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Intragraft iNOS induction during human liver allograft rejection depresses cytochrome p450 activity

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Abstract Allograft function may become impaired during rejection after human liver transplantation. Cytokines induce nitric oxide (NO) production in hepatocytes, Kupffer cells and infiltrating mononuclear cells. NO inhibits cytoplasmatic cytochrome p450 (CYP) enzyme activity in vitro. It is not known whether this mechanism plays a role in vivo. In order to characterize the role of locally produced cytokines in the pathogenesis of liver dysfunction, we analysed human liver transplant biopsy material for the expression of proinflammatory cytokines as well as for NO synthase and we compared these results to the microsomal liver function in vivo [aminopyrine breath test (ABT)] and in vitro (enzymatic analysis of CYP). Microsomal liver function decreased in vivo during rejection while ABT levels decreased by 40% and increased again by 59% after the acute rejection episode. Similarly, CYP 1A2 and 2E1 activity dropped 42% and 24% in rejecting samples, respectively. Competitive reverse transcriptase polymerase chain reaction (RT-PCR) showed a fivefold upregulation of interferon gamma (IFN-γ) gene expression. Inducible, but not constitutive NO-synthase gene expression was upregulated fivefold in samples from rejecting patients suggesting a local induction of NO in response to immune events. Our data show a marked impairment of CYP enzyme activity during allograft rejection which is presumably secondary to an increased intragraft production of proinflammatory cytokines and NO.

Keywords Liver transplantation · Liver function · Nitric oxide · Aminopyrine breath test · Cytokine gene expression

Introduction

It has long been recognized that acute rejection after liver transplantation is mediated by cellular as well as by humoral immune mechanisms. The consequences are intense inflammatory cell infiltration and progressive graft destruction. Characteristic early histopathological changes in rejected human liver grafts include periportal infiltration of mononuclear cells (MNC) [1, 2, 3] which are known to produce proinflammatory cytokines [4, 5]. Thus, portal and sinusoidal T cell and macrophage infiltration stained immunohistochemically positive for interleukin (IL) 1β and IL-6 (20 to 50%) as well as for tumour necrosis factor α (TNF-α) (10 to 20%). These mediators further accelerate cellular graft recruitment and alert secondary effects. Numerous cytokines, interferon and endotoxin induce nitric oxide (NO) biosynthesis, a reaction catalysed by the inducible NO synthase (iNOS, type II NOS) [6, 7, 8, 9, 10, 11]. In addition, it has been shown that the activity of several cytochrome p450

(CYP) isoforms is inhibited by NO in vitro in a concentration-dependent fashion [12, 13, 14, 15, 16, 17, 18]. It has been shown that NO is produced during organ transplant rejection [19, 20], but the underlying mechanisms, the functional consequences and its actual role in graft destruction have not been understood completely.

Nitric oxide is an ubiquitous multifunctional free radical that is synthesized by five-electron oxidation of the substrate L-arginine. This reaction is catalysed by a family of enzymes, the nitric oxide synthases (NOS) including the neural, constitutive and the inducible NOS. NOS is a monooxygenase with substantial sequence homology to cytochrome p450 reductase and has been shown to have enzyme activities similar to those of cytochrome p450 reductase, including reduction of cytochrome p450 [21, 22].

An increase of local intragraft NO production correlating with acute rejection episodes after organ transplantation leads to an increase in systemic serum nitrite/nitrate levels [23]. This effect was also shown by electron paramagnetic resonance spectroscopy. iNOS was immunohistologically localized to the infiltrating inflammatory cells. It was shown, that NO biosynthesis can be induced through stimulation with several immune mediators such as TNF, interferon-gamma (IFN-γ) and type I interferon [23].

Nitric oxide reacts with various molecules, such as superoxide, iron, thiol compounds and various haemoproteins, including the heme moieties within the catalytic centre of CYP enzymes [16, 22, 24, 25]. Although NO interacts reversibly with CYP 450, the interaction leads to a concentration-dependent inhibition of several cytochrome p450 isoforms and has been demonstrated for CYP 1A1, 1A2, 2B1, 2B2, 2E1 and 3A4 [12, 13, 15, 16, 17, 18]. It has been suggested that NO forms dissociated complexes with p450 isoenzymes and the catalytic function of these isoenzymes were irreversibly inactivated [26]. As cytochrome p450 is the key enzyme in detoxification, this may be one pathway leading to progressive liver graft dysfunction in patients who reject the allograft.

Therefore the aim of this study was to investigate the correlation of cytokine induced NO-biosynthesis with the cytochrome function during acute human liver allograft rejection. Our data demonstrate a drastic impairment of the p450 isoforms 1A2, 2E1 but not 3A4. This effect is presumably secondary to an increased intragraft production of NO following iNOS induction by several cytokines.

Materials and methods

Subjects

A total of 104 patients underwent orthotopic liver transplantations (OLT) between April 1991 and August 1997 at Klinikum Rechts Der Isar, Technical University of Munich, Germany, and were subject to follow up. Informed consent was obtained from each patient and the study protocol was approved by the appropriate institutional review committee. The immunosuppressive protocol consisted of either triple-drug therapy using cyclosporine orally (100–200 ng/ml), methylprednisolone (tapered from 2 mg/kg per day to 10 mg per day at day 30) and azathioprine (1–1.5 mg/kg per day) or dual-drug therapy using FK 506-based immunosuppression (8–12 ng/ml) with methylprednisolone. Patients with biochemical or histological evidence of bacterial or viral infection, or clinical complications of unknown aetiology at the time of biopsy, were excluded from the study. The study included 32 patients who had experienced an acute, steroid-sensitive rejection episode. The diagnosis was made by histopathological findings as well as clinically apparent graft malfunction or elevated liver enzymes. All patients with acute rejection received additional immunosuppressive therapy. Episodes of acute rejection were treated by steroid pulses (3×1 g of methylprednisolone). Routine laboratory parameters such as bilirubin, liver cell enzymes, alkaline phosphatase, thromboplastin time and bile flow were measured daily.

Biopsies

Routine percutaneous liver biopsies were performed using a Menghini needle and were taken at one week after transplantation and before discharge. Additional biopsies were taken when rejection was suspected. Each biopsy had a length of 1.5 to 3.0 cm and was immediately divided into three portions. One was placed in formalin for routine clinical histologic staining and examination. The remaining tissue was immediately frozen in liquid nitrogen and used for molecular and biochemical analysis.

Histology

A clinical pathologist did the standard microscopic examination without any information concerning molecular analysis. Acute rejection was graded according to the criteria of the BANFF classification. The Rejection Activity Index in all examined samples was I to II [27]. After additional immunosuppressive therapy as described above, control biopsies showed no histopathological signs of acute rejection.

Aminopyrine breath test

After human liver transplantation, in vivo aminopyrine breath test (ABT) was measured daily from the 1st to the

10th postoperative day and subsequently 2 times a week until the patient was discharged or until the 30th postoperative day. Similar to the biopsy protocol, additional ABTs were performed when rejection was suspected. From these ABT samples those were chosen as 'pre rejection' which best met the criteria at least 5 days after transplantation and at least 3 days before clinical signs of rejection occurred. The 'rejection' breath samples refer to the time point of clinical signs of rejection and were taken from the same patients directly before starting the additional immunosuppressive therapy. The 'post rejection' samples were taken 3 to 5 days after the end of additional immunosuppressive therapy, when the patient no longer showed signs of rejection. ABT samples of the patients were compared and only those with histopathological approved rejection were included.

Breath samples were taken from resting and overnight fasting patients before and 30 min after intravenous injection of 50 kBq [28] aminopyrine. The ^{14}C activity was measured in a beta-counter and expressed as a percentage of the administered dose [normal range: 0.6–1.0 (% dose ×kg/mmol CO_2)] [28]. Entire body radiation exposure was less than 1 mrem per measurement.

Cytochrome p450 isozyme activity

Homogenate of liver biopsy tissue was obtained using an automatic homogenizer (Ultra-Turrax T8, IKA Labortechnik, Staufen, Germany). Cellular protein was measured according to the method of Lowry.

To determine the specific activity of CYP 1A2, 20 µl homogenate of liver biopsy tissue was added to 1068 µl 50 mM Tris (Sigma, Munich Germany,), 12 µl 1 mM Dicumarol (Sigma, Munich, Germany), 50 µl 125 mM $MgSO_4$ (Merck, Darmstadt, Germany) and 50 µl 10 mM NADPH (Boehringer, Munich, Germany). The reaction was started with 50 µl 0.05 mM Ethoxyresorufin-de-ethylase (EROD, Sigma, Munich, Germany), incubated for 30 min at 37°C, stopped with 2.5 ml methanol 100% (Merck, Darmstadt, Germany) and centrifuged. Activity was measured spectrofluorometrically with an excitation at 550 nm and emission at 585 (Spectrofluo JY 3D, Jobin Yvon, Division d' instruments, France).

To determine the specific activity of CYP 2E1, 20 µl homogenate of liver biopsy tissue was added to 260 µl 50 mM potassium-phosphate-buffer pH 7.4, 50 µl 55 mM $MgCl_2$, 10 µl 50 mM ascorbate, 50 µl 13 mM NADPH, 50 µl 33 mM glucose-6-phosphate and 10 µl glucose-6-phosphate-dehydrogenase. The reaction was started with 50 µl 5 mM p-nitrophenol, incubated for 30 min at 37°C, stopped with 100 µl 20% trichloroacetic acid, then incubated again for 3 min at room temperature and centrifuged. The amount of 500 µl of the solution was added to 250 µl 2 N NaOH. The activity was measured spectrofluorometrically at 535 nm extinction (Spectrophotometer UV Gerät Lambda 5, Perkin Elmer, Überlingen, Germany).

To determine the specific activity of CYP 3A4, 10 to 20 µl homogenate (according to 100 µg protein) of liver biopsy tissue was added to 800 µl 50 mM Hepes (Sigma, Munich, Germany)/KCL buffer pH 7.4 (Merck, Darmstadt, Germany) and 100 µl 10 mM NADPH (Boehringer, Munich, Germany). The reaction was started with 100 µl 100 mM Lidocain (Sigma, Munich, Germany), incubated for 30 min. at 37°C, stopped with 10 µl 60% Perchloric Acid and centrifuged. The solution was diluted 1:10 with TD_x-buffer and the activity was measured by a TD_x analyser (Abbott, Wiesbaden, Germany).

All activity values are expressed as pmol of substrate metabolization per mg of total cellular protein and per minute.

Cytokine and NOS reverse transcriptase polymerase chain reaction

A complete series of biopsy material from pre-rejection, rejection and post-rejection time points was available from 12 patients. A 5 to 7 mm portion of the biopsy was homogenized using an automatic homogenizer (Ultra-Turrax T8). mRNA was isolated using a commercial kit (Quick Prep Micro mRNA Purification Kit, Pharmacia Biotech, Freiburg, Germany) and concentrated in a glycogen/potassium acetate solution. Concentration and purity of mRNA was determined by spectrophotometry at 260 nm and 280 nm extinction. 200 ng mRNA was reverse-transcribed into cDNA using Superscript reverse transcriptase, according to the instructions of the commercial kit (Gibco/BRL, Eggenstein, Germany).

For a competitive reverse transcriptase polymerase chain reaction (RT-PCR), a commercial kit was used containing Taq-polymerase (Gene Amp PCR reagent kit, Perkin Elmer Cetus, Norwalk, Conn., USA) and a novel multispecific control fragment for the human system containing primers for cytokines as well as some housekeeping genes as described by Siegling et al. [29]. The control fragments for iNOS and constitutive nitric oxide synthase/type I NOS (cNOS) were designed using the commercial kit PCR MIMIC Construction kit (Clontech, Palo Alto, Calif., USA). The specific primer for iNOS and cNOS was described by Geller et al. [30] and Janssens et al. [31]. According to the varying amplification efficiencies and to the contents of specific cDNA, the samples were subjected for 30 cycles for β-actin, to 40 cycles for IFN-γ and TNF-α, to 45 cycles for iNOS and to 48 cycles for cNOS. Control samples without cDNA were done in all experiments to exclude contamination.

Briefly, for adjusting the different cDNA concentrations, equal amounts of cDNA were co-amplified with tenfold dilutions of the control fragment using β-actin primers. The proportions of PCR products amplified from control fragment and cDNA were calculated after separation on a 1.5% agarose gel by measuring the intensity of ethidium bromide fluorescence with a scanner (Ultra-Scan XL, Pharmacia Biotech, Freiburg, Germany). Based on these data cDNA samples were adjusted to equal input concentrations.

For quantification of cytokines, iNOS and cNOS the adjusted cDNA samples were amplified with tenfold dilutions of control fragment using the specific primers for each cytokine, and the proportion of PCR products was calculated as described above. A typical example of a quantitative PCR is given in Fig. 6 for iNOS induction. Due to the high inter-patient variability in competitive PCR, all results were expressed in relation to the samples harvested at the time of rejection. This value was set at 10 to make the results comparable.

Statistical analysis

The RT-PCR data were evaluated using Kruskal-Wallis analysis comparing rejecting and non-rejecting grafts. When this test showed significance at the 5% level, the groups were compared using Mann-Whitney U test. The enzyme activity and ABT data was evaluated using Mann-Whitney U test with a significance level of 5%. The box plots show the 25th/50th(=median)/75th-percentile.

Results

Aminopyrine breath test

Using the ABT, specific CYP activity of the liver was assessed in vivo daily and after the tenth postoperative day twice a week or additionally when rejection was suspected. A typical case of rejection shows rising liver cell enzymes, fever and a drop in bile production. In 50% of the subjects the drop in ABT levels preceded the clinical parameters by 1 to 2 days. As shown previously, the ABT is a sensitive parameter in the detection of rejection, whereas only less than 50% of the patients presented typical clinical signs as mentioned above [28]. ABT samples of the patients were chosen and compared before and after an episode of acute rejection as described above. Our results show in all cases a marked decrease in microsomal liver function in vivo during acute rejection at ABT levels of 40% (Fig. 1) as compared to levels prior to rejection. The ABT significantly dropped in patients who rejected the grafts from a median of 0.73% of the applied dose to 0.44%

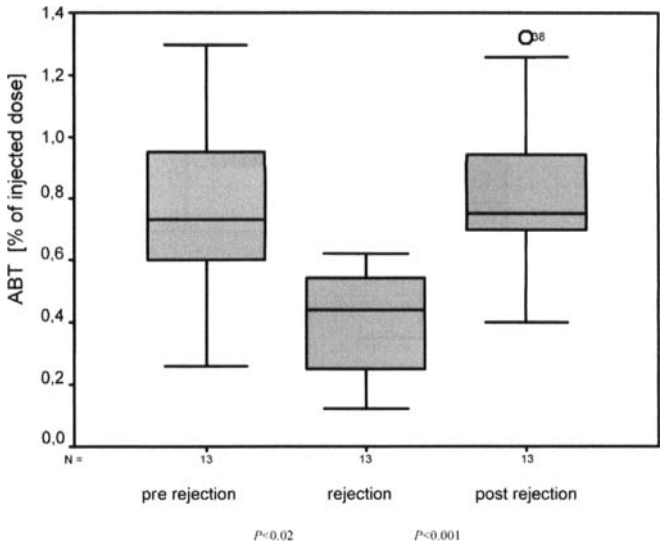

Fig. 1 Liver function in vivo was measured by means of ABT. The results at the time of acute rejection were compared with samples from the same patients before and after rejection

($P < 0.02$). After rejection, the ABT rose again significantly by 59%, from a median of 0.44% to 0.75% ($P < 0.001$).

CYP p450 isozyme activity

Similarly to the ABT, microsomal CYP 1A2 activity dropped by 68% (Fig. 2) and CYP 2E1 by 39% (Fig. 3)

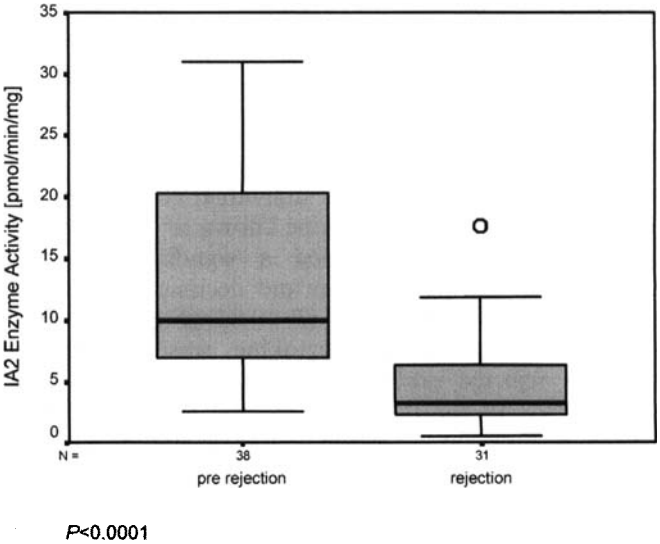

Fig. 2 Effect of acute liver transplant rejection on CYP 1A2 activity. CYP 1A2 activity dropped significantly during rejection by 68% from a median of 10.1 pmol/min per mg to 3.2 pmol/min per mg compared to pre-rejection levels

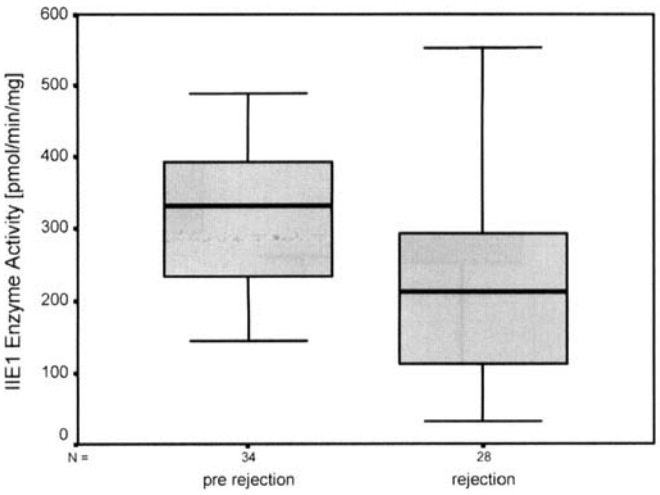

Fig. 3 Effect of acute liver transplant rejection on CYP 2E1 activity. Similar to ABT and CYP 1A2, CYP 2E1 enzyme activity decreased significantly during rejection by 39% from a median of 349 pmol/min per mg to 211 pmol/min per mg

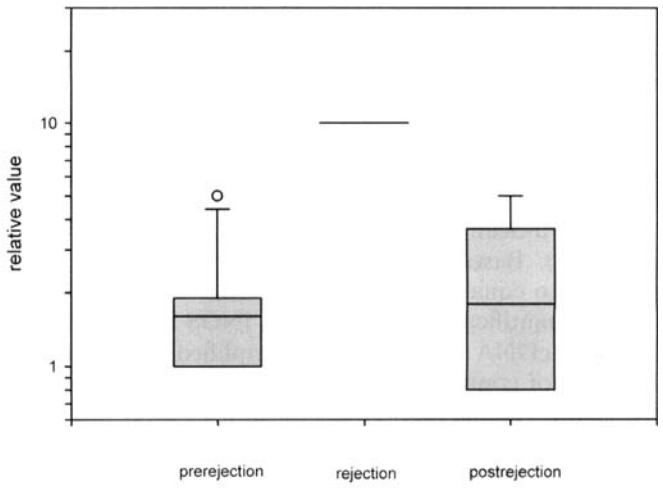

Fig. 4 Intragraft gene expression for IFN-γ before, during and after rejection. The pre- and the post-rejection results were expressed in relation to the samples harvested at the time of rejection, which was set at 10. IFN-γ gene expression was fivefold upregulated during rejection and declined after rejection to the pre-rejection level

in samples of patients who rejected the grafts. Median CYP 1A2 activity declined significantly from 10.1 pmol/min per mg to 3.2 pmol/min per mg ($P < 0.0001$). Median CYP 2E1 activity decreased significantly from 349 pmol/min per mg to 211 pmol/min per mg ($P < 0.001$). CYP 3A4 enzyme activity was not significantly affected (407 pmol/min per mg in control samples vs 391 pmol/min per mg in patients who rejected the grafts; $P = 0.75$).

RT-PCR

The intragraft transcription of TNF, IFN-γ, iNOS and cNOS were analysed before and after rejection by competitive RT-PCR using specific primers after mRNA isolation from biopsy material. Samples taken before and after rejection served as individual controls. Gene expression for IFN-γ, a cytokine known to be associated with graft rejection, showed a significant fivefold upregulation during rejection and declined after rejection to the pre-rejection level ($P < 0.05$) (Fig. 4). TNF-α, a classic proinflammatory cytokine, was twofold elevated in rejected grafts and decreased afterwards, as with IFN-γ, to the pre-rejection level (Fig. 5). TNF-α and IFN-γ are both known to induce iNOS, whereas constitutive nitric oxide synthase (cNOS) remains unaffected by cytokines or endotoxin. Our data showed a fivefold upregulation of iNOS gene expression during rejection versus pre- and post-rejection controls ($P < 0.05$) (Figs. 6 and 7). cNOS mRNA expression remained unchanged (Fig. 8).

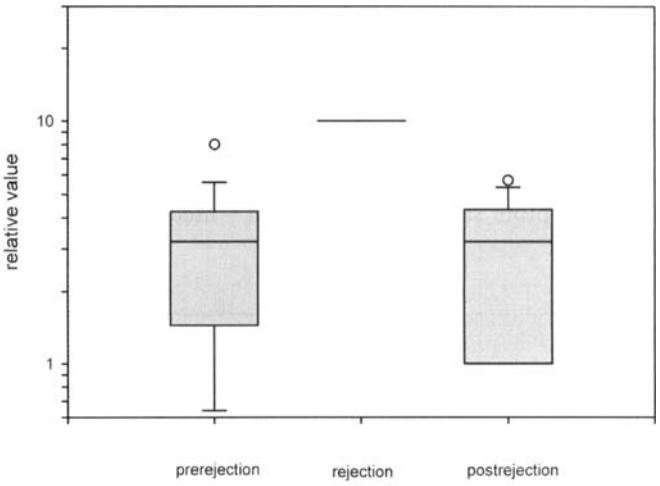

Fig. 5 Intragraft gene expression for TNF-α before, during and after rejection. The pre- and the post-rejection results were expressed in relation to the samples harvested at the time of rejection, which was set at 10. TNF-α gene-expression was twofold elevated in rejecting grafts and decreased afterwards to the pre-rejection level

Discussion

Despite remarkable improvements in clinical organ transplantation, the mechanisms leading to graft malfunction and progressive graft destruction are still not completely understood. Our study shows a drastic impairment in the enzyme activity of p450 isoforms

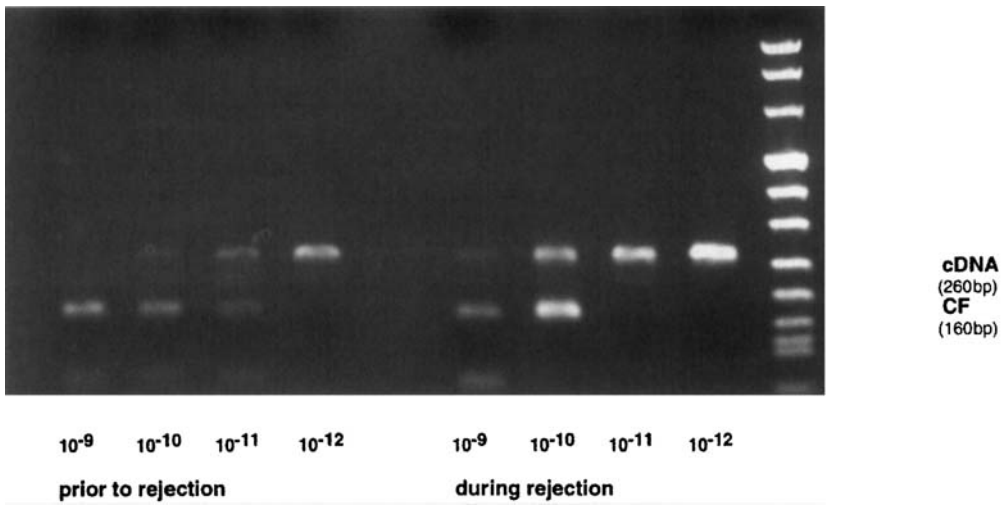

Fig. 6 Example of a competitive iNOS PCR-experiment. Following standardization to β-actin equal concentrations of cDNA compete against a tenfold dilution of the control fragment (*CF*) for the specific iNOS primers. Prior to rejection a 10^{-11} dilution of the CF is required to achieve an equal density of the PCR products on the gel, while during rejection a 10^{-10} dilution of CF is sufficient. Consequently, in this particular patient iNOS gene expression is tenfold upregulated at the time of graft rejection. The overall results are presented in Fig. 7 as median of all 12 patients studied

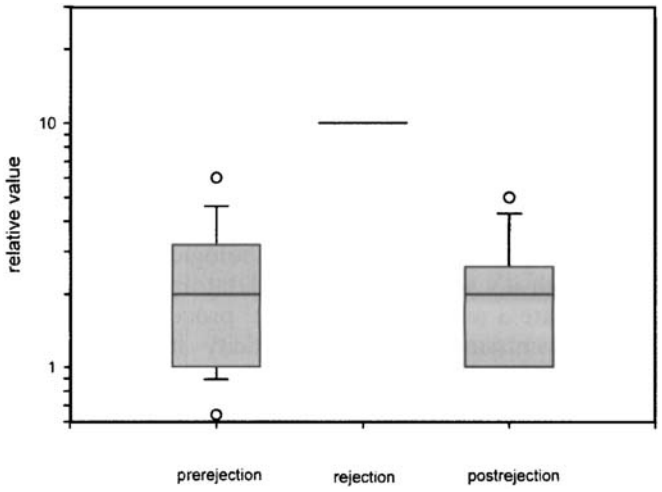

Fig. 7 Intragraft iNOS gene expression before, during and after rejection. The pre- and the post-rejection results were expressed in relation to the samples harvested at the time of rejection, which was set at 10. The results show a fivefold upregulation of iNOS gene expression during graft rejection

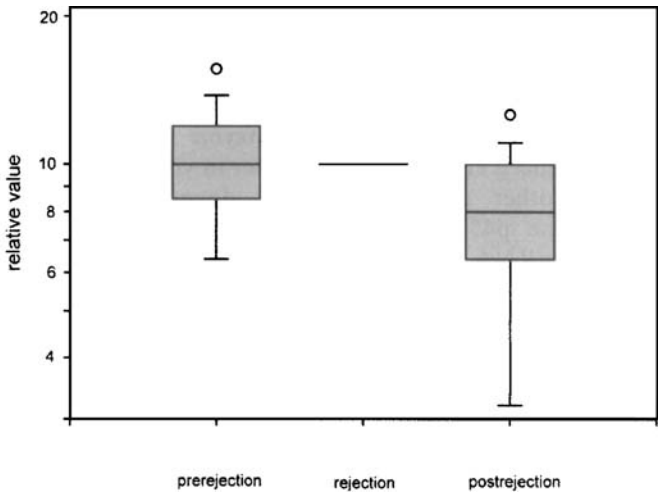

Fig. 8 cNOS mRNA expression before, during and after rejection. The pre- and the post-rejection results were expressed in relation to the samples harvested at the time of rejection, which was set at 10. No alteration during the clinical course

1A2,2E1, but not 3A4, after human liver transplantation. This effect is presumably secondary to an increased intragraft production of NO after iNOS-induction by several cytokines. During rejection, portal and sinusoidal T cell as well as macrophage infiltrates stained immunohistochemically positive for IL-1β, IL-6 (20 to 50%) and TNF (10 to 20%). Follow-up biopsies showed a persistence only of TNF-α expression, whereas later biopsies were essentially normal (data not shown). Our data show a fivefold upregulation of IFN-γ and a twofold upregulation of TNF-α gene expression. Similarly iNOS gene expression shows a fivefold upregulation during graft rejection versus pre- and post-transplant levels.

The ABT is a clinically well-established assay for liver function [28, 32]. It is fast and easy to perform, reproducible and independent of hepatic blood flow. It has been shown as a sensitive parameter for detecting rejection [28]. It must be pointed out, however, that sepsis and, to a lesser degree, viral infections may reduce ABT results, indicating that a drop in ABT values is not specific for rejection. Our studies confirm previous results, showing a decrease in microsomal liver function of 40% from pre-rejection levels during rejection using the ABT. Similarly, our data also show a drastic impairment in enzyme activity of two human isozymes in vitro. Microsomal CYP 1A2 activity dropped by 68% and 2E1 activity by 39% in samples of patients who rejected the grafts. In contrast, CYP 3A4 activity was not sig-

nificantly altered. This may be due to the observation that on the one hand cyclosporine A induces CYP 3A4 enzyme activity, yet on the other hand the enzyme activity is inhibited by NO. Consequently, both effects perhaps balance each other out (personal communication J. Doehmer).

In contrast Donato et al. showed a 200% increase of 3A4 activity after stimulating human hepatocytes with IFN-γ [33]. Theirs and our studies differ in several points. This effect may be due to large amounts of IFN-γ and therefore appears only in cell cultures, but not in vivo. In contrast to our results concerning CYP 450 activity, Western Blot analysis showed that specific isoenzyme protein content remained unchanged (data not shown). This is consistent with the data of Veihelmann et al. [34], who showed stable expression of hepatic CYP 450 1A2 enzyme concentration in a rat model after C. parvum injection or *N*-monomethyl-L-arginine (NMMA) treatment. In contrast, other groups could show a reproducible decrease of hepatic CYP 450 protein after inflammatory stimulation in vitro [17, 35, 36]. To our knowledge a NO-dependent inhibition of protein synthesis has never been shown in vivo, suggesting that an alteration in enzyme activity is not due to reduced enzyme content under in vivo conditions. Though other groups showed a downregulation of cytochrome p450 at a pretranslational level by suppressing mRNA expression [37, 38], our data implicate that the major mechanism responsible for down-regulation of these enzymes by NO is a direct inactivation of enzyme activity.

TNF is a classic proinflammatory cytokine mainly produced by monocytes and T cells. During graft rejection MNC activation is followed by an increase of local, intragraft cytokine production. TNF leads to endothelial cell activation and increases the expression of adhesion molecules. Proinflammatory cytokines cause an unspecific graft infiltration during rejection. IFN-γ is produced during rejection by T cells after stimulation, as well as by natural killer (NK) cells. This leads to macrophage activation and increases their sensitivity for lipopolysaccharide (LPS), TNF and IL-1 [34]. Proinflammatory cytokines, IFN-γ and endotoxin induce iNOS, which leads to an increase in local NO biosynthesis [8, 9, 10, 33], whereas cNOS remains unaffected. Nüssler [23] and Curran [6] showed a maximum of NO production after stimulation of human and rodent hepatocytes with a combination of LPS, IL-1, TNF and IFN-γ. Although various cell types are capable of iNOS gene transcription (endothelium, vascular smooth muscle cells, biliary duct endothelium and mesangial cells [6, 39, 40, 41, 42, 43]), surprisingly iNOS expression at the time of acute graft rejection was shown only in hepatocytes and infiltrating MNC [44, 45].

It is well recognized that acute rejection is accompanied by NO production localized to infiltrating inflammatory cells [46]. Selective iNOS inhibition resulted in prolonged graft survival in a rat heart transplant model, suggesting that NO production in this context was part of the rejection process. In contrast, another group showed in the murine heart transplant model that inhibition of NO synthesis did not alter graft survival, although NO synthesis was a prominent biochemical component of the acute rejection process [47]. A third setting (rat liver transplantation) showed that both acute rejection and spontaneous hyporesponsiveness were associated with increased levels of plasma NO metabolites and allograft expression of the enzyme. iNOS mRNA was localized to both portal inflammatory infiltrate and hepatocytes during acute rejection. Inhibition of NO biosynthesis by systemic administration of NMMA was associated with increased biochemical markers of liver damage and histological signs of mild cellular rejection [45].

To our knowledge this is the first report correlating iNOS upregulation with it's effects on the major detoxification system, cytochrome p450 after human liver transplantation. Nitric oxide is a free radical gas with a half-life time of only a few seconds that triggers a large range of physiological and pathological responses. Along with it's well known vasodilatatory activity, NO can regulate a number of biological processes including neurotransmission, synaptic plasticity in the central nervous system, the pathogenesis of several diseases and non-specific immunity [48]. NO is generated in different cell types through the conversion of L-arginine to L-citrulline by the catalytic enzyme nitric oxide synthase (NOS). It interacts reversibly with iron moieties of heme-containing enzymes, including certain isoforms of cytochrome p450 [16, 22, 24] forming iron-nitrosyl complexes. A nitric-oxide-mediated inhibition has been shown for several CYP isozymes such as 1A1, 1A2, 2B1, 2B2, 2E1 and 3A4 [12, 18]. The possible mechanism of NO-mediated CYP inhibition has been investigated. Minamiyama et al. suggest the thiol modification pathway [26]. Others postulate that the NO inhibition of CYP-mediated O-dealkylase activities involves both binding of NO to the prosthetic group in the catalytic centre and a destruction in the integrity of the primary structure of the haemoprotein [18, 37].

Although the mechanisms of CYP p450-inactivation by NO remain poorly defined, the results presented herein may help in understanding the pathogenesis of liver dysfunction during acute graft rejection.

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