Transporters, enzymes, and enalapril removal in a rat (CC531-induced) liver metastatic model

Lichuan Liu, Huadong Sun, Wiqas Y. Valji, and K. Sandy Pang

Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada

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Liu L, Sun H, Valji WY, Pang KS. Transporters, enzymes, and enalapril removal in a rat (CC531-induced) liver metastatic model. Am J Physiol Gastrointest Liver Physiol 293: G1078–G1088, 2007. First published September 13, 2007; doi:10.1152/ajpgi.00350.2006.—Temporal changes in physiological spaces, protein expression of transporters and enzymes, and enalapril removal were appraised in the metastatic liver tumor model developed from male Wag/Rij rats after the intraportal injection of CC531 colon adenocarcinoma cells; sham-operated preparations received PBS. Liver tissue spaces, investigated with multiple indicator dilution technique in liver perfusion studies, were unchanged at week 3 after tumor induction. At week 4, however, the sinusoidal blood volume and albumin Disse space in tumor-bearing livers were slightly lower compared with those of shams. Increased levels of the canalicular uptake P-glycoprotein, multidrug resistance-associated protein 2 (Mrp2), and bile salt export pump (Bsep) at week 2 (P < 0.05), unchanged levels of Ntcp, Oatp1a1, Oatp1a4, and Mct2, but decreased levels of cytochrome P450 3a2 (Cyp3a2) and glutathione-S-transferase (Gst4–6) at week 4 (P < 0.05) were observed in peritumor vs. sham-operated liver tissues with Western blotting. The steady-state extraction ratio of enalapril, a substrate that enters the liver rapidly via Oatp1a1 and primarily undergoes metabolism by the carboxylesterases, was unaffected by liver metastasis at week 4 regardless of its delivery via the portal vein or hepatic artery into the perfused liver preparations.

COLORECTAL CANCER IS ONE OF THE MOST COMMON SOLID TUMORS IN HUMANS AND APPROXIMATELY 10% OF CANCER DEATH IN THE WESTERN WORLD (26). Depending on the stage of the primary tumor, liver metastases occur in 20 to 70% of colorectal carcinoma patients and distant metastases are major causes of death. Because of the high incidence of mortality, animal models for colorectal metastatic liver tumor were developed to investigate disease mechanism and potential treatment in the mouse (18) and rabbit (7, 38). Some of the currently used mouse models consist of the surgical orthotopic implantation of highly metastatic (human) colon tumor cells in nude mice to result in rapid and efficient formation of metastases in the liver (11). Others include the subcapsular injection of tumor cell suspensions in one or several liver lobe(s) as a solitary tumor (24) and tumor induction by intraportal or intrasplenic injection of CT-26 or C26 colon cancer cells into BALB/c mice (50). Similar methods have been developed to induce metastatic liver tumors in the rat. These include the intraportal or subcapsular injection of a syngeneic rat colon cancer cell line (DHD/K12/TRb) in BD-IX rats (9); other colon cancer RCN-9 cells and its subclone RCN-H4 (21), and KM12SM (human) (17) have been used. The dimethylhydrazine-induced rat colon adenocarcinoma CC531 cell (29), which is syngeneic with the Wag/Rij rat (a Wistar-derived inbred strain), is shown to share characteristics similar to that of human colon adenocarcinoma (44). These cancer models are being utilized to study tumor development (15, 19), assess anticancer treatment (39), and appraise multidrug resistance (12).

The liver is the most important organ responsible for the metabolism and excretion of drugs. The manner in which liver metastasis affects hepatic drug transport and metabolism, however, has not been systematically examined. Factors such as blood flow, binding, transporters, or enzymes that affect drug clearance may be altered. CC531-induced metastatic tumor-bearing rat livers were found to exhibit a reduction in sinusoidal transport but elevation of sulfation activities compared with sham-operated livers (22). Uptregulation of ATP-binding cassette (ABC) transporters in cancer cells that led to drug resistance during cancer chemotherapy in vitro and in vivo was observed (12, 14, 32, 40, 42). Other studies described changes in enzymes and altered drug metabolism in cancer tissues (28) and development of drug resistance (45). Only a few studies evaluated changes of transporters and enzymes in the nontumor or peritumor (liver) tissue (5, 40) and even fewer considered temporal changes in blood flow, binding, transporter, or enzyme that accompany tumor formation. Tumor development may further result in changes of physiological spaces, namely the sinusoidal blood volume, the Disse space, and cellular water space owing either to shunting of flow or changes in the microcirculation associated with the hepatic artery (HA) (23). Since the blood supply is considered to play a critical role in liver metastasis (47), the extraction ratios or clearances of drugs will be altered if there is hepatic arterial bypass and a change in the flow pattern to the tumor-bearing liver.

In the metastatic liver tumor model of direct injection of CC531 cells into the portal vein (PV) of the Wag Rij rat (43), we studied temporal changes in the physiological volumes and variables underlying drug transport and metabolism as well as enalapril removal in the liver. For the identification of tumor development, we devised a system of identifying tumor progression (tumor score) and established a correlation with CC531 in the tumor tissue, determined by immunostaining with MG1, the specific antibody against CC531. In this model, we tested the hypothesis that changes in liver physiological spaces and transporters and enzymes accompanied colon cancer-induced liver tumor metastasis in the rat. The multiple indicator dilution (MID) technique, which involves bolus injection of radiolabeled, noneliminated reference indicators into the PV, was used to estimate the physiological spaces of the tumor-bearing and sham-operated livers in perfusion experi-
ments (34). Western blotting was used to assay for protein levels of transporters and enzymes. [3H]Enalapril, which is transported by the organic anion transporting polypeptide 1a1 (Oatp1a1) and multidrug resistance-associated protein 2 (Mrp2) (25, 36) and metabolized by carboxylesterases to enalaprilat (2) in rat liver, was used as a model compound to investigate whether changes in transporters, enzymes, or mixing of the PV or HA flows (35) had occurred in the tumor liver in single-pass liver perfusion studies. Arteriovenous shunting in tumor livers would reduce the extraction ratio when enalapril, borne by either HA or PV flow, bypasses the hepatocyte.

MATERIALS AND METHODS

Animals and materials. Wag/Rij rats, kind gifts from Leiden University, The Netherlands, were bred in the Division of Comparative Medicine, University of Toronto. Male Wag/Rij rats (250–320 g), were used according to protocols approved by the Animal Committee of the University of Toronto. The CC531 cells were kindly supplied by Dr. Peter J. K. Kuppen, Leiden University. Sources of the primary antibodies were listed in Table 1, and all secondary antibodies were purchased from Bio-Rad Canada (Mississauga, ON). [51Cr]Sodium chromate (3,946 GBq/mg) and 125I-labeled albumin (0.46 MBq/mg) were purchased from Draximage (Montreal, QC, Canada); [3H]Enalapril (specific activity 4.1 TBq/mmol) was synthesized as described previously (8). Bovine chromate (3,946 GBq/mg) and 3H2O (1 mCi/g) were obtained from New Life Science (Boston, MA). D2O (>99.9%) was purchased from Merck Frosst (Montreal, QC, Canada). [3H]Enalapril (specific activity 4.1 TBq/mmol) was synthesized as described previously (8). Bovine blood was a kind gift from Ryding-Regency Meat Packers, (Toronto, ON, Canada). All other reagents were of the highest available grade.

Cell culture. The complete medium for the culture of CC531 cells consisted of RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 20 μg/ml streptomycin, and 20 U/ml penicillin (GIBCO, Burlington, ON). CC531 cells were maintained by serial passages and harvested when 70% confluence was reached. Briefly, cells were trypsinized and centrifuged at 2,700 rpm for 5 min at 4°C. The resultant cell pellet was washed thrice with cold phosphate-buffered saline (PBS) then resuspended in PBS.

The number of CC531 cells in suspension was counted, and cell viability was assessed by Trypan blue exclusion. The cell suspension was kept at 4°C for tumor inoculation (15).

Characterization of physical changes in the rat metastatic liver tumor model. Rats were kept under a 12:12-h light-dark cycle and given food and water ad libitum. After a midline incision for exposure of the PV, male Wag/Rij rats were injected intraperitoneally with either 4 million CC531 cells in PBS (0.5 ml) or PBS (0.5 ml) for the tumor-induced and sham-operated rats, respectively; control rats were not subjected to surgical treatment. Preliminary studies showed that cell counts of less than 4 million were suboptimal in inducing metastatic liver tumors. After inoculation, the abdomen of the rat was sutured and given buprenorphine (0.01 to 0.05 mg/kg, subcutaneously) every 12 h. Then the animal was placed under observation once or twice daily for the next week.

The body weights of the control, sham-operated, and tumor-induced rats were monitored every other day for 4 wk. Livers of control, sham-operated, and tumor rats (1–4 wk) were weighed at death. We measured the tumor size with a ruler and visually detected the frequency; the composite data were developed into a tumor score that related to tumor size and frequency. A correlation was then sought between the tumor score and relative signal intensity of CC531 in the membrane fraction of tumor tissue, detected by Western blotting with the specific antibody, MG1, against CC531 (16). Tumor development was further examined with hematocrit and for assays of alanine aminotransferase (ALT) activity with the ALT kit from Sigma Diagnostics (St. Louis, MO) and total plasma protein by the method of Lowry et al. (27).

Preparation of membrane fraction, microsomes, and cytosol. Peritumour tissue located at least 3–5 mm from the tumor tissue was dissected out; contamination of tumor within this tissue preparation was ruled out by the negative response of the tissue to anti-CC531 antibodies. Tumor development was further examined with hematoxylin-eosin (HE) staining under light microscopy. Blood and the derived plasma from control, sham-operated, and tumor rats were used for the measurement of the hematocrit and for assays of alanine aminotransferase (ALT) activity with the ALT kit from Sigma Diagnostics (St. Louis, MO) and total plasma protein by the method of Lowry et al. (27).

Table 1. Primary and secondary antibodies used in the Western blotting analysis

<table>
<thead>
<tr>
<th>Source of Primary Antibody</th>
<th>Primary and Secondary Antibodies and Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC531 MG1: Antibodies for Research Applications (Gouda, The Netherlands)</td>
<td>(1°) mouse monoclonal, 1:400 dilution</td>
</tr>
<tr>
<td>Oatp1a1</td>
<td>(2°) HRP-anti mouse IgG, 1:2,000 dilution</td>
</tr>
<tr>
<td>Oatp1a4</td>
<td>(1°) rabbit polyclonal, 1:2,500 dilution</td>
</tr>
<tr>
<td>Ntcp</td>
<td>(2°) HRP-anti rabbit IgG, 1:10,000 dilution</td>
</tr>
<tr>
<td>Mct2</td>
<td>(1°) rabbit polyclonal, 1:3,000 dilution</td>
</tr>
<tr>
<td>Pgp C219: Signet (Dedham, MA)</td>
<td>(2°) HRP-anti rabbit IgG, 1:20,000 dilution</td>
</tr>
<tr>
<td>Mrp2 M2III-6; Cedar Lane Laboratories (Burlington, ON)</td>
<td>(1°) rabbit polyclonal, 1:5,000 dilution</td>
</tr>
<tr>
<td>Bsep</td>
<td>(2°) HRP-anti rabbit IgG, 1:10,000 dilution</td>
</tr>
<tr>
<td>Cyp3a2 Daiichi Pure Chemicals (Tokyo, Japan)</td>
<td>(1°) mouse polyclonal, 1:333 dilution</td>
</tr>
<tr>
<td>Sul1e1</td>
<td>(2°) HRP-anti mouse IgG, 1:2,000 dilution</td>
</tr>
<tr>
<td>Gst1-1 Biotrin International (Dublin, Ireland)</td>
<td>(1°) mouse monoclonal, 1:750 dilution</td>
</tr>
<tr>
<td>Gst4-4 Biotrin International (Dublin, Ireland)</td>
<td>(2°) HRP-anti mouse IgG, 1:2,000 dilution</td>
</tr>
</tbody>
</table>

1°, Primary antibody; 2°, secondary antibody. HRP, horseradish peroxidase.
min. The pellet (crude membrane fraction) was placed in resuspension buffer (50 mM mannnitol, 20 mM HEPES, and 20 mM Tris base, pH 7.5). For the preparation of microsomal and cytosolic fractions, tissue was homogenized in the SET buffer (250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4), and subjected to a first centrifugation at 10,000 g for 20 min. The resultant supernatant was further centrifuged at 100,000 g for 60 min to yield the cytosol (supernatant), and the microsomal pellet was resuspended in the SET buffer. All of the procedures were carried at 4°C in the presence of protease inhibitor cocktail (Sigma Aldrich, Canada; Oakville, ON), and samples were stored at −80°C until use. Protein was determined by the method of Lowry et al. (27).

**Western blotting of transporters and enzymes.** Immunodetection of transporter and enzyme proteins was studied with the crude membrane fraction and with microsomal and cytosolic fractions of peritumor and normal tissue, respectively. For CC531 detection with the MG1 antibody, homogenate from the tumor-bearing liver was used (Table 1). Samples (10−20 μg) in the loading buffer were heated at 95°C for 5 min or 37°C for 15 min. Immunoblotting was conducted with SDS-polyacrylamide gel electrophoresis (7.5% or 12% gel), and the proteins were electrophoretically transferred to nitrocellulose membranes (Hybond ECL; Amersham, Oakville, ON, Canada). Before blocking, Ponceau staining was used to confirm that equal amounts of protein were loaded. After blocking with Tris-buffered saline with 0.1% Tween 20 (TBS-T) buffer and 5% nonfat milk, the membrane was incubated first with the primary antibody overnight, then with secondary antibody for 1 h (Table 1) before development for chemiluminescent detection with ECL (Amersham, Little Chalfont, Buckinghamshire, UK). The developed films were scanned and the band intensities were integrated with the NIH Image software. Tissues from control, sham-operated, and tumor livers at the fourth week postinoculation were fixed in 10% neutral buffered formalin for immunohistochemical staining of Pgp and Mrp2. The processing, embedding, and sectioning of paraffin blocks and the avidin-biotin complex method for Pgp and Mrp2 (same primary antibodies as in Table 1) were performed by the Department of Pathology, Mount Sinai Hospital, Toronto, Canada.

**Physiological volumes with the MID method.** The physiological volumes were assessed in the single-pass perfused in situ rat liver preparation with the MID method (34). At the third and fourth week postinoculation, a MID bolus dose consisting of noneliminated reference indicators (51Cr-labeled red blood cells to trace the sinusoidal blood volume, 125I-labeled albumin, and 14C-labeled sucrose for estimation of the albumin and sucrose Disse spaces, respectively, and 3H2O or D2O, to appraise the accessible cell water space) was injected into the PV of the tumor or sham-operated liver preparation after 20 min of equilibration. The outflow perfusate was collected immediately thereafter by a programmed fraction collector at successive 1-, 2-, 3-, and 5-s intervals for up to 215 s. In addition, “sham” experiments (without liver) were conducted to obtain dilution curves to correct for the distortion of the inflow and outflow catheters. The concentrations of the noneliminated indicators in the outflow samples were assayed by gamma (for 51Cr- and 125I-cpm in blood) and beta (for 14C- and 3H-dpm in plasma, upon precipitation with methanol) counting, then normalized by the dose, assayed in like fashion. D2O was assayed by Fourier Transform Infrared Spectroscopy (FTIR) (34). The outflow concentration was normalized to the dose and expressed as the fraction of the injected activity per milliliter. Linear transformation of concentration was normalized to the dose and expressed as the apparent mean transit times (MTTs) of the various indicators were calculated as AUMC/AUC and were corrected for τ0 and the MTT of inflow and outflow catheters from the sham experiment to provide the MTTs of the references. These were multiplied to the appropriate flow rates to estimate the physiological volumes (34). The difference between the total albumin or sucrose space and the vascular plasma space, estimated as the blood volume × (1 − hematocrit), yields the albumin and sucrose Disse spaces, respectively. The cellular water space was given by the difference between the total water space and the water spaces occupied by sucrose and in the red blood cells (34).

**S9 metabolism of enalapril.** The sham-operated and peritumor liver tissues at week 4 postinoculation were homogenized in ice-cold Krebs-Henseleit bicarbonate (KHB) buffer and centrifuged at 9,000 g for 20 min at 4°C. The resultant supernatant (S9 fraction) was used immediately or stored at −70°C until use. Previous studies had shown identical rates of enalapril hydrolysis with freshly prepared and thawed (frozen at −70°C) S9 preparations (2). Thawed S9 preparations were diluted to a concentration of ~2 mg protein/ml and then preincubated in a rotating water bath for 10 min at 37°C. Aliquots (100 μl) of S9 were added to unlabeled and radiolabeled enalapril in KHB to result in concentrations of 50 to 1,000 μM (and 200,000 dpm/ml [3H]enalapril) in 0.2 ml of incubation mixture. Samples (100 μl) were retrieved after 10 min, a predetermined time in which metabolic formation rates were linear with time. The samples were placed into 1.5-ml microfuge tubes containing 300 μl of ice-cold CH3CN to stop the reaction. The tubes were centrifuged, and the supernatant was stored at −20°C until analysis. Then 50 μl of the remaining incubation mixture was removed for liquid scintillation counting (model 5801; Beckman Coulter Canada, Mississauga, ON). [3H]Enalapril and [3H]enalaprilat were separated by thin-layer chromatography (TLC) as described previously (2). The relative amounts of radioactive enalapril and enalaprilat present on the TLC plates and the specific activity of the incubation mixture were used to calculate the enalaprilat formation rate (nmol·min⁻¹·mg⁻¹ S9 protein) normalized to the protein content.

**Single-pass liver perfusion of enalapril in tumor and sham-operated livers.** Single-pass perfusion of livers from the Wag/Rijl rats was performed at the 29th day (4 wk) after inoculation with PBS or CC531 cells. Perfusate consisted of 20% washed, fresh bovine red blood cells, 1% bovine serum albumin (Sigma, St. Louis, MO), 5 mM glucose in Krebs-Henseleit bicarbonate solution (pH = 7.4). The inflow perfusate, containing [3H]enalapril, was used to perfuse the PV at 10 ml/min for 80 min. In other preparations, the inflow perfusate, containing [3H]enalapril, entered the HA, but at a reduced flow rate (6 to 9 ml/min) for 72 min because of the high pressure experienced within the perfusion system. The sampling time in the perfusion preparation and analysis by TLC were similar to those described previously (25).

The steady-state extraction ratio (E) was calculated as (Cin − COut)/CIn, where Cin and COut are the steady-state input and output concentrations of [3H]enalapril, respectively. Total hepatic clearance (CLliver, tot) was calculated as the product of Qh, the plasma flow rate, and inasmuch as enalapril was not distributed into red blood cells (25). The biliary clearance (CLliver, bili) was estimated as the QbileCbile/Cbile, the biliary excretion rate, or the product of the bile flow rate (Qbile) and the concentration of [3H]enalapril in bile (Cbile), normalized to the arterial [3H]enalapril concentration, Cin. The hepatic metabolic bolic clearance (CLliver, met) was given directly as the summed appearance rate of [3H]enalapril at bili and outflow perfusate, divided by Cin. The total intrinsic clearance, CLint, of the liver for enalapril removal in tumor vs. sham-operated livers needed to be compared, especially in light of the fact that different flow rates were used for PV and HA perfusion. Inasmuch as enalapril was transported into the liver at rates much higher than those for metabolism (2), rapid entry of enalapril into the liver tissue may be inferred. Simplified equations may be used to estimate the CLint according to the well-stirred (Eq. 1) and parallel-tube (Eq. 2) models (33):
where $Q_p$ is the plasma flow rate, $f_p$ is the unbound fraction in plasma, and $E$ is the extraction ratio.

**Statistical analysis.** Data were expressed as means ± SD, and the means were compared by the Student’s $t$-test or Wilcoxon Mann-Whitney Test (Systat 11.0, Systat Software, Point Richmond, CA) for MID data. For data on Western blotting for transporters and enzymes, the relative intensities from control and peritumor liver tissues were compared with those from the sham-operated group treated for the same time interval, weeks 2, 3, and 4. Data from the control group were not compared directly with peritumor group since it was recognized that the control group lacked the surgical manipulation and the accompanying inflammatory factors. For the liver perfusion studies, the MID data for tumor vs. sham-operated livers treated for the same time intervals, week 3 or week 4, were compared. It should be emphasized that data from the sham-operated and not control group should be compared with those of the tumor group for appraisal of the changes in enzymes and transporters. Cytokines, in addition to tumor development and inflammation, are found associated with surgery (1, 3, 10, 20, 31). Hence, PV-perfused tumor liver data were compared with those of sham-operated PV-perfused livers; data for HA-perfused tumor liver were compared with those of sham-operated HA-perfused livers. The data were not normalized to liver weight since the tumor burden increased the weights of the liver. The level of $P < 0.05$ was set as the threshold for significance.
A visual detection scheme was developed to relate the size, measured by a ruler, and frequency of tumor to the severity of tumor progression in a tumor score system (Table 2). At week 2, the tumor score was low, but it increased from 2 to 4 wk postinoculation, although large variability existed in the tumor growth pattern (Fig. 3A). A good correlation was found to exist between the assigned tumor score and the CC531 relative intensity detected by Western blotting with the MG1 antibody (Fig. 3B).

**MID liver perfusion studies.** The outflow patterns of the noneliminated reference indicators in the MID studies with the metastatic tumor liver were similar to those from the sham-operated perfused rat liver preparations (Fig. 4). Bimodal patterns for the sucrose and water curves that were characteristics of cirrhotic livers (46) were absent among all of the indicator dilution studies. The dilution profiles were smooth: the red blood cells emerged first and peaked earliest, then decayed rapidly, followed by those for albumin, sucrose, and then water, which were progressively more dispersed, peaking later and decaying slower because of the increasing distribution spaces in the liver. Because the mass of the tumor liver increased beyond 3 wk, physiological spaces estimated for the MID studies were not normalized to the weight of the liver tissue (Table 4). There was no change in the vascular, Disse, and cellular water spaces in the tumor liver at third week postinoculation. At the fourth week, however, the sinusoidal blood volume and the albumin Disse space were less for the tumor-bearing livers compared with those for shams (P <0.05). Although the sucrose Disse space was decreased by 20% for the tumor-bearing livers, the change was not significant because of the large variation among the preparations. There was, however, no change in the accessible cellular water space at fourth week with tumor development (Table 4). A higher sinusoidal blood volume was found for week 4 sham preparations over the week 3 preparations. In view of the small number of samples, it is highly conceivable that data for the sham-operated preparations for week 4 are values in the upper range of the normal values.

**Western blotting and immunohistochemical staining.** Expression levels of apical transporters, known to endow drug resistance, Pgp and Mrp2, in peritumor liver tissue were higher than those of shams at the second week postinoculation (P < 0.05). The same was observed for Bsep (Fig. 5). Immunohistochemical staining showed a copious but similar presence of Pgp and Mrp2 in control, sham, and peritumor liver tissues, but not within the tumor tissue (data not shown). Compared with the sham-operated group, there was no change in the sinusoidal transporters, Oatp1a1, Oatp1a4, Ntcp, and Mct2 in the peritumor liver tissue from 2 to 4 wk postinoculation of tumor-
bearing livers (data not shown). However, cytochrome P450 3a2 (Cyp3a2) and Gst4-4 at week 4 in peritumor tissue were significantly decreased ($P < 0.05$) compared with those of shams (Fig. 6).

Enalapril metabolism in S9 and in sham and tumor-bearing livers in perfusion studies. No significant change was observed in the metabolite formation rate of enalaprilat in S9 fractions from sham and peritumor liver tissues (Fig. 7). Again, because the mass of the tumor liver increased beyond 3 wk, clearance was presented in milliliters per minute, rather than being normalized to the weight of liver tissue (Table 5). In PV-perfused liver preparations, $E$ of enalapril was constant with time during steady state (Fig. 8) and was not different between the sham and tumor livers ($0.78 \pm 0.07$ vs. $0.76 \pm 0.08$, $P > 0.05$). The steady-state biliary (1.64 ± 0.41 vs. 1.96 ± 0.25 ml/min), metabolic (3.28 ± 0.64 vs. 3.49 ± 0.46 ml/min), and total hepatic (6.70 ± 0.53 vs. 7.03 ± 0.07 ml/min) clearances were also similar ($P > 0.05$). In the HA-perfused liver preparations, the biliary ($0.55 \pm 0.66$ vs. $0.47 \pm 0.35$ ml/min), metabolic ($1.44 \pm 1.83$ vs. $0.80 \pm 0.34$ ml/min), and total hepatic ($5.31 \pm 0.83$ vs. $6.03 \pm 1.04$ ml/min) clearances were similar between sham and tumor-bearing livers ($P > 0.05$); the steady-state extraction ratios of enalapril were not significantly different between sham and tumor livers ($0.86 \pm 0.01$ vs. $0.86 \pm 0.01$, $P > 0.05$) (Table 5; Fig. 8).

Another observation was that the sum of the biliary and metabolic clearances did not add up to the total hepatic clearance. The ratios of the metabolic to the biliary clearances, however, remained similar (Table 5). This prompted the measurements of the tissue-to-plasma concentration (CT/CP) ratios of enalapril and enalaprilat. These values in the peritumor liver tissue, perfused via the PV, were found similar to those in sham liver ($21.5 \pm 7.5$ vs. $16.7 \pm 5.3$ for enalapril, and $216 \pm 26.7$ vs. $190 \pm 65.1$ for enalaprilat; $P > 0.05$). However, the CT/CP ratios of enalapril in peritumor liver tissues, perfused via the HA, were significantly lower than those in sham liver ($8.9 \pm 2.1$ vs. $15.8 \pm 3.7$, $P < 0.05$) and different from those of PV-perfused livers. The CT/CP ratios for enalaprilat were
similar for HA- and PV-perfused livers (Table 5). We also
estimated the total hepatic intrinsic clearances according to
Eqs. 1 and 2 and calculated the hepatic intrinsic clearance; all
were unchanged for HA and PV perfused livers and for
sham-operated and tumor livers (Table 5).

**DISCUSSION**

As outlined in this study, the CC531-induced, metastatic
liver tumor model has been successfully established (Fig. 1).
Although others have reported on use of lac-Z-transfected

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**Table 4. Physiological volumes in sham-operated and metastatic tumor Wag/Rij rat livers at 3 and 4 wk postinoculation**

<table>
<thead>
<tr>
<th>Tumor Score</th>
<th>Body Weight, g</th>
<th>Liver Weight, g</th>
<th>Sinusoidal Blood Volume, ml</th>
<th>Albumin Disse Space, ml</th>
<th>Sucrose Disse Space, ml</th>
<th>Accessible Cell Water Space, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor group (n = 4)</td>
<td>2</td>
<td>280±2</td>
<td>8.73±0.70</td>
<td>1.22±0.39</td>
<td>0.83±0.19</td>
<td>1.29±0.09</td>
</tr>
<tr>
<td>Sham group (n = 3)</td>
<td>N/A</td>
<td>280±9</td>
<td>8.66±0.39</td>
<td>1.33±0.12</td>
<td>0.61±0.13</td>
<td>1.04±0.20</td>
</tr>
</tbody>
</table>

| Tumor group (n = 3) | ≥4 | 290±13 | 10.9±0.75* | 1.30±0.36* | 0.50±0.18 | 1.33±0.29 | 4.25±0.49 |
| Sham group (n = 4) | N/A | 290±9 | 8.60±0.76 | 1.85±0.14 | 0.83±0.08 | 1.70±0.14 | 4.00±0.95 |

Values are means ± SD. Data were not normalized to liver weight since the weights of the tumor livers were higher due to tumor burden. N/A, not applicable. *4 wk tumor vs. 4 wk sham (Wilcoxon Mann-Whitney Test, P < 0.05).
CC531 colon carcinoma cells to quantitatively measure tumor cells in the metastatic tumor liver in a chemiluminescence assay (37, 49), progression of the present tumor may be tracked by MG1, the specific CC531 antibody. Moreover, this model has a well-defined end point at \( 7 \) to \( 11 \) wk after inoculation with the CC531 cells. The method would fail only when the tumor score was low (<2) since the tumors would not be readily apparent with visual inspection. The absence of change in the hematocrit, the ALT activity, and total plasma protein up to 4 wk postinoculation of tumor (Table 3) with progression of tumor in this metastatic model mimic the metastatic liver tumor disease in humans.

We then proceeded to document the characteristics and temporal changes of the metastatic tumor model. It has been reported that, in liver tumors, the majority of blood supplying the tumor arises from the artery and not the PV (6, 13). It has also been reported that, in liver diseases, physiological volumes are altered, and bimodal dilution curves resulted from capillarization of the sinusoid, as shown in liver MID studies (46). Such behavior was not observed in our PV-perfused rat liver preparations at weeks 3 and 4 postinoculation, and there was absence of bimodal flow patterns. The physiological volumes of liver accessed by the PV or HA in this metastatic tumor model are virtually unknown. Changes in physiological spaces were absent at week 3 of tumor progression. But at week 4, the sinusoidal blood volume and albumin Disse space were slightly attenuated, although the sucrose Disse space and intracellular water space were unchanged in the metastatic tumor livers compared with normal livers (Table 4). A reduction of sinusoidal space and Disse space is likely explained by an erosion of the peritumor tissue due to the invasion of tumor cells and not bypass of hepatocytes, since the cellular water space was unchanged. Although the same was observed in cirrhotic rat livers (46), the high variation in the liver preparations and the small sample size render these changes less reliable.

Changes in drug disposition are known to be associated with cancer development (22, 48). It is therefore important to
thoroughly examine the changes in enzymes and transporters among tumor and nontumor tissue, since both tissues play critical roles in the disposition of anticancer drugs and success in anticancer therapy (30, 41). The ABC transporters (14) and drug metabolizing enzymes (30) noted in tumors, together with those in peritumor tissue, will affect drug disposition in the metastatic tumor liver. However, with immunoistochemical staining, the expression of Pgp and Mrp2 proteins in tumor tissues was absent in this model, suggesting that the transporters conferring drug resistance are not constitutive but may be induced only with concomitant use of anticancer drugs. For the present tumor model, enzymes (Fig. 6) and canalicular transporters (Fig. 5) but not the sinusoidal transporters were altered in the peritumor liver tissue, and differences were found between the sham-operated and control liver tissues.

For enalapril that enters the liver cell rapidly with Oatp1a1 (2), the carboxylesterase activity was unchanged (Fig. 7); Mrp2 levels and Oatp1a1 levels remained similar at week 4. The direct comparison of clearance, based on the product of flow × extraction ratio among the sham-operated and tumor-bearing livers for the PV- and HA-perfused livers, was not made since a slightly lower and different flow rate was used for HA perfusion due to the development of high pressure. Clearance values for enalapril, a highly extracted compound, would be affected by the flow rate. Indeed, because of the higher flow rate, ANOVA showed that the biliary, metabolic, and total clearances for the PV-perfused livers were higher than those for the HA-perfused livers (P < 0.05, Table 5). The E was expected to increase, albeit slightly, at reduced flow for enalapril that is highly cleared. Values of E for HA-perfused livers

Table 5. Kinetics of enalapril in the single-pass PV and HA perfused liver preparations at 4 wk postinoculation

<table>
<thead>
<tr>
<th></th>
<th>PV-Perfused Livers</th>
<th>HA-Perfused Livers</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham-operated</td>
<td>Tumor-bearing</td>
<td></td>
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<tr>
<td>Number of preparations</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Weight of rats, g</td>
<td>286±23</td>
<td>296±14</td>
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</tr>
</tbody>
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| LIVER TUMOR, TRANSPORTERS, AND ENZYMES

Fig. 7. Rate of enalapril metabolism at week 4 after CC-531 inoculation, presented as enalaprilat formation rate (nmol·min⁻¹·mg⁻¹ S9 protein), in the S9 fraction from the sham and peritumor liver tissues at enalapril concentrations of 50, 500, and 1,000 μM. Data are presented as means ± SD (n = 3).

Fig. 8. Extraction ratios of enalapril at week 4 after CC-531 inoculation, in the sham-operated and tumor-bearing livers via portal vein (PV) perfusion or via hepatic artery (HA) perfusion. Data are presented as means ± SD (n = 3 or 4).

Values are means ± SD. *P < .05, between sham-operated vs. tumor-bearing in portal vein (PV)- or in hepatic artery (HA)-perfused livers, by Student’s t-test. †P < .05, ANOVA of all data. 1Estimated as difference between input and output concentrations of enalapril, during steady state, divided by the input enalapril concentration. 2Estimated as the product of Qp, the plasma flow rate, and E, the extraction ratio. 3Estimated as biliary excretion rate of enalapril, during steady state, divided by the input enalapril concentration. 4Calculated by Eq. 1; unbound fraction in plasma is 0.55 (8); metabolic intrinsic clearance = total intrinsic clearance × [metabolic clearance/(metabolic clearance + biliary clearance)]. 5Calculated by Eq. 2; unbound fraction in plasma is 0.55 (8); metabolic intrinsic clearance = total intrinsic clearance × [metabolic clearance/(metabolic clearance + biliary clearance)].
(0.86 for 7.2 and 8.2 ml/min) were similar to those for PV-perfused livers (0.76 to 0.78 for 10 ml/min) (ANOVA, $P > 0.05$). Because both CL and E are flow dependent, the total hepatic intrinsic clearance (CLint) and the ratio of biliary to metabolic clearances were used to compare whether changes in metabolic and excretory activities of the liver accompanied CC531-induced tumor development. CLint, calculated according to Eq. 1 or 2, were similar (ANOVA, $P > 0.05$) among the PV-perfused experiments among the sham-operated and tumor-bearing livers. The same was observed for the HA-perfused livers (Table 5). When the CLint is proportioned into the biliary and metabolic intrinsic clearances, the metabolic intrinsic clearance so estimated was also unchanged ($P > 0.05$; Table 5). These observations were congruent with the lack of change in carboxylesterase activity (Fig. 7). The calculated values of CLint and metabolic intrinsic clearance were higher for the well-stirred model over the parallel-tube model (Table 5); this observation is consistent with the assumed flow patterns: bulk vs. plug flow for the liver according to the well-stirred and parallel tube models, respectively (34). The observations suggest that there is no change in the hepatic processing activity of enalapril via HA or PV delivery in the metastatic tumor model. If changes in hepatic arterial flow patterns or by-pass of hepatocyte had occurred, these changes might translate as changes in the CLint or metabolic intrinsic clearance; this was not observed.

In summary, we have conducted a systematic study to examine time-dependent changes in transporters and enzymes in the CC531-induced metastatic liver tumor model. Notably, we found a good correlation existing between the CC531 signal and the tumor scores assigned (Table 2; Fig. 3); the correlation would enable us to follow tumor progression or regression readily. At tumor scores <4, metastatic liver tumor model revealed trends for upregulation of canalicular transporters and downregulation of enzymes, but no change in the hepatic elimination of enalapril was observed. However, in other CC531-inoculation studies, we found increased sulfotransferase (Sult1e1) activity but decreased sinusoidal transporter activity (Oatp1a1 and Oatp1b2) toward estradiol-17β d-glucuronide in livers with higher tumor scores (>4 tumor score); this led to increased sulfation activity in tumor-bearing livers (43). Overall, changes in canalicular transporters were observed only at week 2 after inoculation for this CC531-induced metastatic liver tumor model, and changes in enzymes occurred at week 4. However, changes for the ATP canalicular transporters were relatively minor at week 4. Hence the hypothesis that changes in transporters and enzymes accompany liver metastasis was only partially supported by the data. The tumor model at week 2 postinoculation may be useful for the testing of effective therapeutic agents, since dramatic changes in weight loss occur after week 2. In the absence of any effective intervention, tumor development continues unabated to yield higher grades of metastases that will lead eventually to mortality.

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