of Dexa₁₀⁻HSA. Figure 6 shows the disappearance of a tracer amount of ¹²⁵I-Dexa₁₀⁻HSA from the perfusate during a 60-min perfusion period of Con and Cir livers. Con livers displayed a higher uptake rate as compared with Cir livers. After 60 min, 40% of the dose had disappeared from the perfusate of Con livers, whereas Cir livers only showed very little uptake. Of three Cir livers, only one showed some uptake (20% after 60 min). For the Con livers, the rate of uptake was highest in the first 15 min after administration and declined gradually.

Distribution of Dexa₁₀⁻HSA. After 60 min of perfusion with Dexa₁₀⁻HSA, a sinusoidal staining pattern for this conjugate was clearly visible in perfused Con and Cir livers, with no staining in hepatocytes. In both types of livers the conjugate was evenly distributed over the liver lobe, indicating that the lobes were well perfused. Using double immunohistochemical methods, the cells responsible for the uptake of the conjugate in Con livers could be identified as endothelial (Fig. 7A) and Kupffer cells (Fig. 7B). In Cir livers, however, mostly Kupffer cells appeared to have taken up the conjugate (Fig. 7C).

Discussion

The method described here to perfuse parts of human livers offers the possibility to study uptake and distribution of compounds in healthy as well as cirrhotic liver tissue. During a 90-min perfusion period (30 min of preperfusion plus 60 min of perfusion) we found no apparent loss of viability in both Con and Cir livers, as measured by the leakage of the enzymes LDH, GOT, and GPT. The basal levels of the liver enzymes were higher for the Con livers, but this may be explained by the fact that Con livers have less extracellular matrix and consequently much more hepatocytes per gram of liver tissue. This implies a larger reservoir of enzymes that can leak from the cells.

All tests indicated that the liver lobes were still functional. In accordance with the clinical condition of the patients, functional parameters for the Cir livers were significantly lower than for the Con livers. Assessment of taurocholic acid uptake revealed an extraction ratio for Con livers of nearly 1.0, indicating that the uptake of taurocholic acid was limited by the perfusate flow rate through the liver lobes. Cir livers (n = 4) as compared with MEGX formation by human liver slices from the same Con livers (n = 6) after 60 min of incubation.

7-HC glucuronide and sulfate formation after 60 min of perfusion of Con livers (n = 7) and Cir livers (n = 3) as compared with metabolite formation by human liver slices of the same Con livers (n = 7) after 60 min of incubation (*p < 0.05 versus Con).
liver. This corresponds well with data found in humans in vivo and in freshly isolated human hepatocytes (Sandker et al., 1994; Olinga et al., 1995). For the Cir livers, the uptake and processing of taurocholic acid by hepatocytes appeared to be the rate-limiting step, rather than the flow rate. Taurocholic acid is a bile acid taken up by the sodium-dependent taurocholate transport protein NTCP as well as the sodium-independent organic anion transporting polypeptide OATP and is subsequently secreted into bile (Sandker et al., 1994; Satlin et al., 1997; Koopen et al., 1999). In cirrhosis the hepatic uptake of bile acids is impaired (Reichen et al., 1987; de Caestecker et al., 1995), which was confirmed in our perfusion system. After approximately 15 min, taurocholic acid was disappearing from the perfusate at a very low rate. Since the perfused liver lobes do not have a separate outlet for bile, the taurocholic acid may either be secreted from the hepatocytes back into the perfusate, and/or bile fluid may mix with the perfusate, which will finally result in an equilibrium between uptake and secretion. For Con livers, this equilibrium in the medium was established at a level of 20% of the dose, whereas for Cir livers the level was 50%. Since Con livers have more hepatocytes per gram of tissue, they have a higher storage capacity for taurocholic acid (Kroeker et al., 1978); therefore, they will have less taurocholic acid in the perfusate at the equilibrium.

The hepatic lidocaine metabolism is often used in patients to test liver function. In general, the amount of MEGX formed after 15 min after a bolus dose of 1 mg·kg⁻¹ lidocaine is determined, and these data correlate well with liver function (Luketic et al., 1993; Sotaniemi et al., 1995; Testa et al., 1998). Lidocaine is metabolized by cytochrome P450-mediated N-deethylation to MEGX, which can subsequently be metabolized to GX (Parker et al., 1996). It is difficult to compare these in vivo data with the data from our liver lobe perfusions because of the differences in dose. However, the reduction of the MEGX formation (90% reduction) in cirrhotic patients as compared with healthy volunteers (Luketic et al., 1993; Sotaniemi et al., 1995; Testa et al., 1998) is in the same order of magnitude as found in our experimental set-up comparing Con and Cir liver lobes. We also compared MEGX formation in precision-cut liver slices from the same Con livers and found no significant differences between the perfusion system and slices, although the MEGX formation in slices seemed a little higher. Due to the amount of tissue available for the conversion of lidocaine, it was also possible to detect formation of GX by Con livers. In liver slices, GX formation is hard to detect because of the small amount of tissue used during incubation (about 14 mg). GX was not detectable in the perfusate of Cir livers.

The liver tissue in the perfusion set-up was still able to perform phase II conjugations. In both Con and Cir livers 7-HC was sulfated.
and glucuronidated. Again, Cir livers had a much lower metabolizing capacity as compared to Con livers. In patients with liver disease, glucuronidation is spared relative to oxidative drug metabolism (Furlan et al., 1999). However, in advanced cirrhosis this pathway may also be impaired substantially, and there is growing evidence that other conjugation pathways are impaired as well (Morgan and McLean, 1995; Choo et al., 1999). Our results confirm these ideas. Comparison of the perfusion data with data from liver slices of the same Con livers showed a much higher glucuronidation in slices compared with the sultafur, as measured by excretion of these metabolites in the perfusion and incubation medium. This might be explained by the preferential secretion of the glucuronic acid metabolite in bile (Mulder, 1986; Wang and Dickinson, 1998). In slices, metabolites normally secreted in bile will readily diffuse into the incubation medium (Olinga et al., 1998a), whereas in the perfusion set-up the bile is probably retained more within the liver lobe.

Since we found no apparent loss of viability or functionality of liver tissue due to the perfusion set-up, we subsequently used the system to study the uptake and distribution of the liver-targeting preparation Dexa$_{10}$-HSA in human livers. This conjugate has been shown to be taken up by Kupffer and sinusoidal endothelial cells in healthy and cirrhotic rats, most likely via the scavenger receptors (Melgert et al., 2000). We now demonstrate that this conjugate is also taken up by the target cells in human liver tissue, although there is a striking difference between Con and Cir livers. The contribution of endothelial cells in the uptake of Dexa$_{10}$-HSA in Cir livers was small as detected immunohistochemically, whereas in the Con livers these cells account for most of the uptake. Several possibilities explain this. First, the fibrotic process and capillarization of the sinusoids may have partly impaired the endocytotic processes in endothelial cells, and/or it may have changed the scavenger receptor density on the cell membrane (Martinez et al., 1996; Tamaki et al., 1996; Thiele et al., 1999). Second, the endothelial cells of the Cir livers may have been damaged more by the warm ischemia occurring only in Cir livers. This, however, is not very likely because the individual Cir liver lobe with the longest warm ischemic time had the highest uptake of labeled conjugate.

The uptake of Dexa$_{10}$-HSA by the human Con livers was found to be comparable with the uptake of this conjugate by rat livers in vivo. Studies with Dexa$_{10}$-HSA in rats showed the clearance of this conjugate to be 4.0 ml · min$^{-1}$ · kg$^{-1}$, which is approximately 0.1 ml · min$^{-1}$ · g of liver$^{-1}$ (submitted for publication). For human livers in the liver lobe perfusion set-up, the clearance can be estimated using the equation that states that the clearance is equal to the natural log 2 divided by the half-life of Dexa$_{10}$-HSA multiplied by its distribution volume. The distribution volume is equal to the volume of the perfusion set-up, which is 220 ml, and from Fig. 6 we calculated the half-life to be approximately 70 min. For liver lobes of 10 to 60 g the clearance can then be calculated to be 0.04 to 0.22 ml · min$^{-1}$ · g of liver$^{-1}$, which is in the same order of magnitude as found for rats in vivo.

In cirrhotic rats we found no impairment of the clearance of Dexa$_{10}$-HSA (submitted for publication). The human liver lobe perfusion, however, did show a reduced uptake of Dexa$_{10}$-HSA for human cirrhotic livers, which may be explained by the lack of endothelial uptake in these livers, which is in line with our immunohistochemical data showing little uptake in endothelial cells.

In conclusion, during a 90-min perfusion period of human liver lobes, we found no apparent loss of viability or functionality of liver tissue. Phase I and II drug metabolism were still operative, and the bile acid uptake function of the cells did not seem to be impaired. Using this new method we were able to test the concept of drug targeting with neoglycoproteins to nonparenchymal cells in human livers. The drug conjugate Dexa$_{10}$-HSA selectively accumulated in endothelial and Kupffer cells of Con livers, whereas in Cir livers it surprisingly only accumulated in Kupffer cells. This may still lead to relevant dexamethasone effects for the treatment of fibrosis since Kupffer cells are a major source of profibrotic cytokines. The difference in cellular distribution between healthy and cirrhotic livers has not been found in animal studies using rats, showing again that results from animal studies cannot be fully translated to the human situation. Further studies using the present perfusion set-up will include the effectiveness of targeted dexamethasone on endothelin-induced cell activation in human livers.

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