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# Influence of tumor size on the main drug-metabolizing enzyme systems in mouse colon adenocarcinoma Co38

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**Abstract.** Mouse colon adenocarcinoma Co38 is widely used as a screening model for human colon tumors. To understand better the influence of tumor size on the main drug-metabolizing enzyme systems, we tested 15 mouse Co38 tumors at different sizes. The average weight was 917 ± 444 mg (range, 300–1,400 mg). Cytochromes P-450 (1A1/1A2, 2B1/B2, 2C8-10, 2E1, 3A4), epoxide hydrolase (EH), and glutathione-S-transferases (GST-α, -μ, and -π) were assayed by immunoblotting. The activities of the following enzymes or cofactors were determined by spectrophotometric or fluorometric assays: 1-chloro-2,4-dinitrobenzene-GST (CDNB-GST), selenium-independent glutathione peroxidase (GPX), 3,4-dichloronitrobenzene-GST (DCNB-GST), ethacrynic acid-GST (EA-GST), total glutathione (GSH), uridine diphosphate-glucuronosyltransferase (UDP-GT), β-glucuronidase (βG), sulfotransferase (ST), and sulfatase (S). Our results showed the absence of all probed P-450s and EH in Co38 tumors. No relationship was found between the Co38 tumor weights and GPX, GST-α, and EA-GST (regression analysis). However, a significant correlation was found between the tumor weights and all other enzymes investigated. For certain enzymes or cofactors, a linear decrease ($P < 0.05$) was observed as a function of tumor weight (CDNB-GST, DCNB-GST, GST-μ, GST-π, GSH, and βG). Other enzymatic activities (UDP-GT, S, and ST) were found to decrease in medium-size tumors and to increase in large tumors ($P < 0.05$; quadratic correlation). These data demonstrate that the expression of many drug-metabolizing enzyme systems is altered during tumor growth and suggest that tumoral response to chemotherapy could be altered as a function of tumor size.

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## Introduction

Animal tumors are widely used as models to study the etiology, behavior, and treatment of cancer in humans [11]. The mouse colorectal adenocarcinoma Co38, a homogeneous, malignant tissue, is frequently used because of its histological characteristics and metastatic properties, which resemble those of human tumors, and also because of its sensitivity to 5-fluorouracil, a reference drug for this disease [7, 8, 11].

Drug-metabolizing enzymes play an important role in determining the susceptibility of organs or tissues to the toxic effects of drugs or other xenobiotics and may also influence tumoral response to anticancer agents in vivo [9, 12]. The main drug-metabolizing enzymes involved in the metabolism of numerous endogenous and exogenous substrates [21] are phase I (cytochrome P-450s[3]) and phase II enzymes [glutathione-S-transferases (GST), epoxide hydrolase (EH), uridine disphosphate-glucuronosyltransferase (UDP-GT), and sulfotransferase], which correspond to functionalization and conjugation reactions, respectively. The enzymes β-glucuronidase and sulfatase can hydrolyze a metabolite that has been conjugated by UDP-GT and sulfotransferase, respectively. In a previous study, phase I and phase II drug-metabolizing enzymes were compared in the mouse Co38 tumor model and in human colon tumors. It was concluded that the Co38 tumor may not be representative of human colon tumors with regard to many of these enzyme systems [20].

The aim of the present study was to investigate the influence of tumor size on phase I, phase II, and hydrolytic enzymes. Our results suggest that the expression of these enzymes varies during tumor growth, which could influence tumoral response to chemotherapy.

## Materials and methods

*Chemicals and enzymes.* 5,5′-Dithiobis(2-nitrobenzoic acid), reduced nicotinamide adenine dinucleotide phosphate (NADPH), 4-methylumbelliferone, 4-methylumbelliferone sulfate, 4-methylumbelliferone glucuronide, reduced glutathione, 1-chloro-2,4-dinitrobenzene, 3,4-dichloronitrobenzene, ethacrynic acid, β-glucuronidase, and sulfatase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Phenobarbital and 3-methylcholanthrene were purchased from Specia (Rhône-Poulenc, Paris) and from Aldrich (Milwaukee, Wis.), respectively. All other chemicals were of the highest purity available from standard commercial sources.

*Mice.* C57BL/6 female × DBA/2 male mice, hereafter designated B6D2F1 mice, were bred at IFFA Credo (Lyon, France). They received food (URA reference 113, Epinay-sur-Orge, France) and water ad libitum. C57 Bl/6 mouse livers were used as positive controls.

*Tumor models.* Mouse colon adenocarcinoma Co38 tumor fragments were implanted s.c. bilaterally in 30 female B6D2F1 mice on day zero. Tumors were measured with a caliper three times weekly. Co38 tumor weights (in milligrams) were estimated from two-dimensional tumor measurements as follows:

$$\text{Tumor weight (mg)} \approx \text{Tumor volume (mm}^3\text{)} = \frac{\text{length (mm)} \times \text{width (mm)}^2}{2}.$$

Mice were euthanized when tumor weights had reached approximately 300 (ten mice), 1,000 (ten mice), and 1,400 (ten mice) mg, on days 21, 31, and 38, respectively. Tumors were removed, weighed, and then frozen within 30 min at –80° C to allow enzyme preservation. Each tumor was analyzed separately. Part of each tissue sample was saved for histopathological examination, and the remainder was used for the present investigation.

*Tumor-doubling time.* The tumor-doubling time (TD) was estimated in control group tumors (ten mice). TD was estimated from the best-fit straight line from a log-linear growth plot based on control group tumors in exponential growth [5].

*Preparation of microsomes and cytosol.* Mouse livers or Co38 tumors were rapidly excised and rinsed in ice-cold 1.15% potassium chloride solution. Tissues were minced with scissors and homogenized in phosphate buffer (0.1 *M*, pH 7.4) containing ethylenediaminetetraacetic acid (EDTA, 0.1 m*M*). After sonication of mouse tissues, microsomes and cytosols were prepared by differential centrifugation as previously described [16]. Microsomal pellets were suspended in phosphate buffer (0.1 m*M*, pH 7.4) containing 20% glycerol and 10 m*M* $MgCl_2$, then stored at –80° C.

*Western-blot analysis.* Microsomal or cytosolic proteins (10–200 μg) were separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gels as described by Laemmli [17]. Resolved proteins were electrotransferred to nitrocellulose sheets, which were probed with antibodies and stained as previously described [2, 13]. The optical density of each stained band was determined by scanning with a densitometer. The amount of each isoenzyme was expressed in arbitrary units (AU) per milligram of microsomal or cytosolic protein. The threshold of protein detection for all the antibodies used was about 1% of the concentration of each isoenzyme measured in the liver.

The following antibodies were used in this study: monoclonal antihuman P-450 antibody 3A4 [3]; polyclonal antirat P-450 antibodies 1A1/1A2 and 2B1/B2, prepared in Dr. P. H. Beaune's laboratory (INSERM U 75, Paris) and checked against Dr. F. P. Guengerich's preparations of P-450 (Vanderbilt University, Nashville, Tenn.); polyclonal antirat P-450 antibody 2E1, kindly provided by Dr. C. S. Yang [29]; polyclonal antihuman P-450 antibody 2C8-10, previously described by Shimada et al. [24]; polyclonal antihuman epoxide hydrolase antibody and its corresponding polyclonal antibodies, previously described by Beaune et al. [4]; and polyclonal antihuman GST-α, -μ, and -π antibodies, purchased from Bioprep (Stillorgan, Dublin, Ireland).

*Preparation of samples for enzymatic assays.* Mouse samples were homogenized in 2 m*M* TRIS-HCl (pH 8.1), 230 m*M* mannitol, and 70 m*M* sucrose using a Polytron homogenizer. These homogenates were centrifuged at 10,000 *g* for 15 min and the supernatant was used for the determination of the following enzymatic activities: UDP-GT, β-glucuronidase, sulfotransferase, and sulfatase. Total GSH, CDNB-GST, DCNB-GST, EA-GST, and GPX activities were assayed on cytosol preparations.

*1-Chloro-2,4-dinitrobenzene-GST activity.* 1-Chloro-2,4-dinitrobenzene-GST (CDNB-GST) was assayed as described by Habig et al. [14] using CDNB as the substrate. The results were expressed as the quantity (in nanomoles) of CDNB conjugated per minute, per milligram of cytosolic protein.

*Selenium-independent glutathione peroxidase activity.* Selenium-independent glutathione peroxidase (GPX) activity was measured spectrophotometrically (340 nm) in cytosols using NADPH as substrate according to Paglia and Valentine [22]. The results were expressed as the amount (in nanomoles) of NADPH oxidized per minute per milligram of cytosolic protein.

*3,4-Dichloronitrobenzene-GST activity.* 3,4-Dichloronitrobenzene-GST (DCNB-GST) activity was measured in cytosol preparations using DCNB as the substrate [14]. The results were expressed as the quantity (in nanomoles) of DCNB conjugated per minute per milligram of cytosolic protein.

*Ethacrynic acid-GST activity.* The activity of this enzyme was measured in cytosol preparations using ethacrynic acid (EA) as the substrate [14]. The results were expressed as the amount (in nanomoles) of EA conjugated per minute per milligram of cytosolic protein.

*Total glutathione concentration.* The sum of the reduced (GSH) and oxidized (GSSG) forms of glutathione was determined in tissue homogenates using the method of Akerboom and Sies [1]. The results were expressed as the quantity of total glutathione (in nanomoles) per milligram of protein.

*UDP-GT activity.* The activity of this enzyme was assayed in tissue homogenates using 4-methylumbelliferone as the substrate as described by El Mouelhi et al. [9]. The results were expressed as the quantity (in nanomoles) of glucuronide formed per hour per milligram of protein.

*β-Glucuronidase activity.* The activity of this hydrolytic enzyme was assayed in tissue homogenates using 4-methylumbelliferone glucuronide as the substrate [9]. The results were expressed as the amount of β-glucuronidase (in nanomoles) formed per hour per milligram of protein.

*Sulfotransferase activity.* Sulfotransferase was assayed in tissue homogenates using a 3′-phosphate-adenosine 5′-phosphosulfate (PAPS)-generating system [9]. The results were expressed as the amount of sulfotransferase (in nanomoles) formed per hour per milligram of protein.

*Sulfatase activity.* Sulfatase was measured in tissue homogenates in the presence of 4-methylumbelliferone sulfate [9]. The results were expressed the quantity of sulfatase (in nanomoles) formed per hour per milligram of protein.

*Protein assay.* Protein concentrations of microsomes, cytosols, and homogenates were determined by the bicinchoninic acid assay [25] using a commercial preparation (Pierce BCA Protein Assay Reagent).

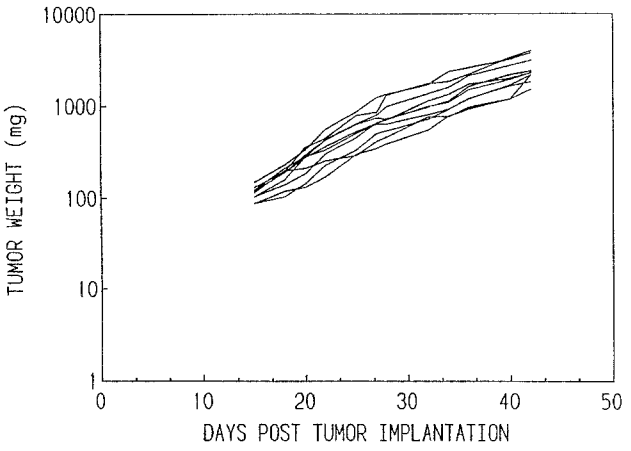


**Fig. 1.** Growth curve generated for Co38 tumors ($n$ = 10). The tumor-doubling time is 4.6 days

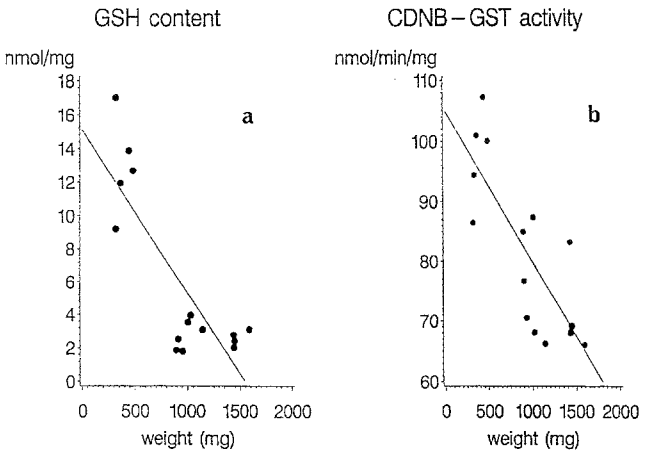


**Fig. 2a, b.** Correlation between tumor weight and the glutathione system. **a** GSH content. **b** CDNB-GST activity. A negative linear correlation was found between the weight of tumors and both CDNB-GST activity and GSH content

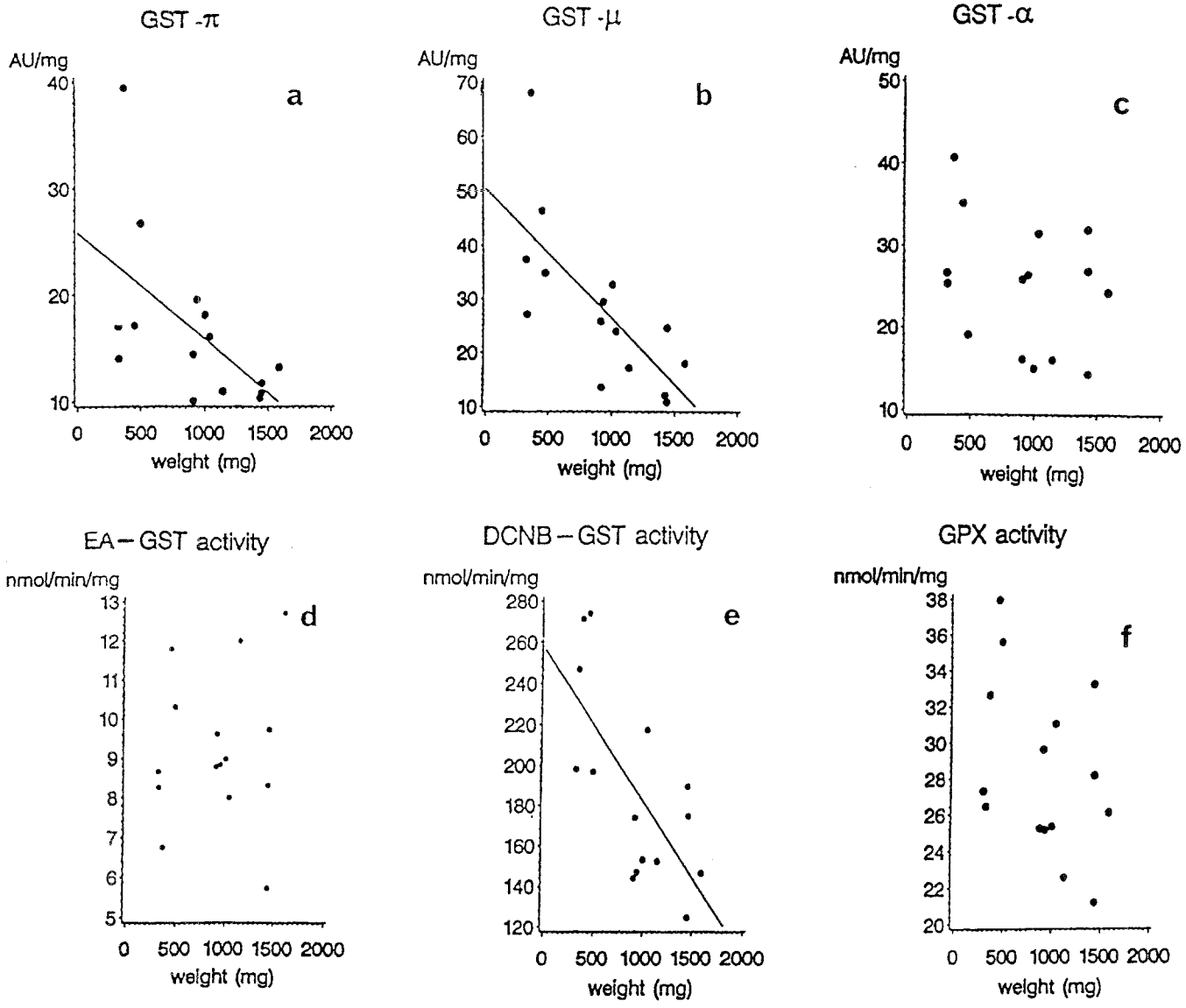


**Fig. 3a–f.** Correlation between tumor weight and the glutathione system. **a** GST-π. **b** GST-μ. **c** GST-α. **d** EA-GST activity. **e** DCNB-GST activity. **f** GPX activity. A negative linear correlation was found between the weight of tumors and the following enzymes or proteins: DCNB-GST, GST-μ, and GST-π

*Statistical analysis.* A regression analysis was performed on Co38 tumor weights and the different enzymes determined by immunoblotting or by spectral assays. A linear correlation and a quadratic correlation were estimated for each enzyme. The best fit was determined on the basis of the model $F$ value. A $P$ value of $<0.05$ was considered significant.

## Results

### *Tumor-doubling time*

The doubling time of the mouse Co38 tumor was 4.6 days as calculated from the best-fit straight line of the linear part of the plot of the log tumor weight as a function of time (Fig. 1). Necrosis was not observed at histological examination of any of the tumors.

### *Cytochrome P-450 isoenzymes*

Cytochromes P-450 (1A1/1A2, 2B1/2B2, 2C8-10, 2E1, 3A4) could not be detected in any of the tumors tested ($n = 15$), whereas mouse livers serving as internal controls showed, as expected, the presence of all cytochrome P-450 isoenzymes. Using five noninduced mouse livers for antibodies 1A1/1A2, 2E1, and 3A4, we obtained values of $142 \pm 48$, $65 \pm 37$, and $67 \pm 10$ AU/mg, respectively; using human liver as the internal control for antibodies 2C8-10 and 2B1/B2, we obtained values of 81 and 84 AU/mg, respectively.

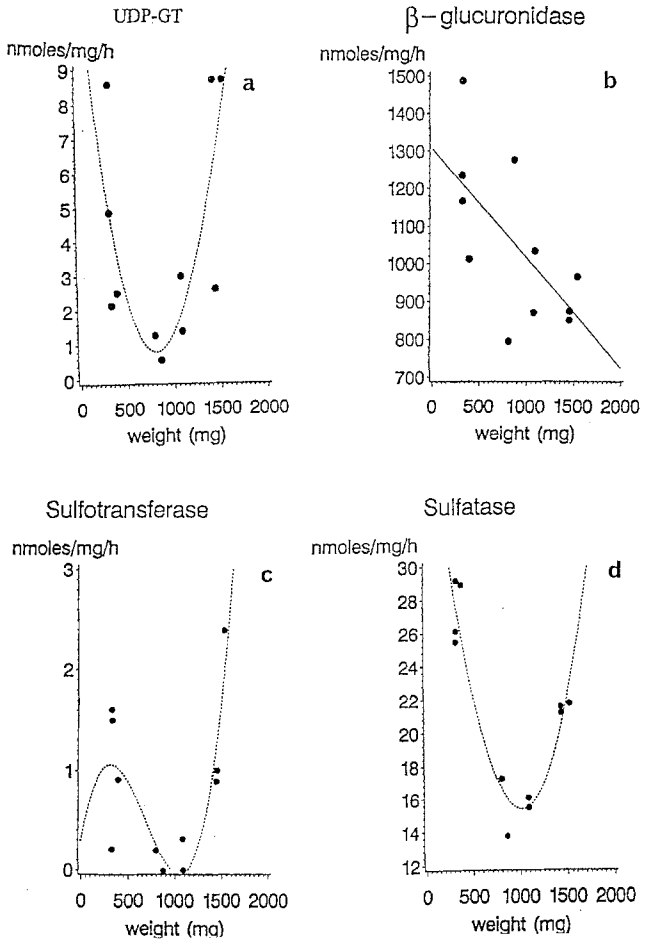


**Fig. 4a–d.** Correlation between tumor weight and glucuronide and sulfate systems. **a** UDP-GT activity. **b** β-Glucuronidase activity. **c** Sulfotransferase activity. **d** Sulfatase activity. A quadratic correlation was found between the weight of tumors and the activities of UDP-GT, sulfotransferase, and sulfatase, whereas a negative linear correlation was found for β-glucuronidase activity

### *Glutathione system*

*Total GSH.* Concentrations of total GSH detected in mouse Co38 tumors are presented in Fig. 2a. A negative linear correlation was found between tumor weights and GSH content ($P < 0.001$).

*CDNB-GST activity.* This activity is representative of all GST isoenzymes. A negative linear correlation was found between tumor weights and CDNB-GST activity ($P < 0.001$; Fig. 2b).

*GST isoenzymes.* GST-α, -μ, and -π were evaluated by Western blotting and were expressed in all the Co38 tumors examined ($n = 15$). A linear correlation was found between tumor weights and GST-π ($P < 0.05$; Fig. 3a) and GST-μ levels ($P < 0.001$; Fig. 3b). No correlation was found between GST-α levels and the Co38 tumor weights (Fig. 3c).

*GST activities.* The GST activities in mouse Co38 tumors at different weights were assayed using representative substrates for the different isoenzymes. A negative linear correlation was found between tumor weights and DCNB-GST activity ($P < 0.01$; Fig. 3e). The correlation found between EA-GST (Fig. 3d) and GPX (Fig. 3f) and tumor weights was not significant.

### *Epoxide hydrolase*

EH could not be detected in any of the Co38 tumors ($n = 15$). Mouse livers serving as internal controls showed the presence of EH ($14 \pm 3$ AU/mg, $n = 5$).

### *Glucuronide system*

UDP-GT was present in all tumors investigated. A quadratic correlation ($P < 0.05$) was observed between tumor weights and UDP-GT activity, with one minimum occurring at about 800 mg (Fig. 4a). β-Glucuronidase was detected in all Co38 tumors studied, and a negative linear correlation ($P < 0.05$) was found between tumor weights and β-glucuronidase activity (Fig. 4b).

### *Sulfate system*

*Sulfotransferase.* This conjugating enzyme was barely detected in all samples analyzed. It was not detected in two tumors. A quadratic correlation was found between tumor

weights and enzymatic activity ($P < 0.05$), with a minimum occurring between 800 and 1,200 mg (Fig. 4c).

*Sulfatase.* This hydrolytic enzyme was detected in all tumors studied. A quadratic correlation was also found between tumor weights and this enzymatic activity ($P < 0.05$), with a minimum occurring at about 1,000 mg (Fig. 4d).

## Discussion

Animal tumor models are used to screen drugs and to evaluate potential therapeutic responses in humans. In an attempt to understand bettter the influence of tumor size on the main drug-metabolizing enzyme systems, we assayed different weights of the frequently used mouse colon adenocarcinoma model Co38. The drug-metabolizing enzyme systems studied can markedly influence the sensitivity of organs or tissues to the cytotoxic and/or carcinogenic properties of drugs and other xenobiotics and could also play a role in tumoral response to anticancer agents by either detoxication or involvement in the toxic effect of these drugs [9, 12].

In accordance with our previous report [20], cytochromes P-450 and EH were not expressed in any of the Co38 tumors investigated. The absence of cytochromes P-450 and EH in Co38 tumors could promote drug retention and cytotoxicity within Co38 tumors if the drug is not metabolized by other enzymes, e.g., phase II conjugating enzymes.

The glutathione system, another major enzymatic pathway involved in the detoxication of exogenous compounds [15], was also studied in mouse colon tumors of different weights. The concentration of the various GST isoenzymes plays a central role in the detoxication of many electrophilic toxic compounds, including carcinogens and cytotoxic drugs. Given the great diversity of their functions, several authors [23, 26] have postulated that the variability in the expression of these enzymes could account for the susceptibility of various tissues to toxins and carcinogens and, if so, have suggested their use as potential prognostic markers in carcinogenesis. In humans, cytosolic GST enzymes have been divided into three distinct groups that are commonly referred to as basic ($\alpha$), neutral ($\mu$), and acidic ($\pi$) transferases according to their isoelectric points [18].

We observed no difference between Co38 tumor weights and GST-$\alpha$ expression as determined either by Western-blot analysis or by GPX activity. These data suggest that tumor size would not influence the response to drugs interacting with GST-$\alpha$ if this enzyme were determinant for response [19].

GST-$\mu$ has been shown to be genetically polymorphic in humans [18] but not in the mouse [20]. GST-$\mu$ and DCNB-GST were detected in all Co38 tumors investigated, and both were negatively correlated with the tumor weights, i.e., levels of GST-$\mu$ and its corresponding activity decreased as the tumor weights increased. It is possible that GST-$\mu$ plays a key role in the malignant phenotype of this mouse tumor and in its response to certain nitrosoureas.

GST-$\pi$ expression also decreased as the tumor weights increased. GST-$\pi$ is also overexpressed in a number of multidrug-resistant cell lines [28] and in human malignant lymphoma and colon carcinoma, two highly resistant tumors [27, 28]. A recent study showed that GST-$\pi$ expression at the time of diagnosis could be a useful marker of clinical resistance to cytostatic drugs in acute nonlymphoblastic leukemia [27]. In Co38 tumors, a decrease was noted in DCNB-GST activity and GSH levels as the tumor weights decreased. However, an increase was previously observed in GSH content and GST activity in 9L rat brain-tumor cell lines pretreated with nitrogen mustard and GSH [10]. This elevated GSH level was interpreted as a mechanism of cellular resistance to nitrogen mustard in the 9L cell line. For Co38 tumors, it can be hypothesized that larger tumors would be less likely to detoxify cytotoxic drugs via GSH conjugation due to their lower GSH levels.

The phase II drug-metabolizing enzymes involved in both glucuronide and sulfate pathways varied according to the weight of the Co38 tumors. In fact, the hydrolytic enzymes ($\beta$-glucuronidase and sulfatase) were more highly expressed than the conjugating enzymes (UPD-GT and sulfotransferase), which were barely detected in the Co38 tumors. Since UDP-GT and sulfotransferase decreased in medium-sized Co38 tumors, drug rentention could be promoted by reducing conjugation in these tissues. These enzymatic pathways would therefore lead to drug retention and, perhaps, to an increase in the sensitivity of mouse colon adenocarcinoma Co38 drugs metabolized via the glucuronide or sulfate pathway.

Taken together, our results provide new information on the main drug-metabolizing enzyme systems in mouse Co38 tumors of different weights. Further studies should focus on drugs that use these specific pathways so as to determine their effects on tumor growth.

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