Localization of apoptosis proteins and lymphocyte subsets in chronic rejection of human liver allograft

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Abstract: In chronic liver rejection lymphocyte mediated processes lead to chronic inflammation, necrosis and repair mechanisms. The aim of the present study was to investigate the expression of apoptosis related proteins (FAS/APO-1, FAS-L, Bcl-2, Bax, TNF-α, and INF-γ). ApopTag reaction and immunohistochemistry were performed on liver samples of chronically rejected allografts and compared with normal donor livers. In chronic rejection, apoptosis was detected in pericentral hepatocytes and in the biliary epithelium. Bcl-2 was strongly expressed on lymphocytes around the bile ducts, but not on the biliary epithelium itself. Bax, FAS, TNF-α and INF-γ were present in pericentral areas. T-cells showed up around bile ducts, whereas macrophages around pericentral areas. In pericentral areas apoptosis seems to be fostered through TNF-α and INF-γ and by the lack of Bcl-2. Based on these results both downregulation and upregulation of apoptotic proteins can be observed in chronic liver allograft rejection: FAS is upregulated in biliary epithelium and zone 2, protein levels of FASL remain unchanged, BAX is upregulated in zone 3, BCL2 is downregulated in both biliary epithelium and zone 1 and both TNFa and IFN are upregulated in zone 3. Our results suggest that the balance between pro- and antiapoptotic patterns was shifted to the proapoptotic side, mainly in the centrilobular area of the hepatic lobule, and in the bile ducts. According to these findings in chronic rejection the predictive sites of apoptosis are the biliary epithelium and the pericentral areas.

Keywords: liver transplantation, chronic rejection, apoptosis, Fas, Bax, Bcl-2, TNF-alpha, biliary, fatty liver

Introduction

Apoptosis described by Kerr in 1972 [1], also called programmed cell death, is one of the most important factors in the pathogenesis of various liver diseases [2–5]. The main features of apoptosis include: deletion of single cells, cell shrinking, and the final formation of apoptotic bodies without inflammatory response and phagocytosis by adjacent normal cells and some macrophages, as well as compaction of chromatin into uniformly dense masses. Apoptosis is present at low rate in the human liver as a physiological part of liver cell homeostasis and at a higher rate in several liver diseases. Three proteins with different activities were found to have major role in apoptosis occurring in the liver: FAS/APO-1 and Bax are located mainly at the surface of hepatocytes and Bcl-2 is located on the biliary epithelium and on the lymphocytes [6–11]. In Fas-mediated apoptosis, Fas-L – expressed either on the cell surface of lymphocytes or intracellularly binds to Fas in a soluble form that induces apoptosis via the caspase-8 cascade. The multistep process is enhanced by Bax and inhibited by Bcl-2. TGF-β, which is a regulatory cytokine of the immune response [12] produced by macrophages, enhances the effect of Bax, while IFN-γ induces apoptosis via the TNF-α and perforin system as well. CD4 and CD8 positive T-cells also influence the effect of Bcl-2 [6–10]. Apoptosis was observed in several different complications following liver transplantation, such as acute rejection [5, 13, 14]. Since chronic liver rejection is characterized by the loss of bile ducts, arteriopathy and fibrosis [10, 14–19], at least two different pathophysiological aspects can be assumed: T-cell medi-

Table I Demographic data of the patients

No	Gender	Age	Disease	Graft survival (months)
1	Male	17	PSC	3
2	Male	25	PSC	96
3	Female	50	Hepatitis B	14
4	Male	50	Hepatitis C	4
5	Female	53	PBC	8
6	Female	48	Non-A non-B hepatitis	10
7	Male	60	PBC	35
8	Male	38	Hepatitis B + HCC	3
9	Male	41	Non-A non-B hepatitis	9
10	Female	39	Non-A non-B hepatitis	3

ated inflammation of the epithelial structures and parenchymatous scarring promoted by macrophages and lymphocytes [12]. Our previous study [15, 21] showed that TGFβ-1 – which plays a role in programmed cell death – is produced in higher rates in chronic allograft rejection by macrophages compared to TGFβ-1 synthesized by normal livers. In order to evaluate both presumed pathways, we investigated the expression patterns of three apoptosis proteins (FAS/APO-1, Bax and Bcl-2) and two cytokines (TNF-α, INF-γ) in comparison with lymphocyte and macrophage distribution, as well as ApopTag Plus reaction in chronically rejected human liver allografts.

Materials and Method

Tissue samples

Specimens from explanted liver allografts were obtained at the time of retransplantation of 10 patients with chronic rejection proven by histology of previous biopsies according to the Banff criteria [22]. Transplantations were performed for primary sclerosing cholangitis in two cases, for primary biliary cirrhosis in two cases and for hepatitis B and C in 6 cases. Median of the graft survival was 5.3 months (min. 3, max. 14) in 9 patient, and 8 years in one patient. Baseline immunosuppression consisted of cyclosporine and low-dose steroid. Acute rejection episodes were treated with daily bolus of methylprednison, antithymocyte globulin or Azathioprine. OKT3 was administered in one case of steroid resistant rejection. Baseline immunosuppression was converted to FK506 in three patients between weeks 15–30 after the OLT (orthotopic liver transplantation). Patient demographics are summarized in Table I. Healthy tissue controls with no morphological abnormalities were obtained from 8 healthy donor livers. All liver samples were snap-frozen in liquid nitrogen, then stored in a freezer at –80 °C until used, as well as fixed in 10% buffered formaldehyde, and embedded into paraffin.

Antibodies

All primary antibodies used in the study are summarized in Table II.

Immunohistochemistry

Immunohistochemical studies were performed as described earlier [15, 21]. Frozen liver specimens were cut into 5-µm-thick sections on a cryostat, fixed in acetone for 10 minutes immediately before use. For immunolabeling experiments, sections were preincubated with heat-inactivated human AB serum, diluted 1:10 in phosphate-buffered saline (PBS) for 20 minutes to block non-specific binding and then covered with a layer of the primary antibody in appropriate dilution for 60 minutes. After three washes in PBS, the secondary antibody (5% peroxydase coupled goat anti-mouse immunoglobulin (Jackson ImmunoResearch Europe Ltd. Soham, Cambridgeshire, UK) and 5% heat-inactivated human serum

Table II Antibodies used for the recent study

Specificity	Clone	Source
FAS/APO-1	Mouse, DX-2	DAKO, Denmark
Bcl-2	Mouse, Bcl-2, 124	DAKO, Denmark
Bax	Rabbit, N-20b	Santa Cruz Biotechnology
CD 3 (T cells)	Mouse, Leu-4	Becton Dickinson
CD 4 (T helper and macrophages)	Mouse Leu-3a	Becton Dickinson
CD 8 (T cytotoxic/suppressor)	Mouse, Leu-2a	Becton Dickinson
CD 20 (B cells)	Mouse, Leu-16	Becton Dickinson
CD 56 (NK cells)	Mouse, MY31	Becton Dickinson
25 F9 (macrophages)	Mouse, 25F9	Prof. Dr. Sorge, Munster
27E10 (macrophages)	Mouse, 27E10	Prof. Dr. Sorge, Munster
TNF-α	6401	Becton Dickinson
INF-γ	25723	Becton Dickinson

in PBS were applied for 60 minutes, followed by further washing steps in PBS. Binding of the secondary antibody was detected by incubation with aminoethyl carbazole (DAKO, Carpinteria, CA, USA) in acetate buffer (pH 5.2) containing hydrogen peroxide or with diaminobenzidine (DAKO, Carpinteria, CA, USA) in imidazol-HCL buffer pH 7.5 containing hydrogen peroxide. After rinsing in tap water, the sections were counterstained with Mayer's hemalum and mounted in glycergel. For negative control samples, primary antibody was omitted and replaced by PBS or irrelevant antibody.

Immunohistochemistry on paraffin embedded samples: In order to retrieve antigens after the standard procedures of formalin fixation, embedding, cutting and deparaffinization, tissue sections were treated by microwave in Antigen Unmasking Solution (Vector Laboratories, Inc.; Burlingame, CA, USA) for 10 minutes. Primary antibodies used on frozen sections were applied overnight at 4 °C in appropriate dilution on the samples. Further processing was the same as for the frozen sections.

Cells undergoing programmed cell death (apoptosis) were detected by *in situ* specific labeling of nuclear DNA fragmentation method (C-terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; TUNEL method) using ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD, USA). Paraffin-embedded thin sections (4 μm) were deparaffinised and digested with 20 μg ml^{-1} proteinase K (DAKO, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked with the treatment of 2.0% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 minutes. The sections were then incubated with working strength TdT enzyme in a humidified chamber at 37 °C for 1 hour, rinsed with working strength stop/wash buffer, and incubated with anti-digoxigenin peroxidase for 30 minutes. Diaminobenzidine (DAKO, Carpinteria, CA, USA) was applied for visualization. For each sample a negative control slide was included, which received identical treatment, except that TdT was omitted from the reaction mixture. Peripheral blood lymphocytes treated with dexamethasone were used as a positive control for apoptosis. TUNEL-positive cells were recognized by means of the following features: chromatin condensation, fragments of positively stained chromatin in apoptotic bodies, and positive staining of single, scattered cells

Semiquantitative evaluation

The samples were analyzed individually by two experienced liver pathologist using brightfield microscopy. According to the commonly used semiquantitative evaluation method [10, 18], immunoreactivity was assessed by a scale rated from negative (–) to strongly positive (+++), corresponding to the intensity of staining or relative to the number of positive stained cells. Positive and negative control slides were examined after each experiment to verify the reliability of the immunohistochemical reaction.

Results

Expression of the different apoptosis proteins was analyzed in three main locations:

1. on the lymphoid infiltrate in the portal tract,
2. on the hepatocytes according to the zonal structure of the hepatic lobule (zones 1–3) and
3. on the bile duct epithelium.

The results are summarized on Tables III and IV. Negative control slides did not reveal positive staining in any case (Figs 6 and 7). *Normal livers* (Table III): Normal liver tissue showed very rare ApopTag positivity on every location, including the biliary epithelium, only zone 3 (pericentral) showed a scattered positivity. Fur-

Table III Expression patterns of pro- and anti-apoptotic antibodies and cytokines in normal livers

		Bile duct epithelium	Pericentral area	Portal tracts
TUNEL		–	+	–/+
Fas/APO-1		–/+	+/++	+
Bax		–	–	–
Bcl-2		+/++	–/+	+
T cells	CD56 (NK)	+/++	–	–
	CD45	–/+	–	++
TNF-α		–	–	–
IFN-γ		–	–	–
IL-6		–/+	–	+
**Macrophages*	*25F9*		+	+
**Monocytes*	*27E10*		–/+	–/+
**TGF-β1*			+	–

*Demirci et al. [21]

Table IV Expression patterns of pro- and anti-apoptotic antibodies and cytokines in chronic rejection of human liver allograft

		Bile duct epithelium	Pericentral area	Portal tracts
TUNEL		++	++	+
Fas/APO-1		+++	++/+++	+++
Bax		–	+	–
Bcl-2		+	–/+	++/+++
T cells	CD56 (NK)	–	–	+
	CD45	++/+++	–	++/+++
TNF-α		+	–	–
IFN-γ		+	–	–
IL-6		+/++	–	++
**Macrophages*	*25F9*		+++	+
**Monocytes*	*27E10*		++/+++	++
**TGF-β1*			++/+++	–/+

*Demirci et al. [21]

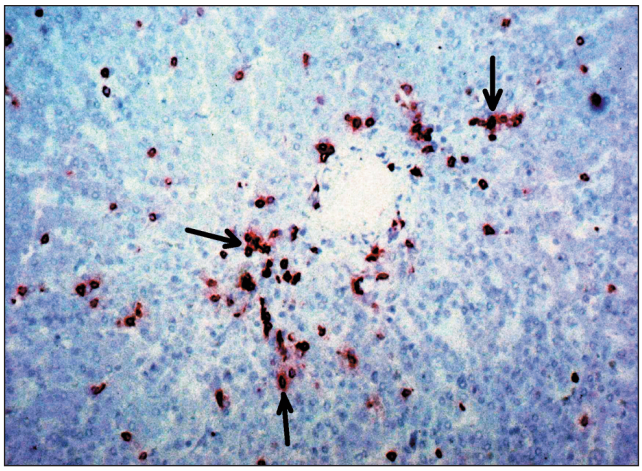

Fig. 1. ApopTag (TUNEL) positive hepatocytes around the pericentral area in chronic rejection (×125 magnification). Black arrows shows the TUNEL positive cells

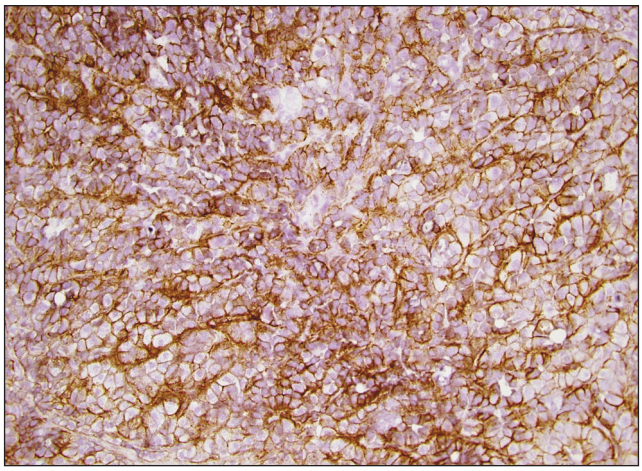

Fig. 2A. FAS/APO-1/CD95 positive hepatocytes in the pericentral/centrilobular area in chronic rejection (×200 magnification)

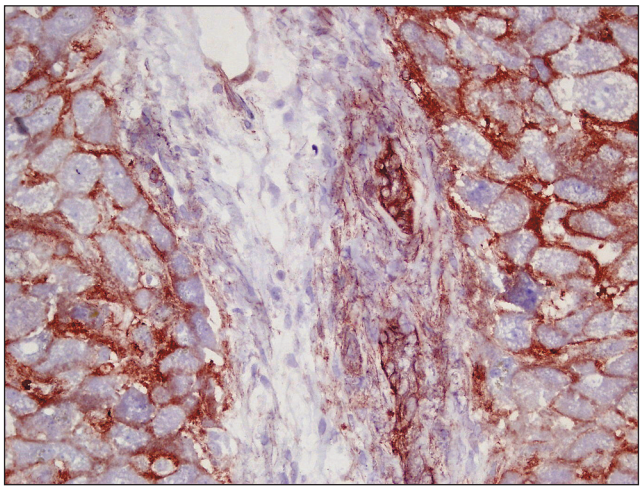

Fig. 2B. Dense FAS/APO-1 expression in the portal zone, and on the biliary epithelium (×400 magnification)

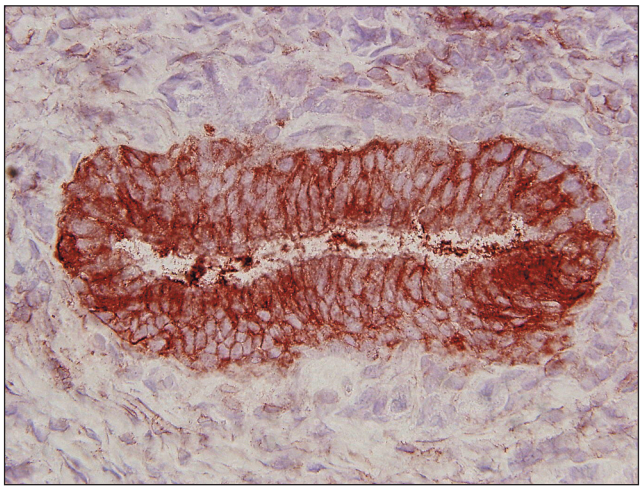

Fig. 2C. High magnification of biliary epithelium (×600 magnification): extensively high expression (FAS/APO-1)

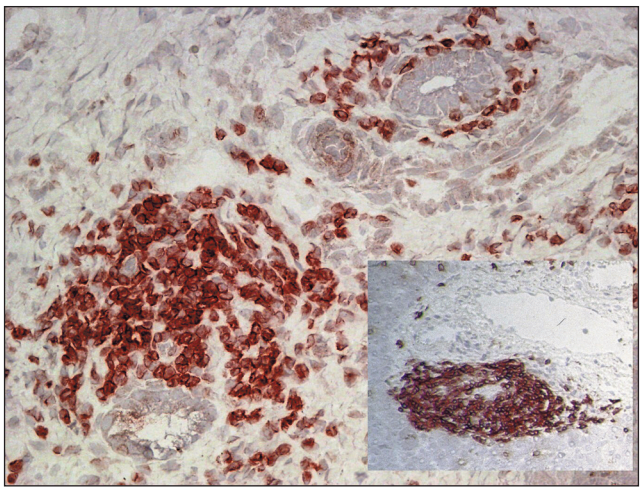

Fig. 3. Bcl-2 expression in the perioportal region, mainly around the bile duct in chronic rejection (×400 magnification). Inserted picture: CD3 positive lymphocytes around the bile ducts (×125 magnification)

ther FAS and FAS-L expressions were generally low in the whole hepatic lobule, moderately strong on the lymphoid infiltrate around the portal tract and the biliary epithelium showed negative result (Fig. 6). Bax immunostaining was negative, while Bcl-2 was positive on the biliary epithelium and in zone 3 (Fig. 7). No expression of TNF-α and IFN-γ was noted. In the normal liver, a scattered occurrence of CD3 and CD8 positive T-lymphocytes was found mainly in the portal tracts or in the sinusoidal lumens. *Chronic rejection* (Table IV): The ApopTag (TUNEL) reaction was positive on hepatic cells, particularly in zone 3 (pericentral, Fig. 1) but it was also moderately strong on the biliary epithelium. FAS/CD95 expression was remarkably high on hepatocytes in all three zones (Fig. 2A and B), particularly strong on the biliary epithelium (Fig. 2C) and was almost negative on the lymphoid infiltrate in the portal tract around the bile ducts. The expression of Fas-Ligand and Bax showed similar characteristics: slight increase on

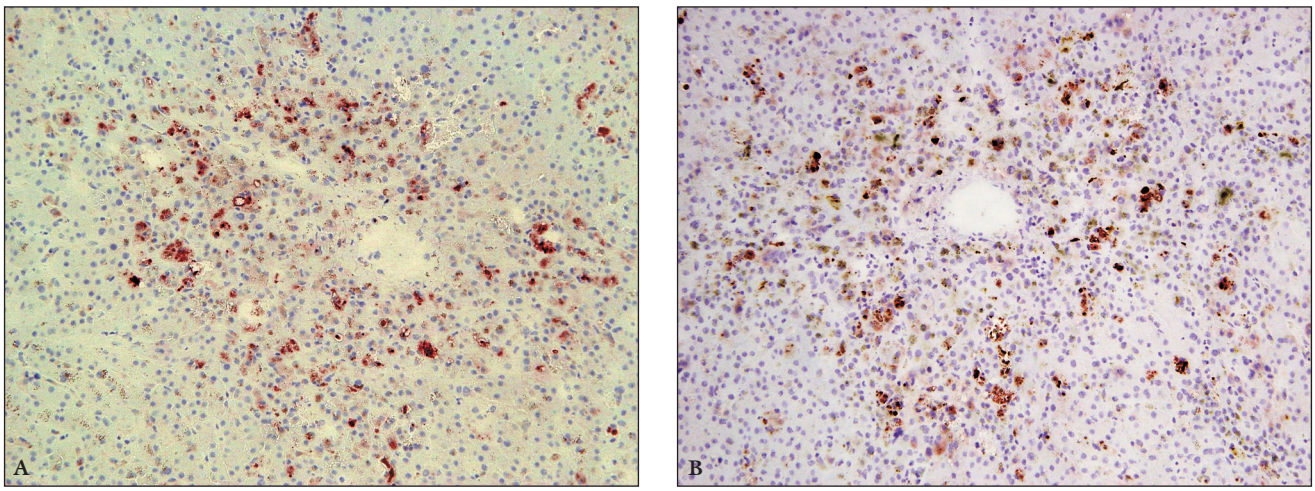

Fig. 4. Scattered BAX (Fig. 4A) and FAS-Ligand, (Fig. 4B) reaction in the centrilobular area of hepatocytes (×200 magnification)

the hepatocytes of zone 3 and negative in all other locations (Fig. 4A and B). Bcl-2 was strongly expressed on the infiltrating lymphocytes in the portal tract around the bile ducts and no expression was noted elsewhere (Fig. 3). In the liver grafts with chronic rejection the CD3/CD8 lymphocytes formed heavy infiltrates around the bile ducts and were present in a scattered pattern in zone 3 (Fig. 3, inserted picture). TNF-α (Fig. 5) and IFN-γ (not shown) were slightly positive on the hepatocytes of zone 3. Normal livers are shown in Figs 6 and 7.

Discussion

The incidence of chronic rejection is around 5–12%, still being a time-dependent major complication affecting long-term outcome after liver transplantation [7, 10, 23, 24]. The mechanism of chronic rejection is not completely understood although main pathways have already been detected and published [15, 17, 19–21, 25–27]. Histopathology of chronic rejection is defined by centrilobular injury with *fibrosis, arteriopathy, perivenulitis* and *progressive loss of intrahepatic bile ducts* (vanishing bile duct syndrome) [10, 15, 16, 19, 20, 28]. *Fibrosis:* Earlier it was demonstrated [15] that livers with chronic rejection show characteristic fibrosis and strong macrophage infiltration at the centrilobular area: (zone 3). TGF-β1 was found highly upregulated in zone 3, while TGF-β3 increased in zones 1 and 2. It was concluded that TGF-β1 acts in the fibrogenesis of chronic liver allograft rejection. It was also demonstrated that chronically rejected livers reveal a remarkably strong expression and accumulation of extracellular matrix proteins, including fibronectin, tenascin, undulin and collagen. Individual integrins show different expression patterns according to the extent of fibrosis suggesting that integrins – supposedly the primary mediators of cell-matrix interactions – might play a central role in the initiation of the fibrotic process itself [21, 29].

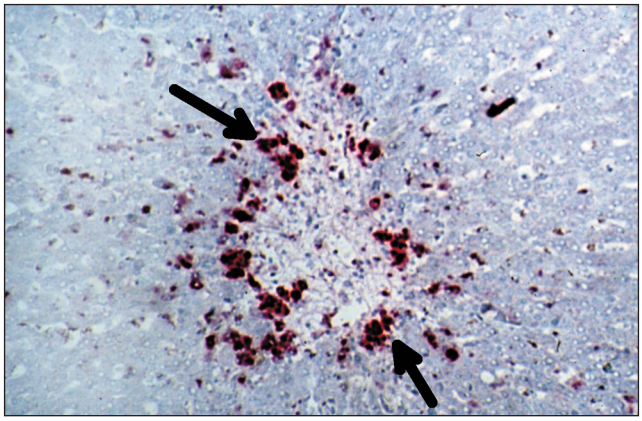

Fig. 5. TNF-alpha positive hepatocytes in the pericentral region in chronic rejection (×200 magnification). Black arrows show the dense area

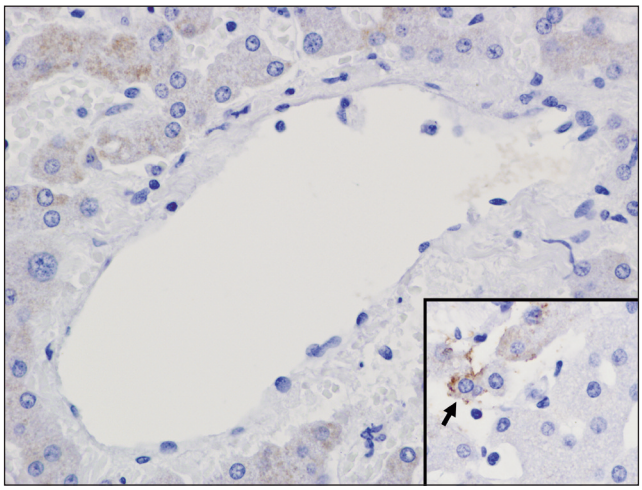

Fig. 6. Normal liver showed only weak non-specific FAS/APO-1 positivity in the cytoplasm of hepatocytes around some of the central veins (main part) and membrane associated moderate positivity was present in only one or two hepatocyte(s) per slide (black arrow, insert). Original magnification: ×600

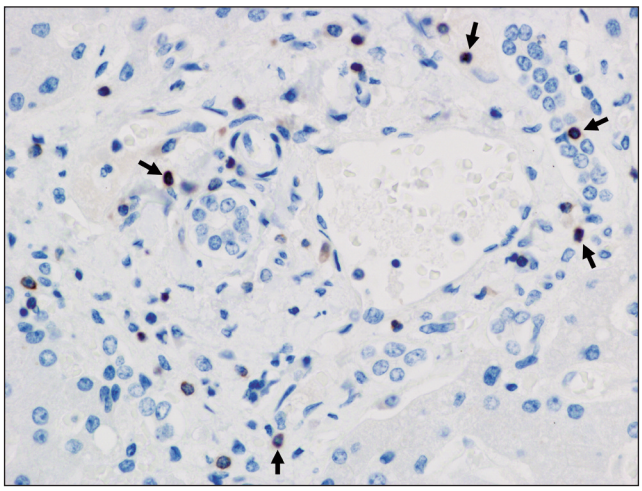

Fig. 7. The normal liver showed positivity for βcl-2 only in normal portal lymphocytes (black arrows) which served as internal positive control for the reaction. Original magnification: ×600

Arteriopathy

Foam cell arteriopathy or chronic obliterative arteriopathy is frequently found in chronic rejection [18, 19, 23, 30, 31]. We have detected *Chlamydia pneumoniae* in chronically rejected livers [18]. The presence of *Chlamydia pneumoniae* was also reported in arterial lesions of non-immunocompromised patients, and is known to be associated with atherosclerosis [32–34].

Intrahepatic bile duct loss

The vanishing of bile ducts is a well-known pathological entity [4]. Bile duct loss is an acquired process, recognized by partial or total absence of bile ducts in certain portal tracts. The underlying process could be immunological, ischemic, infectious, metabolic or toxic [3–5]. The vanishing bile duct syndrome is well known in chronic liver allograft rejection [19] and related to both ischemic and immunological effects [3, 10]. Theoretically both mechanisms could initiate apoptosis [7]. Although the immuno-mechanism of bile duct destruction is not yet discovered completely, it seems to be driven by cytokines and apoptosis [10].

Apoptosis of the biliary epithelium

The biliary epithelium's homeostasis of cell death and proliferation [35, 36] is mainly balanced by Bcl-2 [9, 10, 36]. Bcl-2 expression of the biliary epithelium is generally lower in liver allografts compared to cirrhotic livers and the degree of their apoptosis correlates with the acute rejection as well [9]. IFN-γ was found to be upregulated, while IL-6, TGF-β and TNF-α downregulated in chronically rejecting liver grafts by Hayashi el al. [37]. The Fas/Fas-L system [36], perforin/granzyme B system [33], TNF-α/TNF-α receptor system [39], oxidative stress [40], and the disturbances of Bcl-2 expression on biliary epithelium might all be an induction for biliary apoptosis. The mechanism is similar to PBC, when the proliferation that counteracts the apoptotic loss of biliary epithelium is insufficient. This process is slow and variable in PBC and rapid in allograft rejection. It is a matter of dispute whether there is a substantial apoptosis in the biliary epithelium in ductopenic chronic rejection [8, 10].

The expression of TIAF-1 (*T*GF-β1 *i*nduced *a*nti-apoptotic *f*actor) in the infiltrating T-cells in chronically rejecting kidney and liver samples is high. This might prevent the lymphocytes from apoptosis, concluding to persistent immunoresponse, which results in chronic rejection [39]. This is in accordance with the dense infiltrate of CD3+/CD8+ cells around the bile ducts with high Bcl-2 expression on lymphocytes observed in our present study and also described by others [37]. The similarities in the proinflammatory/immunoregulatory cytokine and apoptotic profile between acute and chronic rejection suggest that prolonged acute rejection could also be a pathogenetic factor for late chronic rejection. This is also in agreement with certain histopathological [42, 43] and clinical findings [23, 44]. Zou et al. did not find differences in Fas expression patterns between normal and fatty livers as well as NASH livers [45]. In our present study we focused on the apoptotic balance of chronic liver rejection. According to our assumption the expression pattern of pro- and anti-apoptotic factors is different in the hepatic lobule and on the biliary epithelium. Correspondingly, allografts with chronic rejection showed increased rate of apoptosis, particularly in the centrilobular (zone 3) areas and on the bile duct epithelium compared to normal livers. Corresponding with the high apoptotic rate, significant FAS/APO-1 expression was found equally in all zones (Fig. 2), but as other parts of the proapoptotic pathways, Fas-L and Bax were expressed in centrilobular location (Fig. 4). Macrophages accumulated in centrilobular areas (Fig. 5), TNF-α and IFN-γ, known as macrophage products were also expressed at the same location. These expression patterns of the proapoptotic factors explain the centrilobular location of apoptosis of the hepatocytes. On the contrary, apoptosis-protective Bcl-2 was mostly detected on lymphocytes surrounding the bile ducts (Fig. 3), therefore these were free to act and destroy bile ducts in all cases of chronic rejection. The infiltrating lymphocytes markedly expressed CD3/CD8, as described earlier [15, 21]. While in the pericentral area the process seems to be fostered through TGF-β [13], TNF-α and IFN-γ produced by the macrophages and also enhanced by the upregulation of Fas/Fas-L and Bax, the mechanism of apoptosis in the biliary epithelium seems to be based on the downregulation of the protective Bcl-2 and the activation of the Fas/Fas-L system. Our findings support the results

of Koukoulis et al. [10], and correlate with standard histopathology as well [22].

Our results suggest that the balance between pro- and antiapoptotic patterns was shifted to the proapoptotic side, mainly in the centrilobular area of the hepatic lobule, and on bile ducts. In conclusion it seems that in chronic rejection both downregulation and upregulation of apoptotic proteins can be observed: FAS is upregulated in biliary epithel and zone 2, proteinlevels of FASL remain unchanged, BAX is upregulated in zone 3, BCL2 is downregulated in both biliary epithel and zone 1 and both TNFa and IFN are upregulated in zone 3. According to these findings in chronic rejection the predictive sites of apoptosis are the biliary epithelium and the pericentral areas.

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