chapter 4

The capability of isolated hepatocytes and liver slices of donor livers to predict graft function after liver transplantation

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Abstract

Background In liver transplantation adequate function tests for donor livers and transplanted livers are of utmost importance to provide an objective basis for decision-making. Isolated hepatocyte and/or slice preparations from human donor liver tissue may be suitable to test the quality of the organ to be transplanted.

Methods Surgical waste material remaining after reduced size or split liver transplantation in children was used to prepare slices and isolated hepatocytes. The viability of these preparations as well as drug transport and metabolism functions were determined and related to graft function in 32 liver recipients.

Results The in vitro tests used in the present study apparently did not select non-viable livers. In vitro preparations of the primary non-function grafts occurred in the investigated group showed normal viability, metabolic and uptake function.

Conclusion These results indicate that either the presently used viability tests are not sensitive enough to detect potential organ failure or that other factors besides the hepatocyte viability at the time of transplantation are of paramount importance to the graft function of the recipient, such as complications during and after transplantation or the viability of the non-parenchymal cells.

Introduction

Liver transplantation is considered the treatment of choice for end-stage liver disease. Liver transplantation programs have expanded over the years, which indirectly has led to donor-liver shortage. Therefore, it is of vital importance to optimize the use of the available donor livers. Consequently, adequate function test(s) are needed to provide an objective basis for decision-making. However, such adequate function tests for donor livers are still lacking\(^1\) and this leads to unnecessary rejection of potential viable livers and the transplantation of non-viable livers, resulting in initial poor function or primary non-function. In some reports a relationship between lidocaine clearance, measured as MEGX test, in the donor and the graft outcome was shown\(^2,4\). However, others did not
find such a relation and therefore the MEGX test did not reach the status of “gold standard”. Recently, it has been investigated whether in vitro preparations of the human liver could be used as predictor of transplantation success. We showed that liver needle biopsies can be used to isolate hepatocytes and subsequently to study the uptake rate of taurocholic acid. However, this parameter did not seem to show correlation with graft function. In the latter study the number of experiments was rather low and no initial poor function or primary non-function of the transplanted livers occurred. In another study from our institute, the metabolism of lidocaine was determined in needle biopsies of human livers. The latter study showed a trend in predicting transplantation success. Groothuis et al. isolated hepatocytes from tissue obtained from reduced size or split liver transplantations and livers discarded for transplantation, but could not detect any differences in viability or yield of the cells. In this study the results of the experiments obtained with hepatocytes could not be correlated with the success of transplantation of the part of the organ that was transplanted, since no dysfunction of the transplanted grafts was observed in this particular group of patients. In the present study, residual tissue remaining after reduced size and split liver transplantations was used to prepare isolated hepatocytes and liver slices. In the liver slices, the original structure of the liver is retained. This integrated in vitro liver system may be a better predictor of the liver in vivo, than are isolated hepatocytes. The viability of these in vitro preparations was compared with the function of the transplanted graft in the host, up to 7 days after transplantation, in order to investigate if any of the tests performed in vitro could be related to graft function measured in the recipient. To give an indication of the viability of the cells present in the liver, the viability was tested by trypan blue exclusion and total yield of hepatocytes isolated from the human liver and the yield of viable cells after Percoll density centrifugation of the obtained cell suspensions was also determined. The slice viability was assessed by the measurement of potassium concentration in the liver slice. The energy status of the hepatocytes was studied by ATP concentration and the ability of the cells to synthesize ATP. The transport of the anionic bile acid taurocholic acid in human hepatocytes was studied to obtain information on the transport capacity of the hepatocytes in the human liver. The metabolism of lidocaine in hepatocytes and liver slices was used to assess the metabolic capacity of the hepatocytes in the donor organ. These in vitro measured parameters may also give more insight into the mechanisms of primary non-function.
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Materials and Methods

Materials
The following compounds were obtained from the indicated sources: collagenase P from Boehringer Mannheim (Mannheim, Germany); bovine serum albumin (BSA) from Organon Teknika (Boxtel, The Netherlands); sodium taurocholate from Fluka (Buchs, Switzerland). Lidocaine was obtained from Centrachemie (Etten-leur, The Netherlands); Percoll from Pharmacia AB (Uppsala, Sweden); \([3H(G)]\) Taurocholic acid (specific activity 3.5 Ci/mmol) from Du Pont NEN Research Products (Boston, USA); University of Wisconsin organ preservation solution (UW) from Du Pont Critical Care (Waukegab, Illinois, USA). Monoethylglycinexylidide (MEGX) was a kind gift of ASTRA (Södertälje, Sweden). The 24 well tissue-culture plates were obtained from Costar (Cambridge, MA, USA). All other chemicals were of analytical grade and were obtained from commercial sources.

Liver material
Human liver tissue was obtained from livers procured from multi-organ donors. Consent from the legal authorities and from the families concerned was obtained for the explantation of organs for transplantation purposes. The donors were 20 males and 13 females in the age of 2 to 63 years (median = 24 years).

The donor livers were split or reduced in volume in order to perform reduced size or split liver transplantation. The donor liver was perfused with cold UW organ storage solution in situ before explantation. The livers were stored in cold UW solution on ice until reduction of the liver. The reduction or splitting of the donor organ was performed while immersed in UW with ice slush. The median time period before a transplantation started was 13 hours after perfusion of UW in situ in the donor. The liver tissue remaining after bipartition was stored again in cold UW solution until the start of the cell-isolation/slicing procedure. Total cold preservation time for the in vitro preparations varied from 6-39 hours (median = 14.5 hours). Liver tissue from each individual was used to prepare both hepatocytes and liver slices. The research protocols were approved by the medical ethical committee of our institution.
Methods

Classification of transplant patients
Recipients were 19 males and 14 females in the age of 3 weeks to 26 years (median= 2.5 years). In all recipients routine liver function tests were performed after transplantation for seven consecutive days. Parameters included in this study were determination in serum of glutamate-oxal-acetate-transaminase (GOT), glutamate-pyruvate-transaminase (GPT), protrombin time (PTT) and anti-thrombin III (ATIII). In addition, the bile production was monitored as well as the amount of blood transfusions needed. Apart from these continuous variables, donor livers were grouped with regard to post-operative liver function according to the method as described by Ploeg et al. Initial poor function was defined as occurring in patients with serum GOT >2000 U/l, PTT >16 sec and ammonia levels >50 µmol/l on post-transplant days 2-7 and in addition all patients with primary non-function. Primary non-function was defined by Ploeg et al. as patient or graft loss in 2-7 days after transplantation. The definition of primary non-function is very strict and implies that also patients that might have suffered from graft loss or died as a result of causes not related to the liver function were included in the group of primary non-function. For instance: according to the definition a patient dying on day 5 from a ruptured splenic artery aneurysm will be classified as a primary non-function, despite the fact that the graft functioned perfectly well. In order not to obscure the possible relationship between our in vitro investigated parameters and graft function, we performed a second evaluation in which patients who died within one week from (in our view) causes evidently not related to the quality of the liver were considered as a separate group of PNF.

Preparation of human hepatocytes and human liver slices

Preparation of human hepatocytes
Human hepatocytes were isolated using a modification of the method as described earlier by Groothuis et al.; the biopsy wedge was cannulated with 2-4 cannulas as was described. The cannulas were filled with ice-cold modified Hanks balanced salt solution (HBSS) without Ca²⁺ (containing 112 mM NaCl, 5.4 mM KCl, 10 mM
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM glucose and 25 mM NaHCO₃, pH 7.42). The biopsy wedge was placed in a cabinet at 37 °C and was perfused single pass for 10 min with modified HBSS without Ca²⁺, saturated with 95 % O₂ : 5 % CO₂ (carbogen), containing 5 mM of [ethylene bis (oxyethylene-nitrilo)] tetraacetic acid (EGTA). The flow rate was adjusted to a flow of 30 ml/cannula/min. Thereafter, the liver tissue was perfused during 2 min single pass with modified HBSS with 5 mM Ca²⁺ (saturated with carbogen). This buffer was supplemented with 0.05 % (w/v) collagenase, and 250 ml of this collagenase buffer solution was perfused in recirculating mode for 30 min. After the recirculating period, the wedge was put in ice-cold modified Krebs-Henseleit buffer (KHB) (containing 118 mM NaCl, 5.0 mM KCl, 1.1 mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM glucose and 10 mM HEPES saturated with carbogen, pH 7.42) supplemented with 1 % (w/v) bovine serum albumin (BSA). The liver capsula was cut and liver tissue was dissociated with the aid of a pair of forceps and subsequently filtered through 250, 100 and 50 µm nylon filters. The cell suspensions were pooled and centrifuged at 50 g for 4 min and washed three times with ice-cold KHB. Non-viable cells were removed by Percoll density centrifugation and the viability was assessed by trypan blue exclusion (final concentration 0.2%).

Preparation of human liver slices
From the pieces of liver tissue, cores (diameter 8 mm) were made as previously described, and stored in ice-cold UW until slicing. The slicing was performed with the Krumdieck slicer. Ice-cold KHB supplemented with glucose to a final concentration of 25 mM was used as slice buffer. Human liver slices (200-300 µm thickness; wet weight 10 -14 mg) were prepared with standard settings (cycle speed 40; interrupted mode). After slicing the human liver slices were stored in UW until the start of the experiment.

Viability and function of hepatocytes
The viability of the hepatocytes was assessed by trypan blue exclusion and ATP content directly after isolation and after 30 minutes of incubation at 37 °C, as described earlier. The uptake experiments with 21 µM of taurocholic acid in isolated human hepatocytes were performed as described by Sandker et al. The rate constant for uptake (k_in) and excretion (k_out) of taurocholic acid were
calculated as described earlier. To determine drug metabolism, hepatocytes (1.5 x 10^6 cells per ml in Krebs-Henseleit buffer (KHB) supplemented with 1 % BSA) were incubated with 5 mM lidocaine during 60 minutes as previously described.

Viability and function of liver slices
Slices were incubated in 1.4 ml KHB supplemented to 25 mM glucose on stainless steel grids in 24 well tissue culture plates at 37 °C with separate magnetic stirrers and carbogen (95 % O₂:5 % CO₂) supply in each well. The potassium content was determined, after 1 hour incubation, as described by Fisher et al.. Lidocaine was added to a final concentration of 5 mM and MEGX formation was measured after 60 minutes of incubation by HPLC. The MEGX formation rate was expressed per 10^6 hepatocytes assuming that 100 x 10^6 hepatocytes are present per gram of human liver.

Statistics
To compare means of the experiments the independent t-test or the nonparametric test (Mann-Whitney test) was used. Linear regression analysis was used to detect possible significance of correlation. The statistical package used was SPSS 6.0 statistical software. A p-value < 0.05 was considered statistically significant.

Results
We studied a group of 33 split or reduced size liver transplantations. Using the classification according to Ploeg et al., nine initial poor functions occurred in the transplanted group. From this group, one patient was diagnosed as brain death shortly after transplantation (although posttransplant graft function was good) and was excluded. The grafts of the eight remaining recipients were all classified as primary non-function. Therefore, 32 split or reduced size liver transplantations were evaluated.

In the second evaluation of the 8 patients with initial poor function, without the patient who was diagnosed as brain death shortly after transplantation, another four patients were excluded because of events not related to graft viability (a modification of the Ploeg et al. classification): one patient suffered from
rejection of the graft after 5 days, two recipients suffered from graft dysfunction because of size-mismatch and compression of the graft and one patient presented with a vena cava thrombosis on day 2. The grafts of the four remaining recipients were classified as primary non-function. Therefore, 28 split or reduced size liver transplantations were evaluated. This second evaluation was used to exclude the possibility that clinical complications that seem evidently unrelated to the initial liver function are obscuring the predictivity of the in vitro tests.

**In vitro results**

From 33 specimens obtained from split livers or reduced size livers, we prepared both hepatocytes and liver slices. In Table 1, the number and viability (trypan blue exclusion) of hepatocytes derived from specimens of these different human livers is shown.

<table>
<thead>
<tr>
<th></th>
<th>Yield</th>
<th>Viability</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Before Percoll</td>
<td>13.4(9.2)</td>
<td>68.9(20.3)</td>
<td>33</td>
</tr>
<tr>
<td>After Percoll</td>
<td>4.3(4.4)</td>
<td>92.0(5.5)</td>
<td>26</td>
</tr>
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</table>

Results are expressed as mean, whilst SD’s are between brackets; n = number of experiments.

**Table 1** Yield of hepatocytes (million cells/g liver) and viability (% trypan blue exclusion) before and after Percoll

Both the yield before and after Percoll density centrifugation (used to remove non-viable cells) is presented. About 33 % (range=0.1-71 %; median=29%) of the totally isolated human hepatocytes were recovered after Percoll centrifugation. These cells showed excellent viability (trypan blue exclusion 92 %).

The rate constants for uptake and excretion of the anionic bile acid taurocholic acid in hepatocytes and the energy status of the hepatocytes determined by the ATP content is depicted in Table 2.

After 1 hour of incubation, the potassium concentration in the slices was 36.7 ± 3.7 nmol/ mg liver slice (n=15).

In Table 3 the metabolic capacity, assessed by the metabolism of 5 mM lidocaine to MEGX, of human hepatocytes and liver slices is shown. The metabolism of
Prediction of liver function after transplantation

| Fractional rate constant for uptake ($k_{in}$) and excretion ($k_{out}$) of 21 µM taurocholic acid in human hepatocytes and ATP directly after isolation ($ATP_{0\ min}$) and after 30 minutes ($ATP_{30\ min}$) incubation |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| $k_{in}$        | $k_{out}$       | $ATP_{0\ min}$ | $ATP_{30\ min}$ | n               | n               | n               |
| 2.6 (0.5)       | 61.8 (10.2)     | 4.0 (0.7)       | 5.5 (1.2)       | 25              | 23              | 13              |

Results are expressed as mean, whilst SEM’s are between brackets. n= number of experiments.

Table 2 Fractional rate constant for uptake ($k_{in}$) and excretion ($k_{out}$) of 21 µM taurocholic acid in human hepatocytes and ATP directly after isolation ($ATP_{0\ min}$) and after 30 minutes ($ATP_{30\ min}$) incubation

Lidocaine in both in vitro preparations was linear up to 60 minutes. Moreover, for each liver the MEGX$_{ratio}$ was calculated as the MEGX formation rate in slices, expressed per $10^6$ hepatocytes divided by the MEGX formation rate in hepatocytes from the same liver.

| Table 3 5 mM lidocaine metabolism to MEGX (in nmol/$10^6$ cells, hr) in human hepatocytes and liver slice, and the MEGX$_{ratio}$ = MEGX$_{slices}$/MEGX$_{cells}$ |
|-----------------|-----------------|
| MEGX$_{cells}$  | 54.0 (30.8)     |
| MEGX$_{slices}$ | 34.8 (32.4)     |
| MEGX$_{ratio}$  | 0.73 (0.56)     |

Results are expressed as mean ±SD; n= number of experiments.

Comparison of in vitro data

The viability of the cells assessed by the trypan blue exclusion test directly after the isolation correlated significantly with the yield of cells after the Percoll density centrifugation ($r^2=0.48$, $p=0.012$). The yield of hepatocytes both before and after Percoll significantly correlated with MEGX$_{ratio}$ (respectively $r^2=0.43$, $p=0.008$ and $r^2=0.49$, $p=0.011$) (Figure 1), but not with the MEGX formation rate in cells or slices (data not shown).
No correlation was found either between taurocholic acid uptake rate or ATP content and the yield of cells. In addition, the MEGX ratio did not correlate with taurocholic acid uptake or ATP content.

Comparison of in vitro with in vivo data

Bile production in the recipients (n=12-16) from day 3-6 correlated significantly with the yield of viable cells after Percoll centrifugation. If a high yield of hepatocytes after Percoll was found, the bile production in the recipient was high. The serum GOT on day 2 and 3 correlated significantly with the yield of cells before (n=28; p=0.042 for day 2 and n=26; p=0.028 for day 3) and after Percoll (n=22; p=0.017 for day 2 and n=20; p=0.02 for day 3). The serum GPT on day 2 correlated significantly with yield of cells after Percoll (n=22; p=0.044). The serum GOT and serum GPT in the recipient were high if the yield of hepatocytes after Percoll was high. All other parameters measured in the recipients during 7 days did not correlate with any of the in vitro parameters.

Figure 1  A. Yield of hepatocytes before Percoll per gram liver versus MEGX\textsubscript{ratio} and B. Yield of hepatocytes after Percoll per gram liver versus MEGX\textsubscript{ratio}. Good function of the graft is represented by the open circles (○), primary non-function according to Ploeg et al. (8) is represented by boxes, where □ are the PNF with impaired liver function and ■ the PNF due to non-liver quality-related failure.
Prediction of primary non-function by in vitro parameters

None of the viability tests of the in vitro preparations of the eight livers, which exhibited primary non-function after transplantation, were significantly different from those livers that showed good graft function after transplantation (Figure 1 and 2). Furthermore, the second evaluation revealed that the four livers, which exhibited primary non-function according to the modified Ploeg et al. classification (Figure 1 and 2), were also not significantly different from those livers with good graft function.

The four livers that were excluded from this modified Ploeg et al. classification were qualified as non-liver quality-related failure and were not significantly different from those livers that showed good graft function after transplantation (Figure 2).

Discussion

Human hepatocytes or liver slices prepared from liver specimen obtained after split or reduced size transplantation offer a unique possibility to assess the in vitro function of the graft. This makes it possible to evaluate the potential
predictive value of in vitro tests for graft function, since the in vitro tests are performed on tissue from the same liver as that is transplanted. In addition, more insight may be given into the mechanisms leading to primary non-function. Due to interindividual differences, MEGX production from lidocaine varies extensively in man, and this complicates the use of the MEGX test as in vivo viability test for donor livers. These interindividual differences in MEGX formation expressed per million hepatocytes are also observed in liver slices and in hepatocytes prepared from the same human liver 9. Therefore, to normalize for the interindividual differences in MEGX formation, the MEGX\textsubscript{ratio} was calculated as the rate of MEGX production in the slice (expressed per million hepatocytes, present in the slice) divided by the MEGX formation in the isolated hepatocytes (expressed per million hepatocytes finally present) as prepared from the same liver. This calculation is based on the assumption that during the isolation of the hepatocytes some sort of selection occurs, in which only the most viable cells of the tissue survive the entire isolation procedure. This assumption is based on the results of the present study and of studies where cells were isolated from livers, discarded for transplantation 7, from liver tissue obtained after partial hepatectomy 14 and cirrhotic livers 5. Therefore, the rate of metabolism in the finally obtained isolated cell population is assumed to represent the metabolic activity of viable cells in this individual liver. Deterioration of metabolic function of the graft will be reflected in a decreased rate of MEGX formation calculated per million hepatocytes in the liver slice as compared to the cells isolated from the same liver. Therefore, the ratio MEGX\textsubscript{slices}/MEGX\textsubscript{cells} may be an indication for the fraction of viable cells in the slices and consequently be a measure for the particular liver graft viability.

The ratio of the rate of MEGX formation in hepatocytes and liver slices from the same liver (MEGX\textsubscript{slices}/MEGX\textsubscript{cells}) correlated significantly with the yield of cells both before and after the Percoll density centrifugation. A low MEGX\textsubscript{ratio} correlated with a low cell yield (\textit{p}<0.05). These data support the hypothesis that the MEGX ratio may give an estimation of the number of viable hepatocytes in the slice and thus in the graft. However, the four livers exhibiting poor function after transplantation did not have a significant lower MEGX\textsubscript{ratio} compared to livers showing good post-operative function (Fig 2).

Interestingly, it was observed that if the bile production was high in the recipient, the yield of viable hepatocytes from the same liver after Percoll separation was
also high. Supposing that the isolation procedure only selects the most viable cells, this high yield suggests a high amount of viable hepatocytes in the graft. This apparent quality of the donor liver may ultimately lead to a better bile production in the graft. However, the serum GOT and GPT levels were significantly higher in the recipients receiving livers from which a high yield of hepatocytes was obtained. The reason for this is unknown and needs further investigation. This residue material of reduced size or split liver tissue offers the unique possibility to assess the functionality of the liver in vitro, thereby giving the opportunity to design more optimal function tests for donor livers. Clearly, during or after transplantation many additional problems may arise that are not related to the viability of the donor liver. For example, the age of the recipient, the operative procedure and the size matching of the graft may all determine the success of transplantation. These factors may hamper the evaluation of in vitro tests for their predictive value of graft functioning. The low incidence of initial poor function and primary non-function of the grafts may indicate that in practice almost exclusively viable donor livers are used in our transplantation center probably due to the strict evaluation of the donors. Nevertheless, the few initial poor function and primary non-function grafts, as determined by both Ploeg et al. classifications, were not predicted with the viability tests used in this study. If such in vitro tests could be made more sensitive and predictive one could potentially increase the number of available donor livers, assuming that some donor livers are incorrectly rejected for trans-plantation at present. For cases where whole livers are transplanted, in vitro tests should be developed with smaller tissue specimens like the needle biopsies. However, major difficulties in correlating results obtained with in vitro tests with those of graft function may be unavoidable due to patient related factors such as age, severity of the disease, and complications due to the operation procedure. In conclusion, the presently used in vitro tests, including the MEGX ratio, did not select non-viable livers. The incidents of primary non-function of the grafts, as determined by both classification occurred in spite of normal in vitro function tests indicating that these particular tests were not able to predict graft function. These results indicate that either the function tests used in this study are not sensitive enough or that other factors than liver viability influenced the graft function of the recipient in this specific group of patients. These results are in
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line with those of Groothuis et al.; they showed that from livers discarded for orthotopic transplantation, a comparable yield of viable hepatocytes was obtained with similar transport capacity for taurocholic acid and ATP content than from livers accepted for transplantation. Non-parenchymal cells seem to be an important factor in reperfusion injury. Therefore, in the future more attention should be paid to the activity of these non-parenchymal cells and the interaction between non-parenchymal and parenchymal cells. In our institute we are now studying the activity of non-parenchymal cells in the human liver slices. Human liver slices prepared from split or reduced size livers could offer an excellent human liver preparation, as this in vitro system represents an integrated liver system, which may be used to gain more insight in the mechanisms of primary non-function, reperfusion injury and to investigate whether predictive function tests should include the probing of non-parenchymal cell function.
References

5. Sandker GW. Isolated human hepatocytes: a research tool for investigations on drug transport, drug metabolism and liver transplantation; University of Groningen, The Netherlands, 1993
13. Olinga P, Merema MT, Meijer DKF, Slooff MJH and Groothuis GMM. Human liver slices express the same lidocaine biotransformation rate as isolated human hepatocytes. ATLA 1993; 21: 466-468
Men mag weer zwijgzaam en wijs zijn
De natte giftige sporen van verbeelding weggewist
Men mag weer stilstaan en staren