Human liver cell lines for the AMC-bioartificial liver

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CHAPTER 7

The effect of rat acute-liver-failure plasma on HepaRG cells

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ABSTRACT

**Background:** Recently, we have demonstrated high liver functionality, including ammonia eliminating capacity of the human liver cell line HepaRG, rendering it a valuable biocomponent of a bioartificial liver (BAL) to support patients with acute liver failure (ALF). This cell line further gains detoxification properties when cultured with dimethyl sulfoxide (DMSO).

**Aim:** In this paper we describe whether its functionality will be compromised by toxic effects of ALF plasma, as has been shown for primary hepatocytes.

**Methods:** We exposed -DMSO and +DMSO HepaRG cultures during 16 hours to healthy- and ALF-rat plasma. The cultures were analyzed for lipid accumulation, cell leakage, apolipoprotein A-1 production, nitrogen metabolism and transcript levels of hepatic genes.

**Results:** The -DMSO cultures showed increased cell leakage after healthy and ALF plasma exposure in contrast to +DMSO cultures, but otherwise the -DMSO and +DMSO cultures were equally affected by exposure to the plasmas. Exposure to both plasmas caused lipid accumulation and decreased transcript levels of various hepatic genes. ALF plasma decreased urea cycle activity, but increased urea production from arginine by upregulated *arginase* 2. However, total ammonia elimination was not affected by exposure to either plasma, indicating its predominant elimination by fixation into amino acids. In addition apolipoprotein A-1 production remained constant.

**Conclusions:** HepaRG cells are negatively affected by rat plasma, even of healthy origin. However, their ammonia eliminating capacity is relatively resistant, underlining their suitability for BAL application. DMSO pre-treatment may increase their viability in plasma.
INTRODUCTION

Bioartificial livers (BALs) have been developed to bridge patients suffering from acute liver failure (ALF) to liver transplantation or liver regeneration. BALs rely on bioreactors with liver cells that compensate for the loss of functional liver-cell mass in ALF patients. The required BAL functions are not specified, however conversion of ammonia into urea, drug-metabolizing capacity and synthesis of blood proteins are probably important.

Ideally, BAL systems contain a biocomponent of human origin with sufficient proliferative and functional capacity. In this respect, the HepaRG cell line is a promising biocomponent. The HepaRG cell line is a bipotent progenitor cell line from a human hepatocellular carcinoma that, in monolayer, starts to differentiate at high confluence (at 14 days after seeding) into a culture with clusters of cuboid and granular hepatocyte-like cells, surrounded by flattened cells. A successive culture phase of 14 days in the presence of 2% dimethyl sulfoxide (DMSO) upregulates components of drug-metabolism, like cytochrome P450 (CYP) 3A4.

Our recent investigation on liver specific functions relevant for BAL application, confirmed a unique high hepatic functionality of HepaRG cells with ammonia elimination and apolipoprotein A-1 (ApoA1) production rates similar to those of primary human hepatocytes. The ammonia elimination occurred probably mainly through fixation into amino acids, particularly through Glutamine Synthetase (GS) activity. However we also showed that DMSO treatment elicited undesirable effects; a reduction in cell mass, galactose elimination and transcript levels of hepatic genes including the rate limiting urea cycle enzyme carbamoyl phosphate synthetase (CPS) and GS.

The abovementioned performance of HepaRG cells was tested in culture medium. However, their suitability as biocomponent for BALs relies on their performance during exposure to ALF plasma, since cytotoxic compounds accumulating in blood during ALF decrease the functionality of liver cells. These toxic compounds in ALF include components of necrotic cells, the activated innate immune system and substrates that are normally metabolized by the liver. Components of ALF plasma with proven cytotoxic effects are ammonia, lactate, bile acids, bacterial lipopolysaccharide and the cytokines IL-6, TNF-α and IL-1β. The cytotoxic effects of ALF plasma on liver cells comprise a disturbance in cell morphology and a reduction in cell viability and function. Importantly, a severe decline in drug-metabolizing and ammonia eliminating activity has been observed in monolayer and in BAL cultures of primary hepatocytes after exposure to ALF plasma. Human liver cell lines have been less studied in this respect; however changes in morphology, a reduction in viability, CYP activity and urea production have been reported.

Moreover, exposure to healthy plasma may already elicit negative effects in hepatocytes, including lipid droplet formation, a reduction in urea, albumin and glutathione production. In contrast, an increase in CYP activity and stable urea production has also been reported. These controversies may relate to differences in cell types and plasmas used, as well as to differences in exposure times.
For HepaRG cells however, the effects of ALF or healthy plasma have not been reported yet. Therefore, the aim of our study was to assess the response of HepaRG cells to exposure to ALF or healthy plasma in the context of BAL therapy. We hypothesized that a high drug metabolizing and ammonia eliminating capacity may yield relatively high resistance to these plasmas.

Therefore, we exposed -DMSO and +DMSO HepaRG cultures to healthy- and ALF-rat plasma, and compared their characteristics with cultures maintained on culture medium, this way isolating the specific responses to healthy plasma and to ALF components in the plasma. ALF plasma from rats was used, as this was available in high quantities and was expected to contain high levels of cytotoxins, being harvested immediately after death from ALF.

**METHODS**

**Cell culture**
HepaRG cells were kindly provided by prof. C. Guguen-Guillouzo (INSERM, Rennes). HepaRG cells were seeded at $10^5$ cells/cm² in 24-well culture plates (Corning, New York, U.S.) and subsequently cultured in 0.5 mL HepaRG culture medium / well for 28 days as described previously.4,6 The HepaRG culture medium was composed of Williams’ E medium (Lonza, Basel, Switzerland) with 10% fetal bovine serum (Lonza), 5 µg / mL insulin (Sigma, St. Louis, U.S.), 50 µM hydrocortisone hemisuccinate (Sigma), 2mM glutamine (Lonza), 50 U / mL penicilline and 50 µg / mL streptomycine (penicilline/streptomycine mix, Lonza). The -DMSO and +DMSO cultures differed in the last 14 days of culture by the absence or presence of 2% DMSO (Sigma), respectively.

**Plasma exposure experiments**
Plasma was derived from 4 healthy rats (combined) and 4 ALF rats (combined). The ALF plasma was collected immediately from rats after death due to complete liver ischemia as previously described.24 The state of ALF was confirmed by assessment of the biochemical profile of the plasmas (Table 1). To harvest the plasma, blood was withdrawn from the healthy and ALF rats and collected in BD Vacutainer® lithium heparin plasma separator tubes (Becton Dickinson, Plymouth, UK). The plasma was collected after centrifugation for 10 min at 1300g and subsequently supplemented with 2 IU/mL dalteparine (low molecular weight heparin; Pfizer, New York, U.S.) and 50 U/mL penicillin with 50 µg/mL streptomycin (Lonza).

The -DMSO and +DMSO HepaRG cultures were exposed to the undiluted plasmas for 16h. Control cultures were maintained on culture medium. After plasma exposure, the cultures were washed twice with phosphate-buffered saline (Fresenius Kabi GmbH Bad Homburg vor der Höhe, Germany). Subsequently, either RNA was immediately harvested for reverse transcription-polymerase chain reaction, or the cultures were subjected to a hepatocyte function test or staining for lipid droplets.
The effect of rat acute-liver-failure plasma on HepaRG cells

Table 1. Biochemical profile of the pooled plasmas (from 4 animals/group) utilized in this study.

<table>
<thead>
<tr>
<th>Source</th>
<th>Ammonia (µM)</th>
<th>Total bilirubin (µM)</th>
<th>AST (U/L)</th>
<th>LDH (U/L)</th>
<th>Triglycerides (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy rat</td>
<td>20</td>
<td>&lt;1</td>
<td>99</td>
<td>631</td>
<td>1.80</td>
</tr>
<tr>
<td>ALF rat</td>
<td>1238</td>
<td>15</td>
<td>&gt;6000</td>
<td>&gt;3000</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Abbreviations: ALF, acute liver failure; AST, Aspartate aminotransferase; LDH, lactate dehydrogenase.

Lipid droplet staining
The cell cultures were fixed in 3.7% formaldehyde solution (Merck, Darmstadt, Germany) and stained with Oil red O for visualization of lipid droplets as previously described.25

Reverse transcription-polymerase chain reaction
RNA was isolated from the cultures by using the RNeasy mini kit (Qiagen, Venlo, the Netherlands). As a reference, two different human liver samples (non-tumour parts of small liver resection samples, immediately frozen with TRIzol® reagent (Life Technologies, Carlsbad, USA) in liquid nitrogen) were included in the analyses as described.26 These samples derived from two female patients, aged 40 and 41 years, with liver adenoma and no elevated liver damage. The patients were not on medication and had no history of drug/alcohol abuse. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation and the Helsinki Declaration of 1975.

First strand cDNA preparation and subsequent real-time polymerase chain reaction were performed as previously described.27 Transcript levels were calculated, subsequently normalized for 18S ribosomal RNA, and expressed as a percentage of the mean transcript levels of two human liver samples as described.28

Assessment biochemical parameters
For the assessment of functionality and cell damage, the cultures were incubated in 1 mL of test medium (HepaRG medium with 1.5 mM $^{15}$NH$_4$Cl (Sigma), 2.27 mM D-galactose (Sigma), 2 mM L-lactate (Sigma) and 2 mM ornithine hydrochloride (Sigma)). Medium samples of 0.5 mL were taken after 45 min and 24 h of incubation, at termination of the test. These time points were chosen in order to acquire homogeneous samples and reproducible data. Subsequently, cultures were washed twice with phosphate-buffered saline and stored at -20 °C for total protein content analysis.

Biochemical assays
The test medium samples were analyzed for function and cell damage parameters. Human APOA1 concentrations were determined by an enzyme-linked immunosorbant assay using a polyclonal rabbit anti-human APOA1 (Merck) in a 1:500 dilution as primary antibody, a monoclonal
mouse anti-human APOA1 (Merck) in a 1:500 dilution as secondary antibody, and horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Dako, Glostrup, Danmark) in a 1:2000 dilution as tertiary antibody. The color reaction was developed by using o-phenylenediamine (Sigma). Ammonia concentrations were determined using the Ammonia (rapid) kit (Megazyme International, Wicklow, Ireland). Urea concentrations were determined according to the blood urea nitrogen test (Sigma). Labeled $^{15}$N urea was measured using gas-chromatography-mass-spectrometry. Amino acid concentrations were measured by gradient reversed-phase high-performance liquid chromatography.

Amino acids that could be determined in this analysis included ornithine, taurine, citrulline and the 20 basic amino acids, except proline and cysteine. Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities were analyzed spectrophotometrically using the P800 Roche Diagnostics analyzer. Triglycerides were analyzed using a glycerol-3-phosphate oxidase-PAP enzymatic test (Roche Diagnostics, Basel, Switzerland). Total protein/well was measured by spectrometry using Coomassie blue (Bio-Rad, Hercules, U.S.). The function and damage parameters were established by calculating the changes in concentration in the $t=45$min and $t=24$h samples and were corrected for protein content/well. The determined biochemical activities were linear within this time frame.

**Statistical analysis**

Student’s t-tests were used to determine statistical differences between two groups (-DMSO vs +DMSO and control vs ALF plasma exposed groups in urea enrichment and arginine consumption tests). One-Way Anova test with Tukey post-hoc analysis were used for statistical analysis of differences between three groups, i.e. the control, healthy plasma and ALF plasma exposed groups. Significance was reached if $p < 0.05$. SPSS 12.0.1 (SPSS Inc., Chicago, USA) was used for statistical analysis. Average values ($\pm$ standard deviation) are reported.

**RESULTS**

**Morphology of HepaRG cultures**

HepaRG cultures (-DMSO and +DMSO) were exposed for 16h to healthy or ALF plasma. The composition of these plasmas is indicated in Table I. The ALF plasmas contained elevated levels of ammonia, total bilirubin, and the cell damage parameters AST and LDH.

The morphology of both cultures was equally affected upon exposure to either the healthy and ALF plasmas, compared to control cultures (Fig. 1). Plasma-exposed cells adapted a more rounded shape and lost cell-cell contacts upon exposure. We observed lipid droplet accumulation in plasma-exposed cultures, again independent on the type of plasma used, as confirmed by Oil red O staining (Fig. 2). This lipid droplet formation may either relate to the high triglyceride levels in plasma (Table I) relative to the culture medium (<0.1 mM) and/or a steatotic response to injury as commonly found in liver.
The effect of rat acute-liver-failure plasma on HepaRG cells

Figure 1. The effect of healthy and ALF plasma on the morphology of -DMSO and + DMSO HepaRG monolayers. Negative controls are included. Bars represent 100 µm.

Figure 2. The effect of healthy and ALF plasma on the lipid accumulation of -DMSO and +DMSO HepaRG monolayers. Negative controls are included. Bars represent 100 µm. For color figure, see page 219.
Biochemical parameters of HepaRG cultures

Exposure to plasma did not change the total protein/well content substantially (Fig. 3A). As previously observed, the protein content was 2-fold lower in +DMSO cultures compared to -DMSO cultures. In contrast, cell damage, as measured by AST and LDH activities in the test medium, increased maximally 5-fold in the -DMSO cultures (Fig. 3B and C) after ALF-plasma exposure. AST leakage was also increased after exposure to healthy plasma and LDH leakage was not significantly different from control conditions. Interestingly, the AST and LDH levels remained unchanged in +DMSO cultures under all conditions, although the AST levels of +DMSO control cultures were already significantly 3 to 4-fold higher to those of -DMSO control cultures as previously observed. There were no significant differences between healthy-plasma- and ALF-plasma-exposed cultures.

The ApoA1 production of the -DMSO and +DMSO cultures (Fig. 3D) was not influenced by plasma exposure and was in the same range as observed previously.

Figure 3. The effect of healthy and ALF plasma on cell mass, cell damage and APOA1 production. (A) total protein/well content, (B) AST leakage, (C) LDH leakage, and (D) APOA1 production of -DMSO (white bars) and +DMSO (gray bars) cultures. Significance: *, p < 0.05 vs control; #, p < 0.05 -DMSO vs +DMSO control cultures (n=12 from 3 independent experiments). No significant differences were present between healthy-plasma and ALF-plasma-exposed cultures after specified exposure times.
The rate of ammonia elimination (Fig. 4A) remained also constant after plasma exposure in both -DMSO and +DMSO cultures. As previously observed, ammonia elimination was 1.4-fold lower in -DMSO compared to +DMSO control cultures. Unexpectedly, the urea production (Fig. 4B) increased 2- to 3-fold in both cultures upon exposure to ALF plasma whereas no changes were observed after healthy plasma exposure. The urea production rates were similar between -DMSO and +DMSO control cultures.

To explain the discrepancy between the relatively stable ammonia elimination and, on the other hand, the increasing urea production in HepaRG cultures after exposure to ALF plasma, we determined the metabolic fate of ammonia after loading the cultures with 1.5 mM $^{15}$NH$_4$Cl. The mass-enriched fraction of urea (Fig. 4C) after exposure to ALF plasma was significantly reduced, namely, 6- and 4-fold in the -DMSO and +DMSO cultures, respectively. Thus, the conversion of ammonia into urea was similarly reduced in both cultures, and therefore, the ALF-plasma-associated increased urea production of both HepaRG cultures was not the result of an increase in elimination of the exogenously-added ammonia.

Figure 4. The effect of healthy and ALF plasmas on nitrogen metabolism. (A) ammonia elimination, (B) urea production, (C) conversion of $^{15}$N ammonia into urea, and (D) arginine consumption of -DMSO (white bars) and +DMSO (gray bars) cultures For C and D only control and ALF plasma treatment groups are included. Significance: *, $p < 0.05$ vs control; §, $p < 0.05$ exposure to healthy plasma vs ALF plasma; #, $p < 0.05$ -DMSO vs +DMSO control cultures (n=12 from 3 independent experiments).
In line with these findings, the transcript levels of CPS, encoding the first enzyme of the urea cycle, and arginase 1 (ARG1), encoding the cytosolic urea cycle enzyme that converts arginine into ornithine and urea as part of the urea cycle, (Fig. 5A and B) remained constant or decreased in plasma-exposed cultures relative to the control cultures. Therefore, these enzymes are likely not associated with the 2- to 3-fold upregulation of urea production upon exposure to ALF plasma.

The transcript level of GS – thought to be responsible for the bulk of ammonia elimination in HepaRG cells – remained constant or decreased upon plasma exposure (Fig. 5C).6 Importantly, the transcript levels of ARG2, the cytosolic enzyme that produces urea from arginine degradation, increased 2- to 5-fold in -DMSO and +DMSO cultures after exposure to ALF plasma (Fig. 5D). These effects were due to ALF components, and not to the plasma background, as indicated by significant differences between healthy-plasma- and ALF-plasma-exposed cultures. Together, these results suggest that the increase in urea production as a consequence of exposure to ALF plasma is explained primarily by an upregulation of ARG2. This was further supported by a trend of increased arginine consumption in both -DMSO and +DMSO cultures after ALF plasma exposure, whereas no consistent and substantial change in metabolism of other amino acids was found (Fig. 4D).

Furthermore, we found a trend that both healthy and ALF plasmas downregulated the CYP3A4 expression (Fig. 5E).

Finally, the differences in transcript levels of hepatic genes between -DMSO and +DMSO control cultures corresponded to a previous description of the cultures, with higher transcript levels of CPS and lower CYP3A4 transcript levels for -DMSO cultures relative to +DMSO cultures.6 No clear differences in the effect of plasma exposure on transcript levels were found between -DMSO and +DMSO cultures.
Figure 5. The effect of healthy and ALF plasmas on transcript levels of various genes. (A) CPS, (B) ARG1, (C) GS, (D) ARG2 and (E) CYP3A4 of -DMSO (white bars) and +DMSO (gray bars) cultures. Transcript levels are indicated as the % of mean transcript levels of two human frozen control liver samples, normalized for 18S ribosomal RNA. Significance: *, p < 0.05 vs control; §, p < 0.05 exposure to healthy plasma vs ALF plasma; #, p < 0.05 -DMSO vs +DMSO control cultures (n=4 from 4 independent experiments).

DISCUSSION

This study shows the effects of healthy-rat plasma and ALF-rat plasma on different aspects of both DMSO treated (+DMSO) and non-DMSO (-DMSO) treated HepaRG cells cultured in monolayer. The cultures were both susceptible to negative effects of plasma from healthy and ALF rats. A protective effect of DMSO treatment was only found with respect to cell leakage. An as-yet unrevealed enhancing effect of ALF plasma on urea production was found, which was urea cycle-independent. Still, APOA1 production and ammonia elimination remained unaffected by exposure to plasma.
The negative effects of healthy and ALF plasma include increased cell leakage, lipid droplet formation and reduced transcript levels of various hepatic genes. The increased enzyme leakage, as a measure for cell damage, was only observed in -DMSO cultures. Since the -DMSO and +DMSO cultures differ to high extent in their detoxification characteristics, it is not unlikely that the differential cell leakage is related to detoxification capacity. On the other hand, the -DMSO and +DMSO cultures also differ in their metabolic activities, cell density, cell leakage and composition of cell types, which may also affect the differential response to the plasmas. Yet, upregulation of drug metabolism by DMSO treatment seems relatively ineffective in maintaining the performance of HepaRG cells after plasma exposure.

Surprisingly, our results imply that most of the negative effects of exposure to ALF plasma are due to the background of plasma, and not to the ALF-related composition of the plasma. The negative effects of healthy plasma may be related to the absence of beneficial compounds normally present in the HepaRG culture medium. Washizu et al. showed that the production of urea and albumin of rat hepatocytes in human plasma could be maintained or upregulated to the level in culture medium by supplementing the plasma with compounds as glucagon, amino acids, hydrocortisone and insulin. Those latter two hormones are also present in non-physiological high concentrations in the HepaRG medium, rendering these as possible candidates implicated in the decline of performance after healthy-plasma exposure. In addition, amino acid concentrations may play a role, as depletion is known to lead to cell damage via autophagy, and amino acids concentrations were higher in culture medium compared to plasma.

The xenogenicity of the plasmas may have contributed to the toxicity of the plasmas, although it is very unlikely that pre-existing antibodies against human liver cells are present in rat plasma. In addition, most observations on cell damage, morphology and hepatic gene transcript levels have been described for hepatocytes exposed to homologous plasma as well.

Only the increase in urea production through \textit{ARG2} upregulation is directly caused by exposure to ALF-related components in the plasma. The physiological role and regulation of \textit{ARG2} in the context of the liver is largely unknown, at least partly due to the high expression of \textit{ARG1}. However in other tissues, \textit{ARG2} and/or urea production can be upregulated by cytokines and lipopolysaccharide (LPS). Furthermore, \textit{ARG2} is associated with the control of nitric oxide synthesis (a potent vasodilator involved in the inflammatory response) and the production of polyamines and proline, which are required for cell proliferation. Therefore, \textit{ARG2} upregulation is in those tissues associated with a protective response to injury. Given the fact that ALF plasma contains high levels of cytokines and LPS, a similar effect may explain the \textit{ARG2} upregulation in HepaRG cells after exposure to ALF plasma. However, no upregulation of urea production by cytokines has been observed in primary rat hepatocytes. On the other hand, HepaRG cells, with a high \textit{ARG2} expression and a low \textit{ARG1} expression relative to human liver, may correspond more to the extra-hepatic tissues with respect to responses to cytokines and urea production. The \textit{ARG2} expression may also be committed to either of the
two subpopulations of the HepaRG cultures and this localization of expression may have been altered after exposure to ALF plasma.

The ammonia eliminating capacity of the HepaRG cultures remained unaffected by exposure to plasma. Even in ALF plasma, despite the reduced urea cycle activity, ammonia elimination remained high, which can be explained by its predominant elimination through fixation into amino acids, particularly glutamine, rather than conversion into urea. Indeed, the glutamine metabolism remained unaffected by plasma exposure. However, since GS transcript levels showed a tendency to decline after exposure to plasma, it can be expected that eventually ammonia elimination will be affected.

In conclusion, cytotoxic effects of ALF plasma have been found in both -DMSO and +DMSO HepaRG cultures. The declined performance is not only due to the ALF-related components of the plasma, but also to the background of the plasma itself. Therefore the maximal time that BAL devices with HepaRG cultures can be connected to ALF patients will be highly similar between patients. However, ammonia elimination, one of the most important functions of a BAL, is relatively resistant to plasma exposure as well as APOA1 production. This supports that HepaRG cells will be suitable for BAL application. When used in a BAL system, their resistance to plasma effects may be increased by DMSO pre-treatment or supplementation of beneficial compounds present in the culture medium. Nevertheless, careful monitoring of BAL functionality during treatment of a patient will be needed to establish the moment that a BAL needs to be refreshed.

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The effect of rat acute-liver-failure plasma on HepaRG cells


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