Haemoperfused liver as an *ex vivo* model for organ invasion of *Candida albicans*

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To study invasion of the human fungal pathogen *Candida albicans*, several infection models have been established. This study describes the successful establishment of an *ex vivo* haemoperfused liver as a model to study invasion of *C. albicans*. Perfused organs from pigs could be kept functional for up to 12 h. By comparing a non-invasive and invasive strain of *C. albicans* and by following a time course of invasion, it was shown that the invasion process in the perfused liver infection model is very similar to the *in vivo* situation after intraperitoneal infection of mice. The advantage of this set-up compared with other models of invasion is discussed.

INTRODUCTION

In hospitals, *Candida albicans* is the leading pathogen causing disseminated fungal infections, but may also cause other life-threatening infections. For example, *C. albicans* causes intra-abdominal mycoses and invasive fungal infections after liver transplantation, with mortality rates of up to 60% (Boos *et al.*, 2005; Fischer & Sterneck, 2005). Furthermore, *C. albicans* may cause peritonitis during continuous ambulatory peritoneal dialysis (Michel *et al.*, 1994). During these infections, the fungus colonizes the surface of parenchymal organs and penetrates into the tissue from the surface, but not from the bloodstream.

The most commonly used infection model for *C. albicans* is murine tail-vein injection for haematogenously disseminated candidosis. However, depending on the size of the inoculum of fungal cells, the outcome of such infections varies (Odds *et al.*, 2000). Furthermore, these models are of limited use in studying the invasion properties of *C. albicans*, as direct injection of the fungal cells into the bloodstream means that the cells do not have to invade actively through host tissue to reach the bloodstream. For this reason, other infection models such as epithelial monolayers, reconstituted human epithelium, chick

chorioallantoic membrane and intraperitoneal (i.p.) infections have been established to study invasion of *C. albicans* (Farrell *et al.*, 1983; Gow *et al.*, 2003; Kretschmar *et al.*, 1999a, b; Schaller *et al.*, 1998). In addition to qualitative histological examination, parameters such as lactate dehydrogenase and alanine aminotransferase (ALT) levels can be analysed to quantify tissue damage and invasion in these models (Kretschmar *et al.*, 1999a; Schaller *et al.*, 1998).

One possible way of avoiding experimentation on living animals is to use models based on *ex vivo* perfused organs. Perfused organs have been used for the investigation of many different physiological, pharmacological and toxicological aspects (Gordon *et al.*, 1972; Groneberg *et al.*, 2002). Following multi-organ harvesting or using organs from slaughtered animals, perfusion has the further advantage that it contributes to the '3R' concept (refinement, reduction, replacement) of animal experimentation by reducing animal usage for research purposes (Grosse-Siestrup *et al.*, 2002a, b). Studies in the mid-1970s used perfused livers from mice as infection models for bacteria and *C. albicans* (Moon *et al.*, 1975; Sawyer *et al.*, 1976) to investigate clearance of the micro-organisms from the bloodstream. The aim of our study was to establish an *ex vivo* haemoperfused liver from pigs as a model to study invasion of *C. albicans*.

METHODS

Strains and media. *C. albicans* strains SC5314 (Gillum *et al.*, 1984) and ATCC 10231 (Kretschmar *et al.*, 1999a) were incubated for 18 h in Sabouraud medium containing 2% glucose. Before

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; i.p., intraperitoneal; p.i., post-infection.

infection, cells were harvested by centrifugation, washed three times with PBS and resuspended in PBS to a final concentration of 2×10^9 cells ml^{-1} .

Animals. White German Landrace pigs (five male, three female; weight 42 ± 13 kg) were used with the approval of the official veterinary institutions. Animals used in this study received humane care and study protocols were in accordance with the guidelines of the German authorities for the care and use of laboratory animals. Animals were killed by final exsanguination during the blood-harvesting procedure.

Organ harvesting. All organs were harvested under general anaesthesia as described previously (Nagel *et al.*, 2005). Directly after preparation of the blood vessels and after blood harvesting, the liver was flushed via the aorta with 4 l pre-warmed (37°C) electrolyte infusion solution 153 (Berlin-Chemie) to wash residual blood from the organ. The liver was removed and the portal vein, hepatic artery, vena cava and bile duct were cannulated. The cannulated organ was used directly for perfusion. Initial liver weight was determined to be 939 ± 208 g.

Blood harvesting. Prior to blood harvesting, one bolus of heparin (10 000 U) was administered to the animals. Collection of blood was performed by cannulation of the cervical vessels on one side immediately before organ harvesting. The collected blood (1.5 l) was further treated with heparin ($10\,000\text{ U l}^{-1}$) and an antibiotic (Augmentan, 1.2 g l^{-1} ; GlaxoSmithKline). Just before connection of the organ, blood was added to the perfusion circuit and pre-warmed to 37°C .

Perfusion. To set up our perfusion model, four livers were perfused for 3 h without the application of fungal cells. Perfusion was carried out as described previously (Grosse-Siestrup *et al.*, 2002b, 2004) with modifications (Fig. 1). The blood for the hepatic artery came directly from the modules for oxygenation and dialysis (oxygen-rich). For the portal vein, a mixture of oxygen-rich (from the dialysis module) and oxygen-poor (venous) blood was chosen to obtain a relatively physiological situation of oxygen for the liver. Roller pumps were used for the dialysis circuit as well as for the blood circuit. The connected liver was put into a sterile plastic bag to create a humid atmosphere and placed on a waterbed kept at 37°C . Perfusion parameters were adjusted and maintained as described previously (Grosse-Siestrup *et al.*, 2002b, 2004). During perfusion, samples of blood and dialysate were taken periodically for standard clinical laboratory analysis, as described previously (Grosse-Siestrup *et al.*, 2002b, 2004). This included determination of the concentrations of the marker enzymes ALT and aspartate aminotransferase (AST) in the blood. The amount of bile production was measured at the indicated time points.

Application of *Candida* cells. After establishing the model, four livers were infected with *C. albicans* and perfused for 12 h (see above). Cells were applied to the liver surface following a protocol modified from that of Kvaal *et al.* (1999). Briefly, a sterile cotton patch (0.5 cm^2) was inoculated with $10\text{ }\mu\text{l}$ *C. albicans* suspension (2×10^7 cells), applied to the surface and covered with a sterile plastic patch (1 cm^2) to protect the fungal cells and prevent a free-oxygen exchange with the environment (microaerophilic to anaerobic conditions). For each strain and each time point, a single cotton patch was used and different strains and different time points were investigated on a single organ.

Histological examination. For histological examination of the invasion process, the fungal patches were removed from the surface at the indicated time points and the underlying region of the liver with adhered and invaded cells was cut out with a sterile scalpel. As a control, a piece of uninfected liver was cut out at the end of the perfusion. The liver pieces were fixed immediately in 2.5 % glutaraldehyde with 2 % paraformaldehyde in PBS (pH 7.4) and stored

Fig. 1. Liver perfusion set-up. (a) Schematic drawing of the blood and dialysate circuit. (b) Photograph of the laboratory set-up. A.h., arteria hepatica circuit; P.v., portal vein circuit.

at room temperature until further use. Embedding, cutting and staining were carried out as described previously (Schaller *et al.*, 1999).

Statistical analysis. All data were analysed using Microsoft Excel. If not indicated otherwise, data are presented as means $\pm$ SD. Significance was calculated using Student's *t*-test.

RESULTS AND DISCUSSION

Several studies have described the extracorporeal haemo-perfused liver as a suitable model to study, for example, hepatotoxicity (Groneberg *et al.*, 2002). Other protocols have shown that a liver can be perfused successfully for up to 72 h (Butler *et al.*, 2002). However, none of the described models have used the perfused organ to study interaction with pathogenic micro-organisms. Here, we describe for the first time the successful infection of extracorporeal haemoperfused porcine liver with the human fungal pathogen *C. albicans*.

Perfusion set-up

To keep the set-up of our model as simple as possible, we developed a protocol based only on general medical

equipment (e.g. roller pumps instead of artificial hearts). To supply the liver with blood with an oxygen level similar to physiological conditions, we mixed the oxygen-poor ('venous') blood in our blood reservoir with oxygen-rich ('arterial') blood and applied this mixture to the organ via the portal vein (in parallel with oxygen-rich blood via the hepatic artery; Fig. 1). With this set-up, we were able to keep the liver functional (see below). In fact, histological examination after 12 h of perfusion showed no lesions and showed that the surface of the organ was intact (see Fig. 3a). Therefore, our set-up was comparable with previously perfused organs (Butler *et al.*, 2002; Nagel *et al.*, 2005).

Perfusion parameters

For functional analysis of the perfused liver, we chose parameters such as bile production, oxygen consumption and liver weight gain, and measured the blood concentration of the marker enzymes ALT and AST to quantify liver damage. ALT and AST are marker enzymes of the liver and an increased level of each of these enzymes in the blood is an established indicator of liver damage (Giannini *et al.*, 2005).

As shown in Table 1, bile was constantly produced over the whole perfusion time in infected livers. Similarly, oxygen was consumed by the infected organs with a slight decrease over time. These data were similar to those obtained from uninfected organs (data not shown) and to other perfusion systems (Butler *et al.*, 2002; Grosse-Siestrup *et al.*, 2002b; Nagel *et al.*, 2005). We observed only a moderate weight gain of the organs after 12 h of perfusion compared with the 3 h perfused livers (Table 1). This gain was possibly due to oedema caused by post-ischaemic injury (Nagel *et al.*, 2005).

For the determination of ALT and AST levels, we compared data from uninfected livers with data from the first 3 h of the infected organ. We also determined the enzyme levels of the infected livers after 3 h; however, at this time point we began to take samples by cutting out liver pieces for histological examination. Therefore, ALT and AST concentrations were increased in the blood due to the cutting process (data not

Fig. 2. Box plot of AST and ALT concentrations in the blood from eight different perfusions. *, Significant difference compared with the initial value ($P < 0.05$).

shown). As shown in Fig. 2, the level of AST increased slightly but significantly during the first 3 h, whereas the ALT level was relatively constant and in the normal porcine range (Butler *et al.*, 2002). The increase in AST level could be explained again by post-ischaemic injury. No difference was detected between infected and uninfected livers within the first 3 h (not shown), showing that the damage of the organ due to the infection process with *Candida* cells is negligible in the early stages of invasion. This finding is in agreement with the *in vivo* situation where an increase in enzyme levels after i.p. infection can only be measured at later time points (after 6 h in mice; Kretschmar *et al.*, 1999a).

Table 1. Bile production, oxygen consumption and liver weight gain during 12 h of perfusion ($n = 4$)

Time (min)	Bile production (ml h ⁻¹)	Oxygen consumption (μmol min ⁻¹ per 100 g)	Liver weight gain (%)*
60	2.55 ± 3.88	81.34 ± 16.99	NA
180	3.13 ± 0.75	87.12 ± 11.56	11.65 ± 11.03†
300	1.99 ± 1.20	81.91 ± 12.30	NA
420	1.43 ± 1.13	68.26 ± 27.78	NA
540	2.70 ± 1.61	77.97 ± 22.06	NA
720	2.35 ± 0.85	66.58 ± 24.83	16.98 ± 21.85

*NA, Not applicable.

†Liver weight gain of the four livers used to set up this model.

Comparison of invasive and non-invasive strains

To study the invasion of *C. albicans* into the perfused pig liver, we used two different approaches: the first approach was a comparison of an invasive strain (SC5314) and a non-invasive strain (ATCC 10231; Kretschmar *et al.*, 1999a, b). The second approach was a time course of invasion of strain SC5314. Based on the results obtained during *in vivo* infection of mice (Felk *et al.*, 2002; Kretschmar *et al.*, 1999a), we took samples for histological examination at time points of 3 (not shown), 5, 7 and 10 h post-infection (p.i.) (Fig. 3).

Fig. 3. Representative pictures of uninfected liver after 12 h of perfusion (a) and of liver infected with *C. albicans* (b–e). Strain ATCC 10231 showed no invasion after 10 h of perfusion (b); strain SC5314 showed no invasion after 5 h (c) but started to invade after 7 h (d) and reached deeper hepatocytes after 10 h of perfusion (e). Bar, 25 μ m.

As shown in Fig. 3, the highly virulent strain SC5314 had invaded deep into the perfused liver after 10 h (Fig. 3e), whereas strain ATCC 10231 was clearly non-invasive (Fig. 3b). The starting inoculum seemed not to play a role during local invasion, as lower cell numbers showed the same differences between the two strains (data not shown). This result is consistent with the findings in i.p.-infected mice (Kretschmar *et al.*, 1999a, b). This non-invasive phenotype of ATCC 10231 is likely to be the main reason for the fact that this particular *C. albicans* strain is avirulent in all animal models tested so far (Balish & Phillips, 1966; Phillips & Balish, 1966; Schmidt & Geschke, 1996).

Time course of invasion

As strain ATCC 10231 was non-invasive in our *ex vivo* perfused model (confirming that our model mimics the situation *in vivo*), we focused our analysis on the temporal progression of invasion of strain SC5314. As shown in Fig. 3(c), no invading cells of strain SC5314 were detected 5 h after infection of the surface of the liver. However, invasion was clearly visible 7 h after infection. Here, *C. albicans* cells had switched to the hyphal mode of growth and penetrated into the liver capsule (Fig. 3d). After 10 h, hyphae had also reached the deeper hepatocytes (Fig. 3e). The invasion process over time was very similar to the observations made by Felk *et al.* (2002) and Kretschmar *et al.* (1999a) with i.p.-infected mice. The only difference observed in our model compared with *in vivo* infection was the fact that invasion into the capsule of the pig liver began later than in mice (~ 4 h p.i. in mice vs ~ 7 h p.i. in perfused livers). One reason for this difference might be the thicker organ capsule of the pig liver compared with the mouse liver.

The perfused liver as a model to study invasion

Several models to study interaction and/or invasion or transmigration of *C. albicans* through mammalian cells have been described in the literature. These models include *in vitro*

monolayers to investigate the interaction of *C. albicans* with human umbilical vein endothelial cells (Filler *et al.*, 1995) or brain microvascular endothelial cells (Jong *et al.*, 2001), transmigration models for transmigration through endothelial layers (Zink *et al.*, 1996) and intestinal cells (Weide & Ernst, 1999), more sophisticated three-dimensional models such as reconstituted human epithelium (Schaller *et al.*, 1998) and *in vivo* models (Kretschmar *et al.*, 1999a, b).

In our study, we showed that the *ex vivo* perfused liver is another suitable model to study invasion of *C. albicans* and possibly other microbes that invade human liver tissue. In contrast to the other models mentioned above, our invasion model has several advantages: (i) The large surface of the pig liver can be used to compare several strains or to investigate several time points of invasion with just one organ; this has the advantage that, for example, a mutant strain can be compared directly with the corresponding wild-type strain leading to a higher reproducibility compared with *in vivo* experiments where each strain or time point has to be tested in a separate animal. (ii) The *ex vivo* perfused liver represents the closest model of the *in vivo* situation (Groneberg *et al.*, 2002). Although limited only to one infection site, this model includes almost the entire repertoire of immune cells and opens up the possibility of investigating interaction of *C. albicans* cells with host cells in an *in vivo*-like situation and analysing both the pathogen and the host simultaneously during infection. (iii) Finally, using perfused organs contributes to the '3R' concept of animal experimentation (Grosse-Siestrup *et al.*, 2002a, b) so that in future the number of animals required to study invasion of *C. albicans* could be reduced.

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