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Increased apoptosis of hepatocytes in vascular occlusion after orthotopic liver transplantation

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Abstract Early vascular occlusion is liable to cause graft failure, and differential diagnosis between this condition and primary nonfunction (PNF) caused by preservation injury may be difficult. Apoptosis has been detected in immunomediated cytotoxicity and is known to be triggered by mild ischemia. In a retrospective analysis we investigated the role of apoptosis in vascular occlusion, PNF, and acute allograft rejection to improve the differential diagnosis of early graft failure. The liver graft histology of 75 patients (46 male, 29 female) a median 47 (1–64) years of age was screened semiquantitatively for the rate of apoptosis on the hematoxylin-eosin stain (HE) and by the in situ end nick labeling technique (TUNEL). This cohort included all patients who developed PNF ($n = 9$) or vascular occlusion ($n = 11$) after orthotopic liver transplantation (OLT) in the years 1992 to 1996. Within this period of time we performed 205 OLTs on 189 patients. We further included 22 patients with early acute rejection and 11 controls. The highest rates of apoptotic hepatocytes were seen in vascular occlusion ($P < 0.001$). Grafts with PNF were explanted 1–3 days after OLT and showed hepatocytes that were 100 % necrotic. Cases of acute early rejection showed a significantly higher apoptotic cell count than did normal controls ($P < 0.003$), increasing in direct proportion to the severity of rejection. Screening biopsies for the rate of apoptosis can improve the efficacy and accuracy of differential diagnosis of early graft failure.

Key words Apoptosis of hepatocytes · Vascular occlusion · Liver transplantation

Introduction

Primary nonfunction (PNF) refers to graft failure during the immediate postoperative period, excluding other causes of graft loss such as blood loss, vascular occlusion, sepsis and hyperacute rejection [26]. Without retransplantation, patients die within 1 week [23]. PNF is caused by parenchymal damage due to disturbances of the microcirculation and thrombosis of the sinusoids, a phenomenon collectively known as preservation injury [4].

Early graft loss is mainly due to PNF or vascular occlusion [26]. Major vessel thromboses may be a result of microvascular preservation injury [6] leading to low flow and hypercoagulability. Clinical presentation and laboratory tests in connection with vascular occlusion are variable and not specific [17, 27]. Therefore, angiography and, to a certain extent, ultrasound examinations are the diagnostic tools of first choice in vascular occlusion [1, 8, 13, 15, 20].

Different forms of cell death of hepatocytes caused either by necrosis or apoptosis have been investigated in the pathogenesis of many hepatobiliary diseases [21,

24]. Apoptosis is encountered in immunomediated allograft rejections, can be triggered by mild ischemia, and was found in time-zero biopsies to be the best predictor of PNF [1, 2, 9, 12, 14, 25, 27, 28].

We investigated apoptosis and necrosis of hepatocytes in vascular occlusion, PNF, and acute allograft rejection to improve the differential diagnosis of early graft loss and to find out whether these results are of prognostic value.

Patients and methods

Patients

The liver graft histology of 75 patients (46 male, 29 female) a median 47 (1–64) years of age was analyzed. All patients who underwent retransplantation for PNF ($n = 9$) or vascular occlusion ($n = 10$) or successful revascularization after hepatic artery thrombosis (HAT) ($n = 1$) between 1992 and 1996 were included. Time-zero biopsies from donor livers and patients with normal graft function ($n = 11$) plus 22 cases of acute early graft rejection were used as controls.

All patients were treated with the same initial immunosuppressive regimen: 2.0 mg/kg body weight rabbit antihuman thymocyte immunoglobulin i.v., 8 mg/kg per day cyclosporine from day 8 on, and a tapering dose of dexamethasone were administered.

In the case of explantation or revascularization, multiple biopsies were taken from both lobes. Acute early rejection occurring within 3 months from grafting and requiring therapy was defined as a deterioration of liver function with a doubling of serum transaminases in two different and subsequently taken blood samples. To confirm the clinical diagnosis, a needle biopsy was performed. The histological grading of rejection was estimated according to Banff's scheme by global assessment and graded into categories of mild, moderate, and severe [3]. Acute viral hepatitis was excluded by serological examination and immunohistochemistry.

The biopsy specimens were screened semiquantitatively for the rate of apoptosis on the hematoxylin-eosin stain (HE) and by the in situ end nick labeling technique (TUNEL) to enable an independent detection of DNA fragmentation by two pathologists.

TUNEL

For the detection of DNA fragmentation, sections of paraffin-embedded tissue were dewaxed by washing in xylene and rehydrated through a graded series of ethanol and dH_2O: the sections were incubated with protein kinase K (20 µg/ml, Sigma, St. Louis, Mo., in 10 mM Tris/HCl, pH 7.4–8.0) for 15 min at 37 °C. The in situ DNA fragmentation assay was performed by applying the in situ cell death detection kit AP (Boehringer Mannheim, Mannheim, Germany; cat. no. 1684809) based on the method by Gavrieli et al. [10]. In humidified chambers (at 37 °C for 60 min), the samples were incubated with the TUNEL reaction mixture of the kit that contains terminal deoxynucleotidyl transferase and fluorescin-labeled dUTP. Then the converter AP solution of the kit (alkaline phosphatase-conjugated anti-fluorescin antibody) was applied for 30 min at 37 °C. After PBS-washing, the slides were treated with an alkaline phosphatase substrate kit (Vectastain, SK-5100; Vector Lab, Burlingame, Calif.) to visualize the alkaline phosphatase and were counterstained with Meyer's haemalum. Negative controls were treated with a TUNEL reaction mixture without terminal transferase.

Quantitative evaluation

An apoptotic index of the TUNEL assay was calculated for each sample by counting the number of positively-stained nuclei and dividing that amount by the total number of cells × 100. A total of 3000 liver cells on each slide were counted. The data were expressed as mean +/– standard deviation. For the semiquantitative assessment of apoptotic bodies (ABs), a scale from 0 to 4+ was used: 0 (no ABs found), 1+ (very few ABs), 2+ (few ABs), 3+ (many ABs, but not seen in every higher-powered field), 4+ (abundant ABs, each higher-powered field containing ABs). Apoptotic cells were identified in HE sections as cells with fragmented nuclei and condensated cytoplasm or as small aggregates of nuclear and/or cytoplasmic fragments replacing one cell.

Statistics

Differences between controls, cases of rejection, and cases of vascular occlusion were tested by variance analysis using the statistical package SAS. When the overall test of a group effect was significant, comparisons between cases with vascular occlusion and the other two groups were made using the multiple comparison adjustment of Dunnett [11]. Associations between cases with vascular occlusion and the grades of rejection were proved by a linear trend test within an ANOVA. All probability values were two-sided and a P value of less than 0.05 was considered statistically significant.

Results

Liver grafts showing PNF were explanted 1–3 days after transplantation. Vascular complications occurred on median day 5 (days 1–14.5), and the recorded rejection episodes 30.5 days (9–90 days) after grafting. Vascular occlusion was diagnosed by sonography and/or angiography and cavography ($n = 9$), or intraoperatively by thrombectomy of the hepatic artery ($n = 2$). Of the patients showing vascular occlusion, 5 had HAT, 2 portal vein thrombosis (PVT), 2 HAT and PVT, and 2 inferior vena cava (IVC) thrombosis.

The highest rates of apoptotic hepatocytes were found in connection with vascular occlusion ($P < 0.001$). Apoptosis first appeared perivenously and then extended to the portal fields with increasing ischemic damage (Fig. 1). No correlation between the rate and pattern of apoptotic hepatocytes and the occluded major vessel was found. A percentage of necrotic hepatocytes of up to 50 % was observed perivenously with increasing ischemic damage (Table 1).

The biopsies taken intraoperatively after successful revascularization in one patient with HAT showed the same rate of apoptotic and necrotic hepatocytes as that observed in explanted grafts after vascular occlusion.

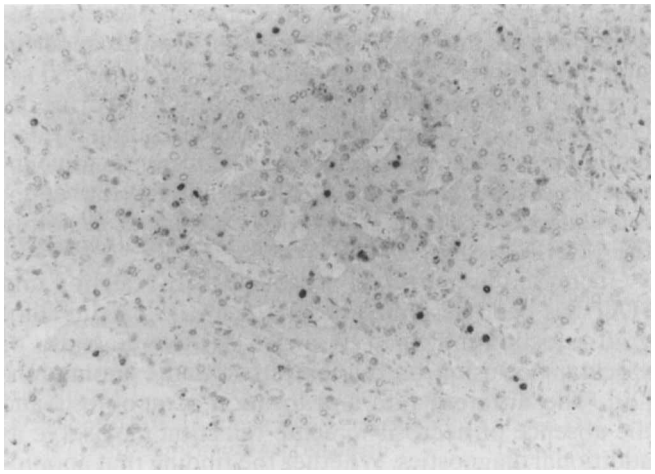

Fig. 1 TUNEL assay stain of ischemic liver graft after hepatic artery thrombosis with abundant positively-stained nuclei of apoptotic hepatocytes (magnification × 100)

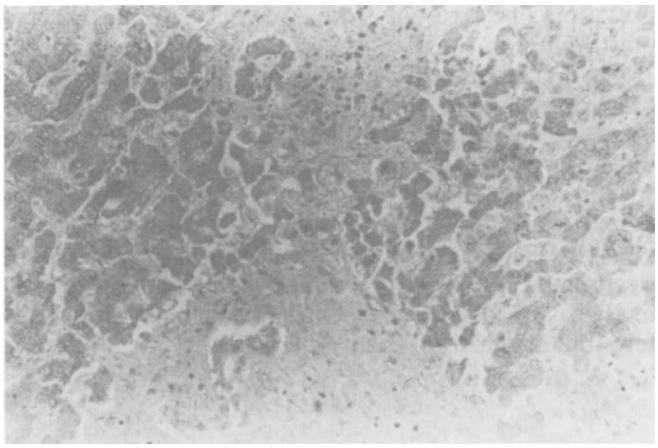

Fig. 2 TUNEL assay stain of a liver graft explanted 2 days after orthotopic liver transplantation because of primary nonfunction showing only necrotic hepatocytes (magnification × 100)

Explanted grafts after PNF showed only necrotic hepatocytes (Fig. 2).

Of the 22 biopsies following acute allograft rejection, 9 were classified as mild, 11 as moderate, and 2 as severe according to Banff's scheme [3]. We identified a significantly higher apoptotic cell count in biopsies following acute rejection than in normal controls ($P < 0.003$). In addition, we found that the rate of apoptotic hepatocytes increased with the severity of rejection. In contrast to vascular occlusions, in the case of acute rejection, apoptotic hepatocytes were seen throughout the lobules.

Table 1 Apoptotic indices of the TUNEL assay. Data are expressed as mean +/− standard deviation. The highest rates of apoptotic hepatocytes were found in the case of vascular occlusion ($P < 0.001$) in comparison to normal controls and cases of acute rejection. In explanted grafts with primary nonfunction *(PNF)*, 100% of the hepatocytes were necrotic. Cases of acute early rejection showed a significantly higher apoptotic cell count than did normal controls ($P < 0.003$), increasing in direct proportion to the severity of rejection

	$n = 75$	Rate of apoptosis per 100 hepatocytes	Percentage of necrotic hepatocytes
Vascular occlusion	11	7.880 +/− 0.533 ($P < 0.001$)	up to 50%
PNF	9	0.0	100%
Acute rejection	22	0.702 +/− 0.378 ($P < 0.003$)	0%
mild	9	0.505 +/− 0.260	
moderate/severe	13	0.866 +/− 0.389	
Controls	11	0.400 +/− 0.400	0%

Discussion

We investigated apoptosis and necrosis of hepatocytes in vascular occlusion, PNF, and acute allograft rejection to improve the differential diagnosis of early graft loss and to find out whether these results are of prognostic value.

Routine laboratory tests and the clinical presentation of patients with vascular occlusion of the liver graft are variable and of little help with respect to differential diagnosis. Angiography is the only reliable diagnostic tool to identify vascular thrombosis [8, 15, 18, 20, 22, 27].

Functioning liver grafts consist to a very large extent of living hepatocytes, but also contain a small number of dead hepatocytes as a result of necrosis or apoptosis. The relation of living to necrotic or apoptotic hepatocytes can be affected by many hepatobiliary diseases, acute allograft rejection, preservation injury, and ischemia. Apoptotic hepatocytes in time-zero biopsies have been described as a predictor for primary graft failure [1]. In grafts explanted for PNF, we only found massive necrosis without signs of programmed cell death. This may be explained by the fact that we performed a retrospective analysis of explanted grafts. Explantation was done 24–72 h after the first transplantation, and no sequential biopsies were available. Within this time, massive necrosis is caused by microcirculatory disturbances due to fibrin deposits in the hepatic sinusoids [4].

Vascular occlusion of major liver vessels does not always lead to extended necrosis as in other organs due to a dual vascular supply of hepatocytes by the hepatic artery and portal vein [5, 16]. Failure of arterial blood supply is compensated by accelerated venous blood flow and vice versa [18, 22]. In addition, mild damage of liver cells, which allows the formation of ATP and

proteins, leads to apoptosis and not to necrosis [21]. In case of a rapid occlusion of major hepatic vessels, compensation via the additional blood supply is insufficient. With increasing ischemic damage, we observed a percentage of necrotic hepatocytes of up to 50 %.

As reported in ultrasound studies, a thrombus in major hepatic vessels can begin as stenosis days before the occlusion [13, 20]. The predominance of zone 3 injury during ischemia is an expression of reduced oxygen delivery [7, 17]. Apoptosis first appeared and then extended to the portal fields with increasing ischemic damage [1, 17, 25]. In contrast to a necrotic cell, apoptotic hepatocytes are removed by phagocytosis within a few hours. The large numbers of apoptotic cells we have observed therefore reflect a continuous process of programmed cell death.

We decided not to exclude IVC thrombosis from the other forms of vascular occlusion of major hepatic vessels because identical high rates of apoptotic hepatocytes were seen in all specimens of explanted grafts with vascular occlusion. So far, we do not know the specificity of the response to insufficient vascular perfusion of the liver graft.

Assessment of graft recovery by the apoptosis index or the extent of necrosis was not possible. The only patient with successful revascularization showed an apoptotic index of 7.5 and a percentage of necrotic hepatocytes of between 30 and 50 % in all specimens taken from both lobes during revascularization. It possibly is not enough to estimate the prognosis of graft function by hypoxic cell damage alone without paying attention to the side effects of revascularization.

It is well known from the rodent model that liver cells differ in terms of their sensitivity to hypoxia and reperfusion damage [19]. Hepatocytes are very sensitive towards anoxia but relatively resistant towards reperfusion injury. In contrast, bile duct cells are damaged severely by oxygen radicals created during reperfusion. Graft function after successful revascularization is often limited by stricture of the bile ducts and consecutive infectious complications [8, 18, 20]. We did not find apoptotic bile duct cells in grafts with vascular occlusion in the TUNEL assay.

Increased apoptosis in acute allograft rejection and HAT has been described before [2, 14, 25]. Acute allograft rejection may lead to a false positive diagnosis of vascular occlusion on duplex ultrasound examinations [20]. The different rate and location of apoptosis and the absence of necrosis in acute rejection may serve as additional diagnostics. Whether the finding that apoptotic hepatocytes increase in direct proportion to the severity of rejection will become an additional diagnostic tool in clinical routine, cannot be answered at the present time. Further investigation of the subject is needed.

Our data seem to be clear but require cautious interpretation since we only looked at the end products of graft failure in the case of vascular occlusion and PNF. A lot of changes occur until a graft has to be explanted or revascularized. Our results coincide with those of other authors [2, 4, 12, 14, 18, 21, 22, 24]. Therefore, our observations have encouraged us to perform a prospective study of early graft failure with sequential biopsies.

As far as we can tell, the screening of liver graft biopsies for the rate of apoptosis/necrosis of hepatocytes is likely to improve the efficiency and accuracy of the differential diagnosis of early graft failure.

References

1. Abraham S, Furth EE (1996) Quantitative evaluation of histological features in "time-zero" liver allograft biopsies as predictor of rejection or graft failure: receiver-operating characteristic analysis application. Hum Pathol 27: 1077–1084
2. Afford S, Hubscher S, Strain A, Adams D, Neuberger J (1995) Apoptosis in the human liver during allograft rejection and end-stage liver disease. J Pathol 176: 373–380
3. An International Panel (1997) Banff schema for grading liver allograft rejection: an international consensus document. Hepatology 25: 658–663
4. Arai M, Mochida S, Ohno A, Fujiwara K (1996) Blood coagulation in the hepatic sinusoids as a contributing factor in liver injury following orthotopic liver transplantation in the rat. Transplantation 62: 1398–1401
5. Burton-Opitz R (1911) The vascularity of the liver. II. The influence of the portal flow upon the flow in the hepatic artery. Q J Exp Physiol 4: 93–102
6. Clavien PA, Harvey PRC, Strasberg SM (1992) Preservation and reperfusion injuries in liver allografts: overview and synthesis of current studies. Transplantation 53: 957–978
7. Colletti LM, Remick DG, Burtch GD, Kunkel SL, Strieter RM, Campbell DA Jr (1990) Role of tumor necrosis factor – α in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. J Clin Invest 85: 1936–1943
8. Drazan K, Shaked A, Olthoff KM, Imagawa D, Jurim O, Kiai K, Shackelton C, Bussutil R (1996) Etiology and management of symptomatic adult hepatic artery thrombosis after orthotopic liver transplantation. Am Surg 62: 237–240
9. Fraser A, Evan G (1996) A license to kill. Cell 85: 781–784
10. Gavrieli Y, Sherman Y, Ben-Sasson S (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119: 493–501
11. Hsu JC (1992) The factor analytic approach to simultaneous confidence interval for multiple comparisons with the best. J Comput Stat Graph 1: 151–168
12. Kerr J (1971) Shrinkage necrosis: a distinct form of cellular death. J Pathol 105: 13–20
13. Kok T, Slooff MJ, Thijn CJ, Peeters PM, Verwer R, Bijleveld CM, Berg AP van den, Haagsma EB (1998) Routine Doppler ultrasound for the detection of clinically unsuspected vascular complications in the early postoperative phase after orthotopic liver transplantation. Transpl Int 11: 272–276

14. Krams SM, Egawa H, Quinn MB, Villanueva JC, Garcia Kennedy R, Martinez OM (1995) Apoptosis as a mechanism of cell death in liver allograft rejection. Transplantation 59: 621–625
15. Langnas AN, Marujo W, Stratta RJ, Wood RP, Shaw BW Jr (1991) Vascular complications after orthotopic liver transplantation. Am J Surg 161: 76–83
16. Lemmens HP, Neumann U, Bechstein WO, Guckelberger O, Lusebrink R, Jonas S, Keck H, Neuhaus P (1998) Incidence and outcome of arterial complications after orthotopic liver transplantation. Transpl Int 11 [Suppl 1]: 251–254
17. Marotto ME, Thurman RG, Lemasters JJ (1988) Early midzonal cell death during low-flow hypoxia in the isolated perfused rat liver: protection by allopurinol. Hepatology 8: 585–590
18. Mor E, Schwartz ME, Sheiner PA, Menesses P, Hytiroglou P, Emre S, Kishikawa K, Chiodini S, Miller CM (1993) Prolonged preservation in University of Wisconsin solution associated with hepatic artery thrombosis after orthotopic liver transplantation. Transplantation 56: 1399–1402
19. Noack K, Bronk SF, Kato A, Gores GJ (1993) The greater vulnerability of bile duct cells to reoxygenation injury than to anoxia. Implications for the pathogenesis of biliary strictures after liver transplantation. Transplantation 56: 495–500
20. Nolten A, Sproat IA (1996) Hepatic artery thrombosis after liver transplantation: temporal accuracy of diagnosis with duplex US and the syndrome of impending thrombosis. Radiology 198: 553–559
21. Patel T, Gores GJ (1995) Apoptosis and hepatobiliary disease. Hepatology 21: 1725–1741
22. Payen DM, Fratacci MD, Dupuy P, Gatecel C, Vigouroux C, Ozier Y, Houssin D, Chapuis Y (1990) Portal and hepatic arterial blood flow measurements of human transplanted liver by implanted Doppler probes: interest for early complications and nutrition. Surgery 107: 417–427
23. Ploeg RJ, D'Alessandro AM, Knechtle SJ, Stegall MD, Pirsch JD, Hoffmann RM, Sasaki T, Sollinger HW, Belzer FO, Kalayoglu M (1993) Risk factors for primary dysfunction after liver transplantation – a multivariate analysis. Transplantation 55: 807–813
24. Rosser BG, Gores GJ (1995) Liver cell necrosis: cellular mechanisms and clinical implications. Gastroenterology 108: 252–275
25. Sedivy R, Gollackner B, Casati B, Mittlböck M, Kaserer K, Steininger R, Wrba F (1998) Apoptotic hepatocytes in rejection and vascular occlusion in liver allograft specimens. Histopathology 32: 503–507
26. Strasberg SM, Howard TK, Molmenti EP, Hertl M (1994) Selecting the donor liver: risk factor for poor function after orthotopic liver transplantation. Hepatology 20: 829–838
27. Tzakis AG, Gordon RD, Shaw BW Jr, Iwatsuki S, Starzl TE (1985) Clinical presentation of hepatic artery thrombosis after liver transplantation in the cyclosporine era. Transplantation 40: 667–671
28. Wyllie AH, Kerr JF, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68: 251–306