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Validation of endothelin (ET) immunoreactivity in human bile by HPLC. Comparison of biliary ET concentration in liver transplant recipients with values obtained during cholecystectomy

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Abstract High endothelin (ET) concentrations were recently detected in human bile after orthotopic liver transplantation (OLT). In the present study we compared biliary ET/big-ET levels measured by radioimmunoassay (RIA) in liver graft recipients ($n = 37$) with levels measured in non-transplant patients during cholecystectomy ($n = 38$) to clarify the influence of transplantation on the levels of biliary ET peptides. HPLC elution profiles of biliary ET were analyzed for characterization of ET peptide composition and validation of RIA analysis in bile extracts. Mean ET/big-ET levels in the common bile duct after OLT were significantly elevated (ET, 20.9 ± 15; big-ET, 39.2 ± 19 fmol/ml) compared to levels in non-transplant patients (ET, 5.7 ± 4.9; big-ET, 12 ± 8 fmol/ml). Highest ET/big-ET levels were measured in the gall bladder during cholecystectomy (ET, 61.7 ± 41; big-ET, 75 ± 28 fmol/ml). ET and big-ET levels were correlated by linear regression. HPLC analysis reveals the presence of high levels of ET/big-ET in human bile. Biliary ET mostly represents ET-1. High biliary ET levels after OLT appear to be derived from active endothelial secretion and probably reflect hepatic endothelial stress after preservation/reperfusion. High biliary ET levels could be involved in the mediation of functional cholestatic syndromes after OLT.

Key words Endothelin/big-endothelin · bile · Liver transplantation · Cholestasis · Motility

Introduction

Endothelin-1 (ET) and its precursor big-endothelin (big-ET) are secreted by endothelial cells and have vascular, non-vascular, autocrine, and hormonal effects. Most striking is a strong contractile effect on smooth muscle cells and vasculature. Multiple unspecific stimuli such as ischemia, hypoxia, mechanical stress or inflammation have been shown to induce ET generation in endothelial cells of ubiquitous location [17, 23]. Hepatic ET secretion has been documented by autoradiographic studies, liver cell cultures, and perfusion experiments. The liver appears to be a major site for ET clearance from systemic circulation by receptor binding and cellular incorporation [1, 5]. Whether biliary excretion of ET peptides is a relevant ET clearance mechanism has not been fully investigated. The existence, source, and physiological relevance of ET peptides in human bile are still controversially discussed in the literature [10]. We have recently published the detection of comparably high concentrations of both ET and big-ET in human bile after orthotopic liver transplantation (OLT), which can be interpreted as a model of severe hepatic endothelial stress [15]. To clarify the influence of liver transplantation on the levels and composition of ET peptides in bile secretion, we have compared biliary ET/big-ET concentrations detected after OLT with levels measured in non-transplant patients. As ET measurement in bile

extracts by radioimmunoassay (RIA) has not been validated in detail, a separation of ET peptides in bile by high-pressure liquid chromatography (HPLC) was performed, comparing elution profiles of bile extracts with purified ET/big-ET peptides.

Materials, patients and methods

Bile samples

Bile samples were collected from two distinct clinical groups (Table 1). Samples were obtained from 37 liver transplant recipients in the early postoperative course (days 1–5) after OLT (two samples per patient) and from 39 patients during elective cholecystectomy for treatment of symptomatic cholecystolithiasis. A total of 113 bile samples was analyzed. Samples in transplant recipients were obtained by sterile syringe aspiration from the bile drain, which was implanted during OLT for stenting of the side-to-side choledocho-choledochostomy. Attention was paid to collect fresh bile only. Bile samples were not taken from the reservoir bag. Samples from non-transplant patients were either obtained by direct puncture of the gall bladder or by aspiration of bile from the common bile duct via canulation of the cystic duct (Table 1).

Patient groups

Indications for OLT included alcoholic cirrhosis ($n = 8$), chronic hepatitis ($n = 12$), cholangio/hepatocellular carcinoma ($n = 7$), primary biliary cirrhosis ($n = 6$), and miscellaneous entities ($n = 4$). Mean patient age was 43 ± 13 years, overall female/male sex ratio was 1:2. UW solution was used for preservation. Mean cold ischemia time was 12 ± 3 h. Postoperative immunosuppression involved methylprednisolone (100 mg/day), azathioprine (2 mg/kg per day), cyclosporine A (blood levels 250–400 mg/dl by HPLC) and FK506 (0.15 mg/kg i.v.). Indication for cholecystectomy was symptomatic cholecystolithiasis. Inflammation of the gall bladder was graded histologically by a pathologist. Patients were differentiated into the presence ($n = 18$) or absence ($n = 13$) of acute inflammation according to the degree of polynuclear cellular infiltration. Mean patient age was 53 ± 13 years: male/female ratio, 2:3. Patients with empyema were excluded.

ET/big-ET analysis

All bile samples were immediately frozen in sterile polystyrene tubes at –40 °C until analysis. ET/big-ET concentration was measured in duplicate in all samples and HPLC fractions by commercial RIA (Biomedica, Austria). Technical details of the RIAs have been published previously [8, 19]. Prior to RIA analysis, ET peptides were extracted from bile samples using preconditioned Sep-Pak-C18 extraction cartridges (Water Millipore) to avoid interference by other plasma components and to enrich the ET peptide component in the sample.

HPLC analysis

Pooled bile from five patients was separated by reversed-phase chromatography. For HPLC injection, dried bile extracts (Speedvac) were redissolved in 0.15 % BSA solution, separated on a Nucleosil 100 5 C18 column (150 × 4 mm; Forschungszentrum Seibersdorf, Austria), and fractions monitored at 1-min intervals with a UV/visible detector (absorption 214 nm). The concentration of ET/big-ET in separated bile extracts was analyzed in all HPLC fractions (volume 1 ml) and compared with elution profiles of purified ET/big-ET peptides. The retention time for ET-1 (fractions 1–38) was 15.5 min, for big-ET (fragments 22–38) was 25.8 min.

Table 1 Mean concentrations ± SD (fmol/ml) of endothelin *(ET-1)* and big ET peptides in human bile in the different clinical groups investigated. All differences were statistically significant. (A versus B $P < 0.001$, A versus C $P < 0.006$, B versus C $P < 0.005$; Wilcoxon test)

Patient groups	Sample aspiration site	ET-1	big-ET
A Liver transplant ($n = 37$)	Common bile duct (bile drain aspiration)	20.9 ± 15	39.2 ± 19
B Cholecystectomy ($n = 8$)	Common bile duct (cystic duct canulation)	5.7 ± 4.9	12.1 ± 8
C Cholecystectomy ($n = 31$)	Gall bladder (direct puncture)	61.7 ± 41	75.4 ± 28

Statistical analysis

Data are presented as mean values ± SD. ET values in different groups were compared by the Wilcoxon signed rank test. Correlation between ET and big-ET was analyzed by linear regression. P values > 0.05 were considered statistically significant.

Results

ET and big-ET peptides were detected in all human bile samples investigated. Mean ET and big-ET levels measured in bile, drained from the common bile duct in the early postoperative course after OLT, were significantly higher compared to levels detected in the common bile duct during cholecystectomy in non-transplant patients. Highest ET/big-ET levels were measured in bile, directly aspirated from the gall bladder. Levels in the gall bladder markedly exceeded levels detected in the common bile duct in both groups. Mean levels of ET and big-ET detected in the different groups investigated are shown in Table 1.

Biliary big-ET concentration always exceeded synchronous biliary ET concentration. Synchronous biliary ET and big-ET levels were correlated by linear regression in all bile compartments and patient groups analyzed (transplant patients, $r = 0.79$, $P > 0.001$; non-transplant patients, bile duct values, $r = 0.6$, $P < 0.04$, common bile duct values, $r = 0.8$, $P < 0.0001$). ET levels in the gall bladder did not correlate with the grade of histological inflammation.

Chromatographic characterization of bile extracts by reversed-phase HPLC revealed identical elution profiles for biliary ET, synthetic human ET, and big-ET (fractions 23–27). Separation of bile extracts by HPLC and analysis of fractions by RIA thus reveals the exis-

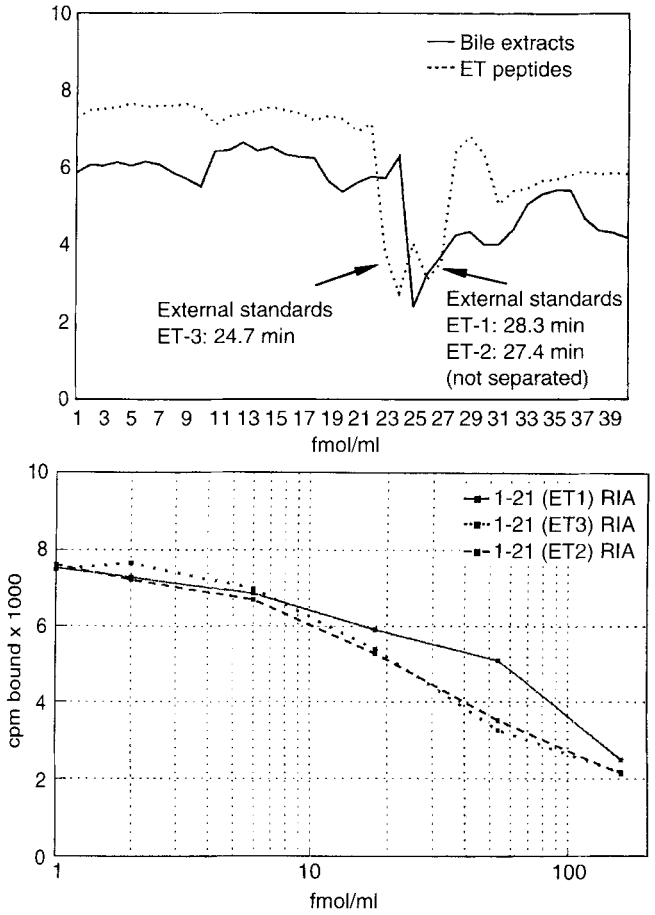

Fig. 1 Elution profiles of purified endothelin *(ET)* peptides *(dotted line)* and ET in bile extracts *(solid line)* after HPLC separation. *Below,* standard curves of bound radioactivity (percentage of total activity) of the radioimmunoassays *(RIAs)* used for ET analysis in HPLC fractions. Total ET immunoreactivity was 135 fmol/ml

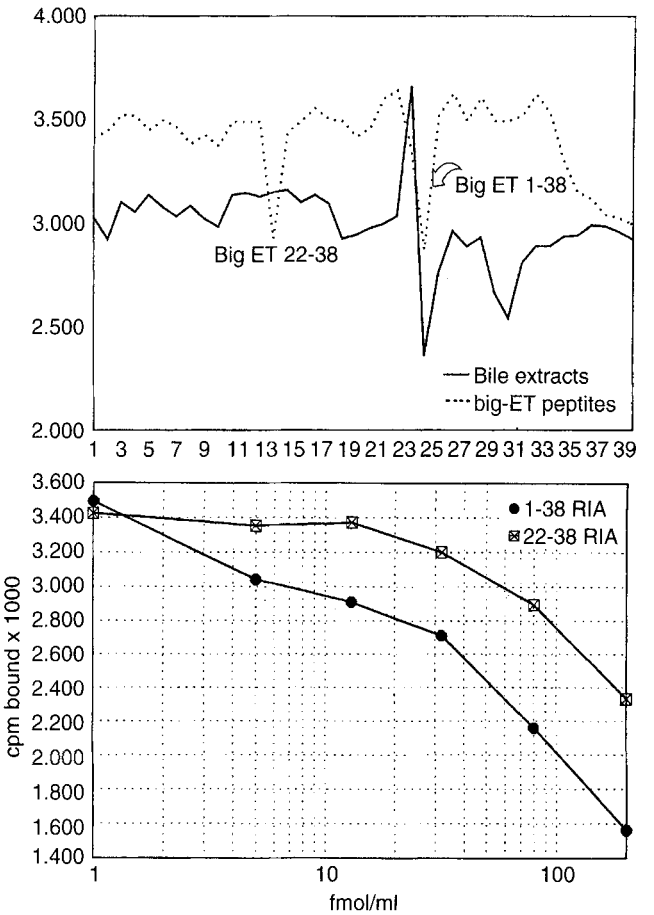

Fig. 2 Elution profiles of purified big-ET *(dotted line)* and big-ET in bile extracts *(solid line)* after HPLC separation. *Below,* standard curves of bound radioactivity (percentage of total activity) of the RIAs used for big-ET/big-ET fragment analysis in HPLC fractions. Total big-ET immunoreactivity was 29 fmol/ml

tence of high amounts of ET and big-ET in human bile. Biliary ET mostly represents ET-1. No big-ET (fragment 22–38) could be detected in human bile. Additional elution signals were found in all assays in fractions 29–34, particularly in the big-ET RIA. Elution profiles of ET and big-ET peptides, including standard curves of the purified peptides are shown in Figs. 1 and 2.

Discussion

Only limited research has so far been focussed on the physiology and source of ET peptides in human bile. We know that cultured human epithelial cells of the gall bladder, intra- and extrahepatic bile ducts, and epithelial lining cells of hepatic cysts are able to synthesize and secrete ET [10]. These findings suggest that ET is locally produced in the biliary tract in vivo and secreted into the bile duct system by a paracrine route. Whether hepatic ET clearance by biliary excretion is a relevant mechanism has not been fully investigated. As all isoforms of ET are potent contractile agonists for smooth muscle cells in a wide variety of tissues, including the gall bladder and bile ducts, biliary ET levels could possess a physiological role in the regulation of choledochal motility and gall bladder contraction [3, 9, 11, 12, 21]. No reference values for ET levels in bile from different clinical groups have been available so far. The specific influence of OLT on biliary ET/big-ET levels could therefore only be estimated. In the current study we have shown that mean ET concentrations in bile collected from the common bile duct during the early postoperative period after OLT significantly exceeded mean ET levels detected during cholecystectomy in non-transplant patients.

Increased biliary levels after OLT could either reflect endothelial stress of the liver graft after cold pres-

ervation and reperfusion [6, 7, 20] or just mirror elevated systemic postoperative ET levels and hepatic ET clearance functions. Systemic ET levels after OLT have been previosly investigated by various authors [13, 14, 16, 22]. Published levels in systemic circulation were far lower than biliary concentrations detected after OLT [15]. The fact that biliary ET levels in patients with symptomatic cholecystolithiasis also proved to be significantly lower, despite the regular presence of marked epithelial inflammation, suggests a dominant sinusoidal endothelial generation of biliary ET peptides within the liver graft. As a marked concentration gradient from gall bladder to the common bile duct was found, any future comparison of biliary ET levels in different groups should always refer to the exact source of the bile sample investigated. Pathological grading of histological gall bladder inflammation did not correlate with biliary ET levels. The remarkable quantity of ET peptides detected in the gall bladder can most probably be explained by a secondary concentration effect, caused by active epithelial water resorbtion. Biliary ET and big-ET levels were significantly correlated by linear regression in transplant and non-transplant patients, both in the common bile duct and gall bladder compartments. As big-ET is the precursor of ET, and in the case of local generation usually found in equimolar concentration [17, 23], this again points to the predominance of local hepatic ET generation. Cyclosporine could have modified biliary ET levels, as it is known to produce cholestasis and, furthermore, has been reported to increase ET levels. ET may thus potentiate the cholestatic action of cyclosporine [2, 4, 14, 18].

According to the HPLC elution profiles of ET peptides in bile extracts, ET peptides measured in human bile probably mostly represent ET-1. A clearcut differentiation between ET-1, -2 and -3 would have required the further separation of fractions 23–27, necessitating the analysis of a further extended bile volume. The additional signals in HPLC fractions 29–34 most probably represent ET propeptides or aggregates and not unidentified ET metabolites, as the peaks could also be detected in a big-ET ELISA system (our unpublished data). The HPLC elution profiles detected demonstrate the validity of the RIAs used for analysis of bile extracts.

Biliary ET secretion could be associated with, if not causative of, a large variety of functional cholestatic syndromes associated with various hepatic disorders or occasionally encountered in the postoperative course after OLT. Whether there is a diagnostic or even prognostic value of single or repetitive ET measurements in bile after OLT for estimation of the individual patient's course or for characterization of graft function and preservation damage, should now be investigated in more detail.

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