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The influence of partial hepatectomy on the pharmacokinetics of preoperatively injected 4'-epidoxorubicin in rats

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Summary. Preoperative administration of 4'-epidoxorubicin (Epi-A) has been suggested as adjuvant therapy in patients undergoing liver resection for hepatocarcinoma. To assess the influence of partial hepatectomy on the pharmacokinetics of Epi-A, an experimental study in rats was undertaken in which 5 mg/kg Epi-A was given i.v. 10 min prior to a 2/3 hepatic resection or sham operation. Epi-A levels in liver tissue and plasma were determined using a sensitive and specific HPLC method. A marked uptake of Epi-A in liver tissue was found at 10 min after injection. The partially hepatectomized rats showed a 2-fold increase in AUC between 4 and 72 h as compared with the sham-operated controls. The terminal half-life from 24 to 72 h was not significantly changed by the partial hepatectomy. The plasma binding of Epi-A was measured at 4 h post-surgery. The fraction of unbound Epi-A was 0.16 in partially hepatectomized animals and 0.20 in sham-operated rats. The results indicate that when Epi-A is given prior to liver resection, a dose reduction might be necessary to avoid increased side effects due to the rise in AUC.

Introduction

Hepatic resection is the only treatment that offers any chance of cure for a primary malignant tumour of the liver [1, 7, 13, 17]. Relapse of the disease after surgery may be due to the growth of undetected small tumours in the unresected liver tissue or to the preoperative presence of extrahepatic metastasis. In addition, failures may be attributable to perioperative tumour seeding due to surgical manipulation.

Only a few studies have reported on chemotherapy as an adjuvant to hepatic resection in humans. Holton et al. [10] obtained long-term survival (28–47 months) in children receiving cyclophosphamide, 5-fluorouracil (5-FU) and vincristine following liver resection for hepatoblastoma. A recent review of patients treated for hepatocellular carcinoma concluded that chemotherapy combined with tumor resection offered the best chances for long-term survival [18]; in 18 patients in whom tumor resection was combined with hepatic arterial infusion of floxuridine, doxorubicin and mitomycin, the median survival was 46 months.

In no previous study has chemotherapy been given i.v. immediately prior to liver resection. We are currently planning a therapeutic program of liver resection combined with preoperative 4'-epidoxorubicin (Epi-Adriamycin; Epi-A) in patients with hepatocarcinoma. Therefore, we performed a study in rats to investigate the influence of partial hepatectomy on the pharmacokinetics of Epi-A.

Materials and methods

A total of 89 inbred male BUF/MOL SPF (Buffalo) rats were used in this study; their age was 36 days and their mean body weight was 110±27 g (mean±SD). The animals were kept in groups of six to eight in 20-dm^3 cages and had free access to water and pellets (Ewos-Alab Brood Stock Feed R 3).

All surgical procedures were carried out between 10 a.m. and 12 a.m. by the same operator. Surgical anesthesia was induced by s.c. injection of a solution of Hypnorm (Janssen: fentanyl dihydrogencitrat, 0.079 mg/ml; fluanisone, 2.5 mg/ml), Dormicum (Roche: midazolam, 1.25 mg/ml) and sterile water (1:1:2, by vol.) at a dose of 0.15 ml/100 g body weight. The liver resection was undertaken by ligation of the hilus of the median and left lateral lobes using the technique described by Higgins and Anderson [8]. In a preliminary experiment on ten rats, the resected portion of the liver was 69%±3% of the total liver wet weight. The sham operation involved cutting the ligaments suspending the median and left lateral lobes without performing the actual resection. All animals were given 2.0 ml 0.9% NaCl s.c. immediately after the abdomen was closed.

4'-Epidoxorubicin HCl for clinical use (Farmitalia, Carlo Erba, Milan, Italy) was dissolved in 5% glucose to give a concentration of 1.75 mg/ml. A dose of 5 mg/kg was injected into a tail vein of the rats during a 2-min period that ended exactly 10 min prior to surgery.

Experimental design

Three sets of experiments were performed. In the first set, Epi-A levels in liver tissue and plasma were determined at the time of operation

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(n = 5) and at 4 h after either partial hepatectomy (n = 4) or sham operation (n = 4). In addition, measurements of haemoglobin and haematocrit as well as albumin and total protein in plasma were carried out at 4 h postsurgery.

In the second set of experiments, plasma concentrations of Epi-A at 4, 12, 24, 48 and 72 h postsurgery were determined. In all, 30 rats were subjected to a partial hepatectomy and 26 rats underwent sham operation; 4–7 animals were killed at each interval for each kind of operation. In the third set of experiments, the plasma binding of Epi-A was determined at 4 h after surgery in partially hepatectomized rats (n = 13) and sham-operated animals (n = 7).

In all experiments the rats were anesthetized with ether before blood sampling. Following laparotomy, a blood sample of 3–4 ml was drawn from the abdominal aorta into a 10-ml heparinized tube, which was immediately put on ice. Plasma was separated within 1 h and then kept frozen in glass tubes at –70° C until measurement of Epi-A values (within 6 weeks). Liver tissue was immediately frozen in liquid nitrogen and stored at –70° C for later analysis (within 1 week).

Measurement of 4'-epidoxorubicin and 4'-epidoxorubicinol in plasma and liver tissue

Chemicals and stock solutions. Pure Epi-A and 4'-epidoxorubicinol (Epi-ol) were provided by Farmitalia, Carlo Erba (Milan, Italy). Daunorubicin hydrochloride was obtained from Rhone-Poulenc (Vitry-Sur-Seine, France) and used as an internal standard for the analysis of Epi-A in liver tissue. Desipramine hydrochloride was obtained from Ciba-Geigy A.G., Basel, and ortho-phosphoric acid (min. 85%, 14.7 mol/l) was supplied by Merck, Darmstadt. Acetonitrile and methanol were HPLC-grade. Stock solutions of Epi-A and Epi-ol were made by dissolution in water and then used to make spiked plasma standards and controls. Plasma standards were made from heparinized, medication-free rat blood and were stable at –70° C within the observation period of 3 months.

Equipment. Chromatography was performed using a Rheodyne Loop Injector 7125 (100 µl), a Waters Model 510 HPLC pump, an LS-5 Luminescence spectrometer (Perkin Elmer) set at an excitation wavelength of 473 nm (slit width, 10 nm) and an emission wavelength of 593 nm (slit width, 20 nm), and an LC 100 Laboratory Computing Integrator (Perkin Elmer). The analytical column comprised a Supelcosil LC-8-DB column (5 µm; 15 cm × 4.6 mm inside diameter) preceded by a 2-cm Supelco LC-8 guard column.

Sample preparation. Duplicate plasma samples from each rat were extracted for chromatography using a modification of the method described by Robert [19]. Instead of Sep-paks, we used Bond Elut solid-phase extraction columns (C8, 100 mg/ml) (Analytichem International). For activation of the bonded silica, 2 ml methanol was passed through the extraction column, followed by 2 ml 0.05 mol/l sodium phosphate buffer (pH 7) containing 10 µg/ml desipramine hydrochloride (to avoid adsorption losses). Next, 300 µl plasma sample was added prior to the addition of 1 ml distilled, deionized water. Following one wash with 1 ml 0.05 mol/l sodium phosphate buffer (pH 7) containing 10 µg/ml desipramine hydrochloride, the sample was eluted from the extraction column with 0.5 ml methanol. After evaporation of the solvent at 37° C under a stream of nitrogen, the residue was redissolved in 300 µl mobile phase, of which 100 µl was injected onto the HPLC column.

Liver-tissue extracts were prepared according to the method of Rose et al. [20]. Epi-A levels were determined in 1–1.5 g tissue and the total amount in liver was calculated according to wet weight.

HPLC analysis. The mobile phase consisted of a phosphoric acid solution (pH 1.8; 0.05 mol/l) and acetonitrile (73 : 27, v/v); the flow rate was 1.5 ml/min. In the chromatograms, Epi-A appeared as a sharp, symmetrical peak, with a retention time of approximately 4.5 min. Chromatograms obtained from medication-free rat plasma did not show any interfering peaks. Using spiked plasma, a standard curve was obtained for each day's run by plotting peak height values against Epi-A concentrations. Linearity was established in the range of 5–500 ng/ml. Epi-A was estimated using peak heights from samples and comparing these with a simultaneously run, standard curve. Spiked plasma controls were included in each run.

Recovery was determined at each run by comparing a stock solution, diluted in mobile phase and injected directly, with a plasma control of the same concentration; the detection limit was 4 ng/ml and recovery was 85% ± 6.5% (n = 10). The interassay coefficient of variation was 5.2% (n = 6) and 4.7% (n = 6) for the 7.5- and 150-ng/ml plasma controls, respectively. Measurement of the peak-height ratio of Epi-A and internal standard was used for the quantification of Epi-A in liver tissue.

In the chromatograms from plasma, the Epi-ol metabolite eluted approximately 1.4 min before Epi-A. At this point, however, an interfering endogenous peak of varying height was detected; thus, it was not possible to determine accurately Epi-ol levels in plasma. However, by subtracting the mean endogenous peak in plasma blanks from the peak found in treated animals, an estimate of the levels of Epi-ol in treated rats could be made. In chromatograms of normal liver tissues this endogenous peak was not detected.

Determination of protein binding

The binding of Epi-A in plasma was studied by equilibrium dialysis using an equilibrium time of 3 h. We used a Dianorm apparatus (Diachema AG, Switzerland) that was kept in a water bath at 37° C. Between the two cell halves, a dialysis membrane (mol. wt. cut-off, 10,000 Da; Diachema AG, Switzerland) was introduced. Then, 1 ml plasma was dialyzed against 1 ml 0.09 mol/l phosphate buffer (pH 7.2) containing 0.03% sodium azide as an antibacterial agent. The buffer compartment was extracted and applied onto the HPLC column following the procedures described above for plasma. Spiked standards were made by dilution of stock solutions with dialysis buffer. Unbound and total Epi-A concentrations were determined; the binding of Epi-A in plasma was expressed as the unbound fraction.

Blood analyses. Albumin and total protein were analyzed on an RA-1000 (Technicon Instruments, Tarrytown, N. Y., USA). Haemoglobin and haematocrit were analyzed on a model $T_6$60 Coulter counter (Coulter Electronic Limited, Luton, England).

Statistical analysis

In the first and third sets of experiments, data from the various groups were characterized as the mean ± SD. Group differences were tested for statistical significance using a two-sided Mann-Whitney U-test. In the second set of experiments, the plasma concentration-time curves from 4 to 72 h were compared by a linear regression analysis on the difference between the mean log concentration data at each time point. Slope and intercept were tested for difference from zero by a two-sided t-test. The half-life corresponding to the terminal phase after 24 h was determined from the slope of the regression lines, log C vs time. The AUC obtained between 4 and 72 h for the partially hepatectomized rats and sham-operated controls was calculated by the trapezoidal rule using the mean concentration values at each time point. The significance level was set at $P < 0.05$ in all tests.

Results

Liver-tissue measurement

The relationship between Epi-A levels in plasma and liver at the time of operation and at 4 h postsurgery is illustrated in Fig. 1. A marked uptake of Epi-A in liver tissue had taken place during the 10-min period after injection. Nonetheless, based on total liver wet weight, we calculated that

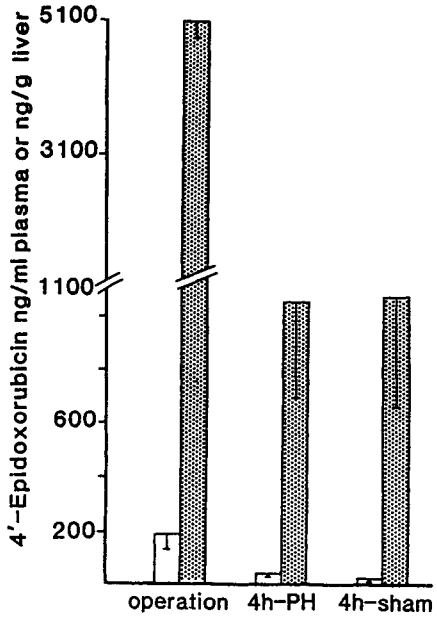

Fig. 1. Bar chart demonstrating Epi-A levels in plasma (□) and liver tissue (▨) at the time of operation and at 4 h postsurgery. Mean values ±SD are given. *PH*, partial hepatectomy

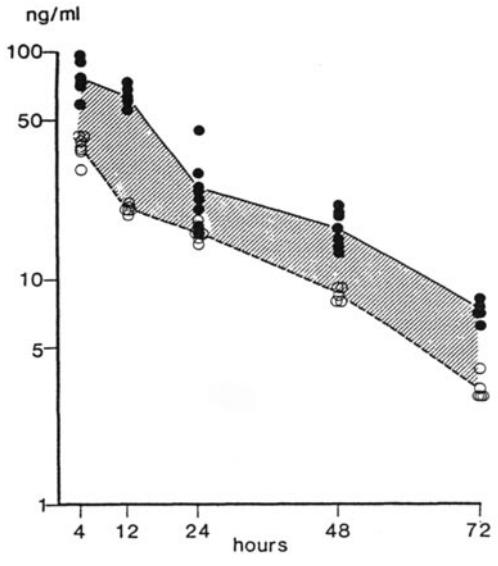

Fig. 2. Plasma concentration vs time curves for Epi-A in partially hepatectomized (●——●) and sham-operated (O----O) rats. The curves are drawn between mean values; points represent measurements in individual animals. *Shaded area:* the difference in AUC between the partially hepatectomized rats and the sham-operated animals

Table 1. Blood values registered 4 h after operation in rats given 4'-epidoxorubicin preoperatively

Group	Haemoglobin (g/dl)	Haematocrit ratio	Total protein (g/l)	Albumin (g/l)
Epi-A + PH ($n = 4$)	12±0.5	35±1	47±1*	30±1
Epi-A + sham ($n = 4$)	12±0.3	35±1	49±1*	31±1

Each value represents the mean ±SD for the number of animals shown in parentheses. PH, partial hepatectomy
* Significant difference between groups ($P < 0.05$)

Table 2. Unbound fraction of 4'-epidoxorubicin in plasma at 4 h postsurgery

Group	Total (ng/ml)	Unbound (ng/ml)	Unbound fraction
Epi-A + PH ($n = 13$)	89±27	14±3	0.16
Epi-A + sham ($n = 7$)	32±11	6±2	0.20

Each value represents the mean ±SD for the number of animals shown in parentheses. PH, partial hepatectomy

only 5%±0.3% of the injected dose was present in the liver at the time of operation. At 4 h postsurgery, a significant decrease in Epi-A levels in liver tissue was registered in partially hepatectomized rats as well as in sham-operated animals ($P < 0.05$; Fig. 1). There was no significant difference between the two groups ($P > 0.05$).

Blood chemistry

The data registered at 4 h after the operation are presented in Table 1. There was no difference between partially hepatectomized and sham-operated rats with regard to haemoglobin level and haematocrit. In the partially hepatectomized rats, plasma albumin was 97% ($P > 0.05$) and total protein, 96% ($P < 0.05$) of the values determined for rats that were subjected to sham operation.

Pharmacokinetics

The plasma concentration-time curves for Epi-A calculated between 4 and 72 h in partially hepatectomized and sham-operated animals are shown in Fig. 2. The difference between the mean log concentration data of the two groups showed no significant time dependence (slope of regression line = −0.0004 log(ng/ml)/h; $P > 0.05$). The mean concentration ratio between the partially hepatectomized rats and the sham-operated animals was 2.17 (significantly >1; $P < 0.05$). This difference is also expressed by the change in AUC estimated for the two groups. The AUC was 1,885 ng/ml × h between 4 and 72 h for the rats subjected to partial hepatectomy as compared with 896 ng/ml × h for the sham-operated animals. The half-lives determined from the slopes of the regression lines between 24 and 72 h was 29 h for the partially hepatectomized rats and 21.1 h for the sham-operated animals ($P > 0.05$).

Plasma protein binding

At 4 h postsurgery, the fraction of unbound Epi-A was 0.16 in animals that were subjected to a partial hepatectomy (Table 2); in the sham-operated rats it was 0.20 ($P = 0.05$).

Epi-ol measurement

Of the 89 rats, 4 had detectable concentrations of Epi-ol in the estimated range of 5–9 ng/ml plasma. No Epi-ol could be detected in liver tissue at 10 min or at 4 h after injection.

Discussion

Clinical experience has shown that doxorubicin (Adriamycin) has provided the best therapeutic effect in single-drug therapy of hepatocellular carcinoma [6, 16]. The therapeutic response of the analogue 4'-epidoxorubicin (Epi-A) has been shown to be equivalent to that of Adriamycin, although its haematological and cardiac toxicity would appear to be lower [9]. On these grounds, Epi-A was chosen as an adjuvant to hepatic resection.

The purpose of this study was to investigate whether partial hepatectomy alters the pharmacokinetics of Epi-A given in a single dose shortly before surgery. Since Epi-A is mainly eliminated by the hepatobiliary tract [3, 21], the elimination of the drug might be expected to be influenced by liver resection. This could have important implications with respect to the cytocidal effect of the drug on tumour cells and the toxic effects on vital organs in patients receiving Epi-A immediately before liver resection for hepatocarcinoma.

The finding of a high liver-tissue/plasma concentration ratio for Epi-A at 10 min after injection clearly demonstrates the ability of the liver to extract Epi-A from plasma. However, only 5% of the total Epi-A dose given was present in the liver at the time of operation. The amount of drug removed by the partial hepatectomy was therefore of minor quantitative significance. At 4 h postsurgery, the amount of Epi-A per gram of liver tissue showed no difference between the two groups of animals.

The hepatectomized rats showed a 2-fold increase in AUC (4–72 h) as compared with the sham-operated animals. This was probably caused by a reduction in the hepatic eliminating capacity. Removal of liver tissue caused a reduction in the amount of enzymes responsible for the metabolism of Epi-A. Based on this, we would expect a change in AUC for the partially hepatectomized rats of approximately the same magnitude as the reduction in liver tissue mass (approximately 70%). Since the increase in AUC was only 52%, other factors influencing the AUC must be considered. One such factor is suggested by previous findings, which show that liver-tissue blood flow increases in the remaining liver after resection [2, 11]. It may well be that an increase in blood flow raised the eliminating capacity of the remaining liver.

A decrease in the apparent volume of distribution in hepatectomized rats as compared with sham-operated animals could contribute to the increase in AUC. One of the mechanisms underlying such a reduction might be an enhanced binding of Epi-A to the plasma of rats subjected to partial hepatectomy. The finding of a lower unbound fraction of Epi-A in partially hepatectomized rats (0.16) as compared with sham-operated animals (0.20) lends support to this explanation.

Eksborg et al. [5] have demonstrated that the percentage of binding of Epi-A to human plasma is about 75%, with albumin binding constituting approximately 60%. In the present study, plasma albumin remained unchanged and total protein was slightly decreased in partially hepatectomized rats. Thus, the increase in Epi-A binding probably reflects an increase in the concentration of other Epi-A binding macromolecules.

A potential source of error inherent in our study design is the possibility that partial hepatectomy might induce a greater degree of haemorrhage and hypotension than a sham operation. This would result in changes in peripheral blood-flow distribution, with unpredictable influence on the pharmacokinetics of the drug. However, the lack of difference between the groups in mean values of blood haematocrit and haemoglobin at 4 h postsurgery lead us to believe that large haemodynamic differences between the groups were not present. Moreover, we never observed macroscopic bleeding during the operation.

In humans given Epi-A, the metabolite 4'-epidoxorubicinol (Epi-ol), aglycones and glucuronides are found in plasma [22]; of these, Epi-ol is supposed to be cytotoxic [15]. Our estimates of this metabolite showed that only 4 of the 89 rats had detectable Epi-ol levels (range, 5–9 ng/ml). It cannot be excluded, however, that the chromatographic peak detected in these rats merely reflected the variability of the endogenous peak. Support for this contention is given by the findings of Maessen et al. [14], who investigated the metabolism of Epi-A in rats and found that maximal Epi-ol concentrations in plasma were <1% of the Epi-A concentrations.

Several HPLC methods for Epi-A measurement have been described [4, 12, 22]. We found Supelcosil LC-8-DB to be the superior analytical column in terms of peak sharpness. The use of Bond Elut solid-phase extraction columns enabled a fast and simple procedure as well as the simultaneous extraction of several plasma samples. The assay was shown to be sufficiently specific, sensitive and reproducible for therapeutic monitoring and pharmacokinetic studies of Epi-A.

Plasma peak concentration, AUC and time of exposure above a threshold concentration are parameters that have been considered to be of importance for both the therapeutic effect of and the toxic reactions induced by cytostatic drugs. Unfortunately, the optimal dose or plasma-concentration range of Epi-A that should be applied in single-dose treatment is not known. However, our results show that partial hepatectomy results in an increase in the AUC for this drug. Consequently, inasmuch as the AUC is the determinant of deleterious side effects, a dose reduction is preferable in patients undergoing partial hepatectomy.

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