Cyclosporine A Augments P-Glycoprotein Expression in the Regenerating Rat Liver

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In the liver, the multidrug resistance (MDR) protein P-glycoprotein (P-gp) is physiologically expressed at the bile canalicular membrane, where it participates in the biliary excretion of various lipophilic drugs and xenobiotics. Previous studies showed that the immunosuppressive agent cyclosporine A (CsA) modulates P-gp and exerts a hepatotrophic influence in the regenerating liver. Hepatocytes isolated from regenerating rat liver, after 2/3 partial hepatectomy (PH 2/3), were used as an *in vivo* experimental model of cells with high proliferating activity in order to investigate whether CsA influences cellular levels of P-gp in those cells. Male Wistar rats were treated with CsA (20 mg/kg body weight) for 4 d preoperatively and 1 d postoperatively, and regenerating hepatocytes were isolated by collagenase perfusion 12, 24 and 48 h after PH 2/3. Flow cytometry and Western blotting studies with the monoclonal antibodies C494 and C219 showed that after PH 2/3, cellular levels of P-gp were initially suppressed, 12 h after PH 2/3, by 23%, but were significantly elevated thereafter, 24 and 48 h after PH 2/3 by 28% and 73%, respectively. In CsA pretreated animals, P-gp levels were increased even in normal hepatocytes by 34%, and an additional augmentation was seen in hepatocytes from 24 and 48 h regenerating livers (60% and 56%, respectively). In summary, we demonstrate for the first time that CsA has an additive effect on the expression of P-glycoprotein during liver regeneration in the rat. Therefore, induction of P-gp might also be considered in patients receiving CsA after liver transplantation for hepatocellular carcinoma and chemotherapy as an adjuvant treatment for the prevention of tumor recurrence.

Key words cyclosporine; P-glycoprotein; liver regeneration; flow cytometry; hepatocytes; 2/3 partial hepatectomy

The energy-dependent drug efflux pump P-glycoprotein (P-gp) confers multidrug resistance (MDR) on various cancer cells, but also on normal cells, including hepatocytes.[1,2] MDR is a complex phenotype and was originally identified as cross-resistance of mammalian tumor cells *in vitro* to many structurally and functionally unrelated cytostatic agents.[3,4] Generally, MDR cells have a reduced ability to accumulate drugs, which is often associated with an overexpression of one or more members of the ABC-transporter family, *e.g.* P-gp.[5,6] In humans, MDR 1 gene encodes the glycosylated plasma membrane efflux pump P-gp,[3,7] whereas in rodents two P-gp isoforms have been identified encoded by the *mdr1a* and *mdr1b* genes.[8] P-gp is expressed at the luminal surface in excretory cells from normal tissue such as colon, small intestine, kidney and liver.[7,9] In the liver, the exact physiological function and the natural substrate of P-gp are still unknown; however, its location suggests a fundamental role in the excretion of ingested xenobiotics and physiological metabolites.[3,9] The transport function of P-gp can be restored by application of chemosensitizers[5] including the immunosupressant cyclosporine A (CsA). CsA interacts with P-gp, leading to inhibition of P-gp transport function.[10,11] There is also evidence that CsA itself is transported by P-gp.[12]

The liver is a unique organ, given its inherent capacity to restore itself fully after significant hepatic tissue loss either from acute liver injury or partial hepatectomy. After 2/3 partial hepatectomy (PH 2/3), liver cells start to proliferate and the remnant grows until it reaches its original volume.[13] Previous studies showed that the expression of transporters present in hepatocyte plasma membrane, including P-gp, is increased during liver regeneration after PH 2/3 in rats.[14,15] The upregulation of the mdr1 gene during liver regeneration[16,17] can be attributed, according to Ros *et al.*,[18] to NF-κB activation, induced by TNF-α which plays a pivotal role in liver regeneration.[13] The NF-κB trancription factor is involved in the regulation of genes in the rat mdr family.[18]

As liver regeneration is augmented by the P-gp chemosensitizer CsA,[19,20] we investigated whether this hepatotropic agent might also affect cellular levels of this transporter. The influence of CsA on cellular P-gp content in hepatocytes isolated from both normal and regenerating rat livers, was determined by flow cytometry and Western blotting, using the monoclonal antibodies C494 and C219.[21—23] Significantly augmented P-gp levels were found in hepatocytes derived from regenerating livers of CsA-treated rats.

MATERIALS AND METHODS

Animals Male Wistar rats, weighing 250—300 g, were used as liver donors. All animals were allowed to acclimatize for two weeks prior to experimentation in temperature-controlled and light-controlled rooms (12 h light cycle).

Surgery Rats were randomly assigned to one of eight experimental groups (see below). Ten animals in each group ($n=10$) were submitted to either standard 2/3 partial hepatectomy, according to the method of Higgins and Anderson,[24] or to sham operation. Their livers were isolated 12, 24 and 48 h postoperatively. The operations were carried out under light ether anesthesia between 06:00—08:00.

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CsA-Treatment All animals received equal volumes of either CsA (Novartis, Basel, Switzerland) 20 mg/kg body weight or the CsA vehicle (olive oil) for 4 d preoperativeley and 1 d postoperatively. This dose has previously been proved to be effective to stimulate liver regeneration.[19] For this purpose, CsA was dissolved in olive oil (5 mg/ml) and the appropriate dose was administered by gavage, using an orogastric stainless steel animal feeding tube (Popper and Sons Inc., New Hyde Park, NY, U.S.A.). Throughout the study, all animals had free access to food (standard rat pellet chow) and water *ad libitum*.

Groups Group A: animals were submitted to sham operation and received the vehicle. Group B: animals underwent 2/3 partial hepatectomy and received the vehicle. Group C: animals were submitted to sham operation and received CsA. Group D: animals received CsA and underwent 2/3 partial hepatectomy. Animals from groups B and D were further divided in order to study liver regeneration at 12 (Bi, Di), 24 (Bii, Dii) and 48 (Biii, Diii) h.

Blood Chemistry In order to monitor the cytotoxic effects of CsA, blood samples were collected during the operation for isolation of hepatocytes and were analyzed for glucose, serum creatinine, blood urea nitrogen, activity of serum aspartate transaminase (AST), serum alanine transaminase (ALT) and alkaline phosphatase (ALP), in the clinical laboratory with an Olympus AU500 analyzer and the Olympus system reagent 500 kit.[25] Total bilirubin levels were measured with a BT224 A.P.C. spectrophotometer (Biotechnica Instruments, U.S.A.).

CsA Measurement During the operation for isolation of hepatocytes, whole blood samples were collected and cyclosporine A levels were measured with the TDx analyzer[26] using a polyclonal antibody and the fluorescence polarization immunoassay (FPIA) (Abbot).

Hepatocyte Isolation Livers were removed from 2/3 hepatectomized and sham operated animals by the surgical procedure described previously.[27] In order to isolate hepatocytes from the sham operated whole organs or the regenerated remnants, livers were perfused through the portal vein with a roller pump (Ergo Medical TN 400) at a rate of 3.5 ml/g liver/min, for 30 min with modified Krebs Henseleit bicarbonate buffer (NaCl 118.41 mM, KCl 4.75 mM, $CaCl_2$ 2.57 mM, KH_2PO_4 1.19 mM, $MgSO_4 \cdot 7H_2O$ 1.185 mM, $NaHCO_3$ 25.0 mM), and, thereafter, for 10 min with modified Hank's balanced salt buffer solution (HBBS) (0.05 M EDTA in 400 ml of HBBS). The solutions were kept at 370 °C under continuous oxygenation (95% O_2–5% CO_2). The pH of all solutions was 7.4. The portal vein pressure, which varied between 8 and 14 cm H_2O, was measured by the fluid level in a vertical tube inserted before the portal vein and was controlled by adjusting the perfusion rate.

Finally, the liver was perfused for another 10 min in a recirculating system with 100 ml of HBBS to which 15 mg calcium chloride and 80 mg collagenase type IV (Sigma, St. Louis, MO, U.S.A.) were added. When the capsule began to detach, the perfusion was stopped and the lysed liver was raked gently, and washed with ice-cold Leibovitz medium (Gibco, Grand Island, NY, U.S.A.). The released hepatocytes were collected and filtered though a nylon mesh (60 μm). Afterwards, cells were pelleted by centrifugation at 50 g for 5 min at 4 °C.

Hepatocytes of regenerating and non-regenerating livers were resuspended in Leibovitz medium, counted and viability was determined by trypan blue dye exclusion. The experiments were performed with cells showing >90% viability.

Isolation of Crude Membranes from Isolated Hepatocytes Freshly isolated hepatocytes were washed in phosphate buffered saline (PBS, pH 7.4), to which 0.2 mM phenylmethylsulfonylfluoride, 0.1 mM aprotinin, 1 mM leupeptin and 1 mM pepstatin were added, homogenized with 4 volumes of PBS using a Dounce homogenizer.[10] After removing the nuclear and mitochondrial fraction (10000 ***g*** for 20 min) the crude membrane fraction was isolated by centrifugation at 100000 ***g*** for 60 min. The pellets were resuspended in KCl (1.15%) and centrifuged at 100000 ***g*** for another 20 min. The crude membrane samples were stored in glycerine/PBS at −70 °C until use. Protein content was determined using a kit from Biorad (Hercules, CA, U.S.A.).

Antibodies P-gp detection was performed with two monoclonal anibodies C219 and C494 (Alexis Corporation, San Diego, CA, U.S.A.), which both recognize cytoplasmic epitopes of the protein. C219 recognizes a conserved cytoplasmic epitope close to the ATP-binding cassette in all known members of the P-gp subfamily, whereas C494 specifically recognizes a cytoplasmic epitope of the MDR1 gene product.[10,21] Both antibodies were used for flow cytometry and Western blotting analyses. Irrelevant mouse IgG2a at the same concentration as that of monoclonal antibodies was used in isotypic control samples.

Western Blotting Proteins of the crude membrane fraction were separated under reducing conditions on a 6% PAGE using 4% urea in the sample buffer and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, U.S.A.). P-gp was identified in Western blots using the monoclonal antibody C494 at a concentration of 0.02 mg/ml, which detects the MDR1 gene product. A horseradish peroxidase-conjugated anti-mouse IgG (Sigma) was used as a secondary antibody. Immunoreactive proteins were detected by chemoluminescence (ECL kit; Amersham, U.K.). On immunoblots, the monoclonal antibodies C494 and C219 recognize a 170 kD protein in the particulate fraction.[10]

FLOW CYTOMETRY

Cell Preparation, Fixation and Staining Hepatocytes isolated from regenerating or non-regenerating livers were washed in PBS, pelleted and fixed sequentially with 70% methanol and 4% formaldehyde (Sigma). The protocol used for fixation of hepatocytes preserves their normal light scatter properties needed for flow cytometry analysis. The methanol formaldehyde fixed hepatocytes were then treated with 50% acetone for 10 min at −20 °C to achieve cell membrane permeabilization, because the antibodies recognize cytoplasmic epitopes. The cells were washed three times with PBS containing 1% ovalbumin (Sigma) and 0.05% polyoxyethylene sorbital monolaurate (Tween-20, Sigma). 1×10^6 cells per ml of PBS (supplemented with 1% ovalbumin), were allowed to react with 5 μl of the monoclonal antibodies C219 and C494 (final concentrations of 10 μl/ml and 6 μl/ml respectively) for 45 min at 4 °C. After washing the excess of the primary antibody, a fluorescein (FITC)-conjugated anti-mouse IgG (Sigma) was used as a secondary antibody. Im-

munoreactive cells were detected by flow cytometry. Isotypic controls were prepared using fluorescein-conjugated goat anti-mouse IgG alone.

Analysis Cells were analyzed in a FACStar Plus flow cytometer (Becton Dickinson) equipped with an argon laser. Data were registered and stored in list mode. Debris and damaged cells were excluded by gating on a forward and side scatter dot plot. FITC fluorescence was detected in the FL1 channel and stored using logarithmic amplification. Data obtained were evaluated with the FACStar Plus Software (Becton-Dickinson). Results are expressed in histogram form, with the ordinate representing cell number and the abscissa representing fluorescence intensity in arbitrary units. For each histogram, 1×10^4 viable cells were analyzed. Results are expressed as the mean channel fluorescence of the population of cells expressing P-gp. In order to estimate the expression of P-gp, the mean fluorescence intensity (F) was calculated, taking also into consideration the mean fluorescence value of the isotypic control which was substracted from the mean fluorescence intensity of the corresponding sample.

Statistics Data are expressed as mean±S.D. ($n=10$). Comparisons between groups were performed with the Student t-test for independent samples. p-values <0.05 were considered as significant.

RESULTS

The present study was aimed at determining changes in P-gp expression induced by hepatic regeneration and the P-gp modulator and hepatotrophic agent CsA.

Analysis of blood samples from CsA-treated animals showed that glucose, creatinine, blood urea nitogen, AST, ALT, ALP and total bilirubin levels were in the normal range, indicating that CsA did not exert toxic effects on the liver. The measured values of CsA in sham operated (C) and partial hepatectomized rats after 12 h (Di), 24 h (Dii) and 48 h (Diii) are given in Table 1. Although the CsA values did not differ significantly between the groups, the generally higher values for hepatectomized animals, and particularly group Diii, might be due to the decreased capability of the resected liver for biliary drug excretion. Cyclosporine A is primarily eliminated *via* biotransformation by cytochrome P450 (CYP)3A in the liver, and the reduction of liver mass by 2/3 partial hepatectomy reduces its capability to do so.[28] Moreover, there is a marked reduction of CYP3A activity at 24 to 48 h after partial hepatectomy, which is not influenced by the administration of CsA.[29,30]

Cellular levels of P-gp protein were determined in hepatocytes isolated from normal and regenerating liver of rats treated with CsA or the CsA vehicle by flow cytometry and Western blotting. Expression patterns of P-gp were analyzed 12, 24 and 48 h after 2/3 PH using the two monoclonal antibodies C494 and C219.

In Western blots, both antibodies recognized a 170 kD band when crude membrane fractions prepared from isolated hepatocytes were applied.[10] A representative blot is shown in Fig. 1. This immunoreactive band corresponded to the P-gp band in membranes prepared from MDR1-transfected leukemia cells (data not shown).

Single-cell suspensions of hepatocytes were subjected to flow cytometry in order to detect P-gp expression on their plasma membranes. Since the two antibodies recognize cytoplasmic epitopes of P-gp, the cells had to be permeabilized before analysis. During the procedure, cells kept their light scattering characteristics for the flow cytometry analysis. The reaction pattern of both antibodies was comparable, but the C494 antibody, which specifically detects the mdr1 gene product, generally showed a higher degree of reactivity and less background staining than the C219 monoclonal antibody. Therefore, the C494 antibody was used in the majority of experiments.

Table 1. Cyclosporine Levels (ng/ml)

	Group C	Group Di	Group Dii	Group Diii
MV	2221	2630	2547	2914
S.D.	249	347	691	374

Cyclosporine levels (ng/ml) were measured in blood samples, derived from all groups of animals, which were collected during the operation for isolation of hepatocytes. Data are expressed as mean±S.D. of ($n=10$).

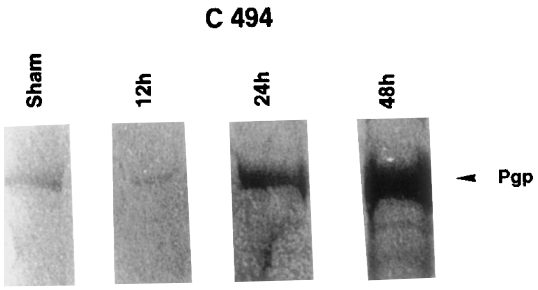

Fig. 1. Representative Western Blot Analysis of P-gp Expression in Isolated Liver Plasma Membrane Using the Monoclonal Anibody C494 and the ECL Detection System

A 170 kDa immunoreactive band (P-glycoprotein) is seen in the canalicular membrane fraction isolated from a) "normal" (sham operated) hepatocytes, b) 12 h regenerating hepatocytes, c) 24 h regenerating hepatoytes, d) 48 h regenerating hepatocytes.

Distinct changes in P-gp expression occured during hepatic regeneration (Fig. 2), with a reduction after 12 h and an increase after 24 and 48 h. As expected, in "normal" hepatocytes, derived from sham-operated animals, no statistically significant alteration in P-gp expression was observed compared with hepatocytes derived from non-operated controls ($F=95+22$).

Quantitative evaluation of the data (summarized in Table 2; mean±S.D., $n=10$) revealed a decreased level of P-gp in 12 h regenerating hepatocytes (Group Bi; expression $F=86\pm25$) compared to P-gp in "normal" hepatocytes (Group A; $F=111\pm18$). Hepatocytes isolated from 24 and 48 h regenerating livers (Groups Bii, and Biii) revealed expression of P-gp with $F=149\pm24$ and $F=192\pm44$, respectively. This pattern of reactivity was confirmed with Western blotting, where a slight reduction in the P-gp after 12 h, is followed by an approximately two-fold increase after 24 and 48 h (Fig. 1).

Treatment with the immunosuppressant CsA further increased membrane levels of P-gp as assessed by flow cytometry. The quantitative evaluation is given in Table 2. Compared to "normal" hepatocytes (Group A), in cells from sham-operated, CsA-treated animals (Group C), a statistically significant increase in the P-gp labelling was demonstrated. In addition, CsA induced a statistically significant increase in the labelling of P-gp in hepatocytes, isolated from

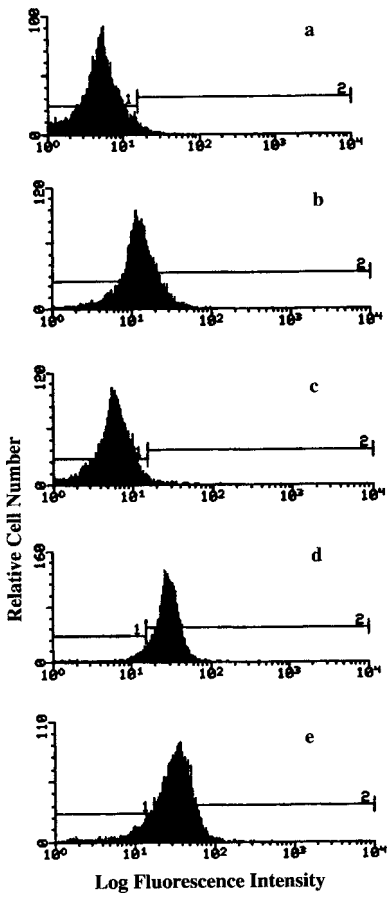

Fig. 2. Representative Fluorescence Histograms Demonstrating Changes in the Expression of P-Glycoprotein

a) Isotypic control, b) "normal" (sham operated) hepatocytes, c) 12 h regenerating hepatocytes, d) 24 h regenerating hepatocytes, e) 48 h regenerating hepatocytes. Data were obtained using the monoclonal antibody C494.

Table 2. P Glycoprotein Expression in Hepatocytes

	Untreated	CsA treated	*p* value
Sham operation	(Group A) 111±18[a)]	(Group C) 149±24	<0.01
12 h regeneration	(Group Bi) 86±25	(Group Di) ND	
24 h regeneration	(Group Bii) 142±17	(Group Dii) 227±50	<0.01
48 h regeneration	(Group Biii) 192±44	(Group Diii) 300±23	<0.001

Mean values of fluorescence intensities of populations expressing P-gp in different groups (normal and regenerating hepatocytes in CsA treated and control animals). The values were corrected to show only significant digits. *a*) Mean±S.D., (n=10). ND= not determined. Control hepatocytes F=95±22.

24 and 48 h regenerating livers Group Dii: F=227±50 and Group Diii: F=300±23, respectively. These results are in good agreement with the observed increase in P-gp expression seen by Western blotting (Fig. 1). Moreover, using isolated plasma membranes the increase in P-expression becomes even more pronounced.

DISCUSSION

We demonstrated that CsA induces P-gp in hepatocytes isolated from sham operated or 2/3 hepatectomized rats. Consistent with data from the literature,[31—34)] low expression of P-gp is found in liver from rats that underwent sham operation. The increased expression of P-gp in regenerating hepatocytes 24 and 48 h post hepatectomy, is in aggreement with Marino *et al.*,[14)] who, by Northern blot analysis, showed a greater than 20-fold increase in mdr1 mRNA levels in 4—72 h regenerating hepatocytes. There was little or no increase in mdr1 gene transcription when measured by nuclear run-on analyses, which attributed the increase in mdr1 mRNA levels to a posttranscriptional event. In 12 h regenerating hepatocytes, we observed suppression of P-gp expression, which might be explained by destabilization of mRNA in the early stage of hepatic regeneration. Teeter *et al.*,[15)] and Vos *et al.*,[17)] found highly elevated levels of hepatic mdr1b mRNA but only moderately increased levels of mdr1a. We demonstrated elevated cellular levels of P-gp in regenerating hepatocytes, 24 and 48 h after 2/3 PH, which could be due to an upregulation of mdr1b. This induced expression of rat *mdr1b* can be attributed to stress by endogenous stimuli,[35,36)] produced during hepatic regeneration but the mechanism involved in this upregulation of this gene is still unclear.

In the regenerating liver, TNF-α has been implicated as a major regulator in the regenerative response.[35)] According to Taub[35)] the upregulation of the rat mdr1 gene is TNF-α dependent,[37)] which regulates gene expression through the activation of the transcription factor NF-κB.[37—39)] Elevated NF-κB activities that have been observed during liver regeneration are attributed to TNF-α,[35)] which induces a signalling cascade leading to activation of NF-κB.[40)] This transcription factor is involved in the basal and in the inducible transcriptional regulation of mdr1.[39,41)] This is mediated by a κB-element (mdr-κB) in the promoter of the rat mdr1b gene.[37,41)] Therefore, TNF-α binding to hepatocytes, which induces a signaling cascade, leads to the rapid translocation of NF-κB to the nucleus.[38—40)] This activation by TNF-α might, therefore, be at least in part responsible for the upregulation of mdr1b.[37)]

Furthermore, our results are supported by data from Yasumiba and coworkers,[42)] who also showed an increased expression of P-gp in hepatocytes isolated from CsA treated animals. Our results suggest that CsA, apart from inhibiting P-gp function by direct binding to the transporter,[43)] augments cellular levels of hepatic P-gp. This is particularly pronounced during liver regeneration. The observed increase could be explained by the activation of NF-κB due to the hepatotrophic effect of CsA and additional stimulation *via* the TNF-α induced signaling during hepatic regeneration.[44)]

In summary, we demonstrate for the first time that CsA has an additive effect on the expression of P-gp during liver regeneration in the rat. Therefore, P-gp induction might be considered in patients receiving CsA after cadaveric or living donor liver transplantation for hepatocellular carcinoma and chemotherapy as an adjuvant treatment for the prevention of tumor recurrence.[45,46)] CsA has an adverse effect on tumor recurrence after transplantation,[47)] On the other hand, hepatocellular carcinomas express P-gp and the response to systemic chemotherapy is inversely related to the level of P-gp expression in patients with inoperable tumors.[48)] CsA inhibits the function of P-gp in several tumors and in hepatocellular carcinoma cell lines as well.[49)] The relationship between the increase of P-gp expression and the inhibition of P-gp func-

tion in proliferating malignant hepatocytes and the clinical importance of these actions, after liver transplantation for hepatocellular carcinoma, has not been elucidated. Additional experiments are also required to elucidate if the induction of P-glycoprotein expression during liver regeneration occurs at therapeutic levels of CsA after liver transplantation (100—300 ng/ml).[28)]

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