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The function of transgenic human DAF-expressing porcine livers during hemoperfusion with human blood

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Abstract Extracorporal pig liver perfusion could bridge the deadly problem of acute human liver failure. However, preformed natural antibodies and complement activation (CA) are the predominant mechanisms of hyperacute xenogeneic rejection. The blockade of both pathways of CA in the xenograft, using transgenic livers expressing human decay accelerating factor on the endothelial surface results in prolonged graft survival and lower release of mediators.

Introduction

In the search for a temporary substitute for a non-functioning liver during hepatic coma, animal organs such as pig livers were connected to the patients' circulation [4]. These xenografts were rejected hyperacutely without major effect. New means have been found recently to improve this situation. Beside the absorption of multireactive preformed natural antibodies (PNAb) by plasmaphereses or apheresis, the breeding of transgenic pigs expressing human complement regulatory proteins such as decay accelerating factor (DAF) on the endothelial cell surface is the most advanced technique [8]. The combination of these methods seems to be able to prolong the survival time (SVT) of extracorporeal livers due to inhibition or reduction of antibody binding, complement activation, cell adherence and, finally, cytokine release.

Applying these means it seems to be possible to substitute metabolic liver failures after intoxication for a clinically relevant time [1, 2, 6]. In case the patient's own liver recovers, allogeneic transplantation could be avoided. We constructed and tested, therefore, an extracorporeal hepatic perfusion device, which allows investigation of the function of porcine livers when perfused with native, heparinized human blood undergoing hyperacute rejection (HXR). This system allows monitoring of the immediate reaction of the xenogeneic human blood elements when contacting the endothelium or the sinusoidal cells of the porcine liver [7].

Materials and methods

Three transgenic pigs (TP) (body weight 16.3 ± 1.5 kg) and six ordinary landrace pigs (NP) (body weight 25.0 ± 1.6 kg) were heparinized and underwent hepatectomy. The livers (TP: 535 ± 42 g; NP: 689 ± 29 g) were washed clear by infusion of 2000 ml of UW solution at 4 °C via catheters in the portal vein and hepatic artery. After a cold ischemia time of 92 ± 8 min the livers were perfused

Table 1 Data presented (mean ± SEM) of the transgenic human decay accelerating factor (DAF)-expressing pig livers (*n* = 3) perfused with human blood. (*Rah* Vascular resistance of the hepatic artery, *Rvp* vascular resistance of the portal vein, *WBC* white blood cells, *GLDH* glutamate dehydrogenase, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase, *CK* creatinine kinase, *Hggt* hemagglutination, *TNFα* tumor necrosis factor α, *IL-6* interleukin-6, *IFNγ* interferon-γ)

| Parameter measured	Perfusate sampling time (min)								
	0	EF	5	15	30	60	90	120	180
Rah (mm Hg/ml per min)	0.76 ± 0.23	0.66 ± 0.22	0.87 ± 0.57	0.87 ± 0.57	0.80 ± 0.47	0.43 ± 0.03	0.44 ± 0.01	0.41 ± 0.02	0.40 ± 0.01
Rvp (mm Hg/ml per min)	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	0.13 ± 0.01	0.13 ± 0.01
WBC (1000/µl)	3.23 ± 0.09	2.70 ± 0.61	1.07 ± 0.15	1.37 ± 0.27	1.30 ± 0.26	1.27 ± 0.29	1.33 ± 0.32	1.33 ± 0.32	1.23 ± 0.17
GLDH (U/l)	0.27 ± 0.13	8.87 ± 3.25	3.77 ± 1.31	4.47 ± 1.54	5.57 ± 1.28	6.07+2.02	6.87 ± 1.56	8.53 ± 2.27	9.20 ± 2.88
AST (U/l)	4 ± 1	198 ± 131	71 ± 43	83 ± 41	92 ± 42	103 ± 48	118 ± 52	139 ± 56	168 ± 60
ALT (U/l)	4 ± 1	12 ± 5	6 ± 2	7 ± 2	8 ± 2	8 ± 2	10 ± 3	10 ± 3	11 ± 3
LDH (U/l)	62 ± 4	487 ± 279	208 ± 97	242 ± 84	293 ± 89	335 ± 113	396 ± 124	489 ± 136	651 ± 158
CK (U/l)	18 ± 3	94 ± 24	58 ± 27	101 ± 32	135 ± 38	178 ± 62	232 ± 79	276 ± 96	342 ± 119
Hggt (%)	100.0 ± 0	4.67 ± 3.94	1.53 ± 0.90	1.53 ± 0.90	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
CH50 (%)	100.0 ± 0	–	–	–	–	81.5 ± 2.4	–	81.5 ± 1.0	77.7 ± 4.5
AP50 (%)	100.0 ± 0	–	–	–	–	85.7 ± 5.9	–	83.4 ± 5.3	81.2 ± 10.4
C4 (%)	100.0 ± 0	–	–	–	–	84.1 ± 2.5	–	83.5 ± 2.7	86.8 ± 3.1
C3 (%)	100.0 ± 0	–	–	–	–	85.2 ± 5.1	–	85.1 ± 3.9	86.1 ± 4.9
TNFα (pg/ml)	1.0 ± 0.3	4.7 ± 4.2	2.0 ± 1.2	3.7 ± 2.7	39.3 ± 15.4	266.7 ± 114.4	496.0 ± 98.8	666.0 ± 140.0	1003.0 ± 115.9
IL-6 (pg/ml)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.0 ± 0.5	8.0 ± 2.9	39.0 ± 19.7	275.3 ± 50.2
IFNγ (pg/ml)	0 ± 0	48.0 ± 36.2	28.3 ± 14.7	38.3 ± 19.2	43.7 ± 30.0	71.0 ± 34.4	92.0 ± 41.5	117.0 ± 23.5	114.0 ± 19.9
6kPGF1α (pg/ml)	22 ± 3	1230 ± 400	625 ± 80	1087 ± 317	974 ± 321	1120 ± 161	2815 ± 182	3995 ± 848	5118 ± 743

Principle of Perfusion
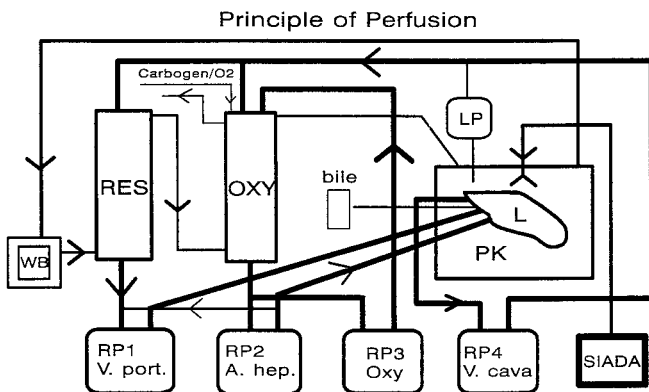
Fig. 1 Scheme of the pig liver perfusion model. (*WB* Warm water bath at 38 °C, *RES* heated blood reservoir, *OXY* heated oxygenator, *RP1–4* roller pumps for the portal vein, hepatic artery, oxygenator, and vena cava, respectively, *LP* Lenz pump to recirculate extrahepatic bleeding, *L* liver packed in a bag, *PK* perfusion chamber filled with 38 °C NaCl solution, *SIADA* simulation of the intraabdominal pressure amplitude)

with heparinized blood in which the pH was adjusted to 7.4 and the temperature to 37 °C. Fresh human blood (1.6 l) from three donors of identical blood groups was diluted to a hematocrit of 30 % with a multielectrolyte solution. The blood was oxygenized and applied under a flow rate of 1.0 ml/g per min (25–30 % over the hepatic artery and 70–75 % over the portal vein. Steady pressure was controlled electronically. The livers were mounted in a plastic bag which was fitted into a transparent perfusion chamber. Around the plastic bag, phosphate-buffered saline (PBS) of 38 °C was circulating. The liver was placed in physiological saline in a vertical position in the air-fed chamber. This fixation allowed changes during the respiratory cycle to be mimicked (Fig. 1).

The metabolic capacity of the xenoperfused liver was determined measuring bile production and oxygen consumption (artery-vein oxygen difference). Perfusate was sampled at times 0, EF, 5, 15, 30, 45, 90, 120, and 180 min. In order to monitor the mechanisms of HXR, liver weights (g), liver-specific enzymes (U/l), electrolyte (mmol/l), white blood cells and platelets (1000 cells/µl), and vascular resistance (as a quotient of perfusion pressure to flow) of the hepatic artery and of the portal vein (mm Hg/min per ml) were determined. Human tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and interferon-γ (IFNγ) (pg/ml) were detected by enzyme-linked immunosorbent assays, the prostaglandin I₂ metabolite, 6kPGF1α (pg/ml), by radioimmunoassay. The quantity of PNAb was titrated in a hemagglutination assay using pig red blood cells [5]. Complement consumption was

Table 2 Data (mean ± SEM) from ordinary pig livers (n = 6) hemoperfused with human blood. For abbreviations, see Table 1

Parameter measured	Perfusate sampling time (min)								
	0	EF	5	15	30	60	90	120	180
Rah (mm Hg/ml per min)	1.17 ± 0.12	1.16 ± 0.12	1.17 ± 0.09	1.24 ± 0.10	1.22 ± 0.09	1.25 ± 0.10	1.29 ± 0.08	1.33 ± 0.08	1.32 ± 0.07
Rvp (mm Hg/ml per min)	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
WBC (1000/μl)	4.88 ± 0.16	6.95 ± 0.94	2.90 ± 0.43	2.15 ± 0.29	2.13 ± 0.18	2.08 ± 0.62	2.87 ± 0.49	3.22 ± 1.10	3.78 ± 1.76
GLDH (U/l)	2 ± 1	6 ± 2	7 ± 3	8 ± 2	8 ± 4	9 ± 3	10 ± 3	10 ± 3	12 ± 4
AST (U/l)	7 ± 1	256 ± 104	148 ± 44	194 ± 56	215 ± 51	241 ± 54	311 ± 80	353 ± 93	591 ± 157
ALT (U/l)	5 ± 1	14 ± 4	11 ± 2	13 ± 3	14 ± 2	15 ± 3	18 ± 4	20 ± 5	29 ± 8
LDH (U/l)	144 ± 9	541 ± 230	406 ± 121	473 ± 110	609 ± 152	682 ± 146	782 ± 144	850 ± 139	1218 ± 260
CK (U/l)	26 ± 4	79 ± 23	143 ± 40	228 ± 62	273 ± 67	340 ± 81	427 ± 136	434 ± 118	512 ± 130
Hggt (%)	100.0 ± 0	1.05 ± 0.43	0.30 ± 0.15	0.40 ± 0.18	0.17 ± 0.13	0 ± 0	0 ± 0	0 ± 0	0 ± 0
CH50 (%)	100.0 ± 0	–	–	–	–	66.4 ± 4.0	–	63.1 ± 8.5	57 ± 7.3
AP50 (%)	100.0 ± 0	–	–	–	–	67.9 ± 10.5	–	61.3 ± 7.5	60.1 ± 6.5
C4 (%)	100.0 ± 0	–	–	–	–	71.2 ± 7.8	–	60.6 ± 14.4	59.0 ± 13.9
C3 (%)	100.0 ± 0	–	–	–	–	78.3 ± 2.3	–	79.2 ± 9.4	71.1 ± 8.03
TNFα (pg/ml)	8.8 ± 5.9	65.3 ± 11.5	38.3 ± 13.8	58.7 ± 15.4	178.0 ± 31.6	419.8 ± 65.1	561.8 ± 73.0	861.2 ± 126.3	1103.2 ± 179.4
IL-6 (pg/ml)	2.0 ± 1.0	1.0 ± 0.5	1.8 ± 0.7	2.0 ± 0.7	2.3 ± 0.6	11.7 ± 1.6	46.8 ± 5.8	146.8 ± 20.0	370.0 ± 58.8
IFNγ (pg/ml)	2.5 ± 2.5	4.5 ± 4.5	6.0 ± 3.9	14.7 ± 11.6	19.5 ± 14.3	45.8 ± 16.7	72.2 ± 13.6	137.2 ± 17.4	146.8 ± 25.1
6kPGF1α (pg/ml)	0 ± 0	303 ± 62	359 ± 21	364 ± 52	435 ± 69	1042 ± 152	1403 ± 122	1499 ± 163	1720 ± 198

measured using the CH50 (%) and AP50 (%) tests. C4 and C3 (mg/l) were directly determined in the plasma aliquots. At completion of the perfusion, tissue sections were snap-frozen for histological and immunohistological examination.

PNAb of IgM and IgG types and the complement components of the classical and alternative complement pathways were stained using the appropriate secondary detecting antibodies.

Results

All data were tested by the Wilcoxon test in each group and by variance analysis and LS means test between groups (P < 0.05). Because n = 3 in the TP group, statistical tests did not reach significant levels, but the trend was positive. Data can be compared in Tables 1–3.

Physical characteristics

In the NP group, the mean liver weight increased by 14.1 % in contrast to only 9 % in the TP group. Bile flow (expressed in ml/h) increased by 28.6 % during reperfusion but did not change in the TP group. Resistance values showed marked differences between the hepatic artery and the portal vein, with a very high initial resistance in the portal vein. Resistance in the hepatic artery showed no particular pattern. All values were found to be lower in the TP than in the NP group. A traumatic leukocyte depletion, directly after starting reperfusion, was found to be identical in both groups.

Biochemical characteristics

A significant difference existed in plasma levels of creatinine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), [glutamate oxaloacetate transferase (GOT], alanine aminotransferase (ALT) [glutamate phosphotransferase (GPT)], and glutamate dehydrogenase (GLDH) immediately after reperfusion and at the end of the experiment between the two groups. The perfusing system only had no impact on biochemical results or white blood cell counts and cytokines, nor did the titers of PNAb or the amount of complement change.

Immunological parameters

Both the TP and NP groups showed a rapid decrease of white blood cells and thrombocytes after circulating

Table 3 Data (mean ± SEM) of the controls (human blood circulating the system without a graft; $n = 5$). For abbreviations, see Table 1

Parameter measured	Perfusate sampling time (min)							
	0	5	15	30	60	90	120	180
WBC (1000/μl)	4.28 ± 0.48	4.04 ± 0.48	3.78 ± 0.36	3.76 ± 0.37	3.20 ± 0.28	3.34 ± 0.47	3.40 ± 0.42	3.27 ± 0.30
GLDH (U/l)	0.62 ± 0.23	0.48 ± 0.16	0.46 ± 0.07	0.58 ± 0.17	0.62 ± 0.16	0.80 ± 0.20	0.84 ± 0.20	0.72 ± 0.24
AST (U/l)	5 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 1	6 ± 1	6 ± 1
ALT (U/l)	3 ± 1	3 ± 1	3 ± 1	3 ± 1	3 ± 1	3 ± 1	3 ± 1	4 ± 1
LDH (U/l)	122 ± 15	119 ± 10	127 ± 10	141 ± 13	155 ± 20	180 ± 29	191 ± 35	230 ± 45
CK (U/l)	25 ± 7	25 ± 7	26 ± 8	26 ± 8	25 ± 7	25 ± 7	25 ± 7	25 ± 7
Hggt (%)	100.0 ± 0	100.0 ± 0	100.0 ± 0	100.0 ± 0	100.0 ± 0	100.0 ± 0	100.0 ± 0	100.0 ± 0
CH50 (%)	100.0 ± 0	–	–	–	99.4 ± 0.6	–	96.8 ± 2.4	98.0 ± 2.0
AP50 (%)	100.0 ± 0	–	–	–	96.1 ± 3.9	–	99.4 ± 0.6	100.0 ± 0
C4 (%)	100.0 ± 0	–	–	–	98.8 ± 1.2	–	100.0 ± 0	100.0 ± 0
C3 (%)	100.0 ± 0	–	–	–	100.0 ± 0	–	100.0 ± 0	96.3 ± 3.7
TNFα (pg/ml)	14.4 ± 11.8	9.8 ± 7.9	10.0 ± 8.1	12.0 ± 8.7	200.8 ± 87.4	642.6 ± 324.2	957.8 ± 324.2	1318.0 ± 289.8
IL-6 (pg/ml)	4.0 ± 1.4	2.2 ± 0.4	2.6 ± 0.9	3.8 ± 0.7	6.8 ± 3.6	72.6 ± 41.3	312.4 ± 93.4	519.8 ± 65.4
IFNγ (pg/ml)	2.0 ± 1.1	25.2 ± 13.3	23.4 ± 10.6	21.6 ± 9.1	27.4 ± 12.5	38.2 ± 20.3	23.4 ± 8.9	31.6 ± 12.7
6kPGF1α (pg/ml)	30 ± 28	36 ± 32	36 ± 25	7 ± 3	7 ± 3	6 ± 2	16 ± 8	46 ± 21

the xenograft. PNAb were depleted rapidly from the circulating blood in both groups.

Marked differences were found in complement consumption. In the NP group, the CH50 and AP50 tests revealed a 70% decrease but this was only 20% in the transgenic group. In plasma, C4 and C3 showed no decrease in the peripheral blood of the TP group but a significant reduction in the NP group. This was reflected by immunohistology showing no staining for C4/d/d and C3/c/d in the NP group but marked staining in the NP group.

Levels of the proinflammatory cytokines, TNFα, IL-6, and IFNγ, increased significantly during the first 60 min of reperfusion without noticeable differences, however, between the two groups. Values of 6kPGF1α were found to increase significantly during hemoperfusion of livers of both groups with a much higher release in the TP group.

even more, the modification of the transgenic pigs, [3, 9] improved the perfusion parameters significantly. Dramatic differences were seen in the release of specific enzymes, prostaglandins, and cytokines. This might be due to the significantly lower consumption of complement and its activation on the endothelial cells surfaces, as shown by immunohistology.

The expression of human DAF on endothelial cells seems to reduce the early endothelial cell stimulation by preventing the extreme hemorrhage and edema seen in normal livers. The significantly reduced complement activation by these complement regulator proteins also prevents endothelial cell activation, as shown by the low release of cytokines and high release of prostaglandins. In the event that the observed increase of resistance can be inhibited by DAF antagonists and antibody aphereses, this temporary approach could be the first clinically relevant application of a discordant xenograft.

Discussion

Until recently the temporary xenogeneic hemoperfusion of porcine livers was one of the only clinical applications of discordant xenografts. New developments in many directions, especially in producing transgenic pigs, could further improve the beneficial results achieved.

Our reperfusion system allows a careful management of the organ in applying physiological conditions. The specially prepared tubing system and oxygenator but,

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