Transcriptional Repression of Hepatic *Cytochrome P450 3A4* Gene in the Presence of Cancer

Kellie A. Charles,[1,3] Laurent P. Rivory,[5] Sandie L. Brown,[4] Christopher Liddle,[2,3] Stephen J. Clarke,[6,7] and Graham R. Robertson[3,7]

Abstract

Purpose: Many chemotherapeutic drugs have an inherent lack of safety due to interindividual variability of hepatic cytochrome *P*450 (CYP) 3A4 drug metabolism. This reduction in CYP3A4 in cancer patients is possibly mediated by cytokines associated with tumor-derived inflammation. We sought to examine this link by using an explant sarcoma in a novel transgenic mouse model of human CYP3A4 regulation.

Experimental Design: Engelbreth-Holm-Swarm sarcoma cells were injected into the hindlimb of transgenic CYP3A4/lacZ mice. Hepatic expression of the human *CYP3A4* transgene was analyzed by direct measurement of the reporter gene product, β-galactosidase enzyme activity. Hepatic expression of murine Cyp3a was analyzed at the mRNA, protein, and function levels. The acute phase response was assessed by examining cytokines [interleukin-6 (IL-6) and tumor necrosis factor] in serum, liver, or tumor as well as hepatic expression of serum amyloid protein P.

Results: Engelbreth-Holm-Swarm sarcoma elicited an acute phase response that coincided with down-regulation of the human *CYP3A4* transgene in the liver as well as the mouse orthologue *Cyp3a11*. The reduction of murine hepatic *Cyp3a* gene expression in tumor-bearing mice resulted in decreased Cyp3a protein expression and consequently a significant reduction in Cyp3a-mediated metabolism of midazolam. Circulating IL-6 was elevated and IL-6 protein was only detected in tumor tissue but not in hepatic tissue.

Conclusions: The current study provides a mechanistic link between cancer-associated inflammation and impaired drug metabolism *in vivo*. Targeted therapy to reduce inflammation may provide improved clinical benefit for chemotherapy drugs metabolized by hepatic CYP3A4 by improving their pharmacokinetic profile.

The pharmacokinetics of anticancer drugs vary substantially between patients and are an important causative factor in their inherent lack of safety. Much of the interpatient variability in the clearance of anticancer drugs can be attributed to differences in the levels of drug metabolizing enzymes, especially cytochrome *P*450 (CYP) 3A4. CYP3A4 is responsible for the metabolism of many important classes of anticancer drugs, including the taxanes, *Vinca* alkaloids, and camptothecins (1). We have observed previously that patients with advanced cancer have significantly reduced CYP3A4 activity (2) associated with increased plasma concentrations of inflammatory mediators, in particular interleukin-6 (IL-6) and C-reactive protein (3). Importantly, impaired CYP3A4-mediated clearance was correlated with increased toxicity to docetaxel and vinorelbine (2). IL-6 is one of several proinflammatory cytokines with proven involvement in tumor growth, invasion, and metastasis in several malignancies (4) and is associated with initiation of many clinical features of malignancy (i.e., fevers, weight loss, and fatigue; ref. 5). A systemic inflammatory response, as indicated by elevated C-reactive protein, is a prognostic indicator of poor outcome in numerous malignancies, including adenocarcinoma of the pancreas, bladder cancer, lymphoma, colorectal cancer, and esophageal cancer (6–11).

The relationship between inflammation and repression of CYP activity, including CYP3A, has been extensively studied in various *in vitro* and animal models of acute inflammation, including infection and trauma, but also following administration of endotoxin or individual cytokines. These studies have shown that the inflammatory response leads to reduced CYP mRNA and protein synthesis (12, 13), resulting in decreased microsomal metabolism and CYP-mediated drug clearance.

Studies conducted in the late 1960s consistently found that tumor-bearing rodents had reduced CYP protein levels (14) and altered drug pharmacokinetics (15). The changes in liver metabolism in tumor-bearing animals were virtually identical to those associated with inflammatory stimuli. However, no mechanism was proposed for the repression of CYP function in the tumor-bearing animals beyond the presence of a "toxohormone" (16). We postulated that the reduction of CYP activity in the presence of cancer may be due to down-regulation of *CYP3A4* gene expression by tumor-derived inflammatory cytokines.

Due to the difficulties associated with obtaining human liver tissue to investigate such an hypothesis, we used a novel transgenic mouse model incorporating the 13-kb upstream regulatory region of human *CYP3A4* linked to the convenient *lacZ* reporter gene (17). This model recreates most aspects of human *CYP3A4* regulation, including tissue specificity, xenobiotic inducibility, and appropriate pathophysiologic regulation (18). The findings of this study establish a relationship between inflammation and *CYP3A* expression in transgenic CYP3A4 mice in the presence of a localized sarcoma.

Materials and Methods

Materials

Biochemical reagents were mainly purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside was supplied by Astral Pty., Ltd. (Gymea, New South Wales, Australia). Anesthetic agents ketamine and xylazine were purchased from Ilium (Smithfield, New South Wales, Australia). Molecular grade chloroform, isopropanol, and diethylpyrocarbonate were supplied by Crown Scientific (Moorebank, New South Wales, Australia). Antibiotics penicillin and streptomycin and FCS were from Life Technologies (Mulgrave, Victoria, Australia).

Molecular reagents were primarily purchased from Invitrogen (Mulgrave, Victoria, Australia). These included SuperScript II first-strand synthesis system kits, random hexamers, deoxynucleotides, and DTT. Other molecular reagents, such as Trizol, were supplied by Life Technologies, Inc. (Grand Island, NY). The Taqman Universal PCR Master Mix and Taqman probes for real-time reverse transcription-PCR were purchased from Applied Biosystems (Foster City, CA).

Rabbit anti-rat CYP3A1 primary antibody was purchased from Biotrend (Cologne, Germany) and β-actin and IL-6 primary antibodies were from Santa Cruz Biotechnology (Clayton, Victoria, Australia). Horseradish peroxidase–conjugated goat anti-rabbit IgG secondary antibodies were obtained from Sigma-Aldrich. Alexa-Fluor 488–tagged goat anti-rabbit IgG secondary antibody was purchased from Molecular Probes (Eugene, OR). Immobilon-P polyvinylidene difluoride membranes were from Millipore (Bedford, MA). Enhanced chemiluminescence reagents were from Pierce Pablo (Rockford, IL).

Methods

Tumor transplantation. Eight- to 10-week-old male mice carrying the −13CYP3A4/lacZ transgene (9/4 line, see ref. 17) or FVB strain (only for midazolam sleep test experiments) were aseptically inoculated, using a 14-gauge needle, with 0.3 mL suspension of Engelbreth-Holm-Swarm (EHS) sarcoma in sterile 0.9% saline into the quadriceps muscle of right hind leg. Control animals were injected using a 14-gauge needle with 0.3 mL sterile 0.9% saline. Tumor mass reached ∼3 g or 10% of total body weight over 17 to 21 days. The mice were then euthanized, blood was collected, and liver and tumor were harvested. Whole body, liver, and tumor weight were measured to the nearest 0.01 g on a top-load balance. All animal work was approved by the Western Sydney Area Health Service Animal Ethics Committee.

Blood collection and serum cytokine analysis. Serum was prepared from clotted whole blood by centrifugation at $5,000 \times g$ for 10 min at 4°C and stored at −70°C until analysis of serum cytokines. Serum concentrations of IL-6 and tumor necrosis factor-α were determined by commercially available ELISA kits (Quantikine ELISA kits, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Fluorescent immunohistochemical detection of IL-6. Cryostat sections (10 or 18 μm) of frozen liver and tumor tissue were prepared by conventional techniques before incubation with rabbit anti-mouse IL-6 antibody (1:20) in a humidified chamber for 1 h at room temperature, washing in PBS and incubation with Alexa Fluor 488–tagged goat anti-rabbit IgG secondary antibody (1:1,000). Liver and tumor sections were viewed and images were digitally captured using digital camera-mounted fluorescent microscope and the Advanced Spot software (Diagnostic Instruments, Sterling Heights, MI).

Hepatic CYP3A and acute phase reactant gene analysis. Macroscopic detection and quantification of the *CYP3A4* transgene was analyzed using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of liver wedges and the *O*-nitrophenyl-β-D-galactopyranoside assay, respectively, as described previously (17). The mRNA expression of liver-derived genes of interest was measured using real-time PCR and Taqman technology (17). The target genes were a representative mouse *Cyp3a* gene, *Cyp3a11*, and the major mouse acute phase reactant, *serum amyloid protein P* (*SAP*). Cyp3a11 and SAP mRNA expressions were normalized using the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). The 5′ to 3′ sequences for forward and reverse primers and Taqman probes used in this study were as follows: (*a*) Cyp3a11, TGCTCCTAGCAATCACTGG (forward), GTGCCTAAAAATG-GCAGAGGTT (reverse), and FAM-CCTCTACCGATATGGGACTCGTAA-ACATGAACTT-TAMRA (probe); mSAP, TACTGCTTTGGATGTTTGTCTT-CAC (forward), TCAGCTTCACATGATTTTCAG (reverse), and FAM-CAGAAGCCTTTTGTCAGACAGACCTCAAGAGT-TAMRA (probe); and mGAPDH, GTCGTGGATCTGACGTGCC (forward), TGCCTGCTTCAC-CACCTTCT (reverse), and VIC-CCTGGAGAAACCTGCCAAGTATGAT-GACA-TAMRA (probe).

Hepatic CYP3A protein analysis. Extraction and preparation of reduced proteins from liver tissue was done as described previously (19). Protein extracts (20 μg total liver protein per lane) were run on 7% SDS-PAGE gel electrophoresis for 2 h under reducing conditions. After transfer to polyvinylidene difluoride membranes and blocking with nonfat skim milk, membranes were incubated in a polyclonal rabbit anti-rat antibody (1:2,000) or polyclonal goat anti-mouse antibody (1:2,000) raised against rat CYP3A or β-actin, respectively. Membranes were rinsed in three 10-min washes of TBST and then subjected to horseradish peroxidase–tagged goat anti-rabbit IgG secondary antibodies (1:25,000 and 1:2,000, respectively) at room temperature with agitation for 60 min. Proteins were visualized by enhanced chemiluminescence and quantified using densitometric analysis.

Hepatic CYP3A activity analysis. CYP3A enzyme function was assessed using the midazolam sleep test. Tumor-bearing and control male FVB mice (background strain of the transgenic CYP3A4 mice) were given 60 mg/kg midazolam i.p. The length of sleep was measured from the initiation of sleep to awakening (as determined by movement from a central spot thrice in 30 s).

Data analysis and statistics. Quantitative data are expressed as mean ± SE for six mice in all groups. For comparison of gene and protein expression in control and tumor-bearing mice, the raw data were transformed to reflect fold inhibition in tumor-bearing animals compared with control animals. Statistical analyses between control and tumor groups were done using Student's *t* test. Significance was set at $P \leq 0.05$.

Results

The expression of *CYP3A4/lacZ* transgene–derived β-galactosidase enzyme showed considerable variability in both saline-treated animals and EHS tumor-bearing animals. Despite the heterogeneity of the *CYP3A4* transgene expression in the

transgenic mice, it was consistently observed that in the presence of the EHS tumor, the hepatic expression of the *CYP3A4* transgene was reduced compared with saline-treated controls (Fig. 1A). Furthermore, β-galactosidase activity in liver lysates was significantly reduced by ~60% in tumor-bearing mice ($P = 0.025$; Fig. 1B). The expression of the murine *Cyp3a11* gene showed similar decrements in the presence of cancer ($P = 0.003$; Fig. 2A). Furthermore, immunoblotting with CYP3A-specific antibodies showed that murine CYP3A protein was also significantly reduced by 50% in tumor-bearing transgenic mice ($P = 0.025$; Fig. 2B).

To determine whether the transcriptional repression of murine *Cyp3a* genes and protein by the EHS tumor resulted in altered CYP3A function, we measured CYP3A activity using the midazolam sleep time assay (20). The major pathway of elimination of midazolam is hepatic CYP3A-mediated 1′-hydroxylation in mice. Therefore, the length of sedation following treatment with midazolam (60 mg/kg ip) is indicative of CYP3A activity. As expected, the decrease in CYP3A protein led to reduced CYP3A activity as observed by the significantly longer midazolam-induced sedation in tumor-bearing mice ($P = 0.02$; Fig. 2C).

Accompanying the down-regulation of *CYP3A* gene expression was evidence of an acute phase reaction in mice with the EHS sarcoma. In tumor-bearing mice, there were increased mRNA levels of the major murine acute phase reactant SAP in hepatic tissue and elevated serum concentrations of IL-6 ($P = 0.002$ for both; Fig. 3A and B). In contrast to IL-6, there was no detectable tumor necrosis factor-α in the serum of control or tumor-bearing mice. IL-6 was readily detected in the cytoplasm of tumor tissue (Fig. 3C) by fluorescent immunohistochemical detection but not in liver tissue from tumor-bearing mice (data not shown).

Discussion

We have shown for the first time *in vivo* that the repression of a human *CYP3A4* transgene and murine *Cyp3a* gene expression in the presence of cancer occurs at the transcriptional level. The down-regulation of *CYP3A* genes in the liver was associated with IL-6 production in the tumor and an acute phase response. Furthermore, the repression of *CYP3A* genes led to increased midazolam-induced sleep times, confirming that the tumor-mediated down-regulation of *CYP3A* genes results in a functionally relevant alteration in the pharmacokinetics/pharmacodynamics of a CYP3A substrate. We have observed a similar repression of CYP3A expression in the colon 38, melanoma B16, and breast EO771 mouse tumor models in conjunction with inflammation (data not shown). Therefore, the links between tumor-derived inflammatory responses and reduced hepatic drug metabolism may be a common feature of several different malignancies.

These results are consistent with the clinical observations of reduced CYP3A4 activity, as determined with the erythromycin breath test, in patients with advanced cancer (2). Similarly, the repression of CYP3A4 activity in the clinical study was correlated with an ongoing inflammatory response, characterized by increased serum concentrations of the major human acute phase reactant, C reactive protein, and IL-6 (3). These findings are clinically relevant with the reduction in CYP3A4 activity resulting in decreased clearance of weekly docetaxel (2).

Other studies have examined genetic differences in human *CYP3A* genes as the basis for interindividual variability in CYP3A-mediated drug metabolism. However, they have consistently failed to establish a link between single nucleotide polymorphisms in *CYP3A4* gene and variable CYP3A activities (21). Indeed, recent studies in cancer patients have found that genetic diversity is unlikely to make a contribution to differences in CYP3A activity, as assessed by erythromycin breath test, midazolam clearance, or paclitaxel pharmacokinetics (22–24). Therefore, the findings from the current study reinforce the concept that repression of CYP3A-mediated drug metabolism by tumor-derived inflammation, rather than genetic variants, is involved in the excessive toxicity experienced by some cancer patients.
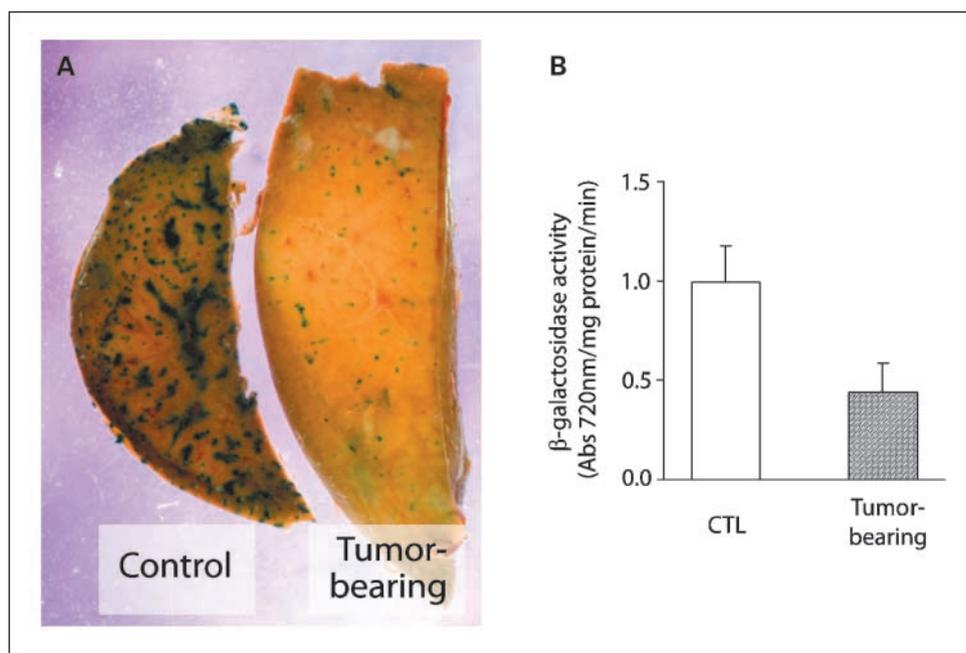

Fig. 1. The *in vivo* effect of EHS sarcoma on the human *CYP3A4/lacZ* transgene in the livers of tumor-bearing mice. *A,* macroscopic detection of human *CYP3A4* transgene using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining in a representative liver wedge from a control (*left*) and tumor-bearing (*right*) transgenic mouse. Magnification, ×10. Liver wedges were fixed with glutaraldehyde overnight at 4°C, washed in a PBS solution for 1 h, and then stained in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside at 37°C for 30 min. Hepatocytes exhibiting the human *CYP3A4* transgene expression are visualized by dark blue staining on the cut surface of the liver. *B,* quantification of the human *CYP3A4/lacZ* transgene expression in the liver of tumor-bearing transgenic and control mice. *CYP3A4/lacZ* expression was quantified via indirect measurement of β-galactosidase activity using the *O*-nitrophenyl-β-D-galactopyranoside assay. Columns, average fold inhibition for six mice; bars, SE.
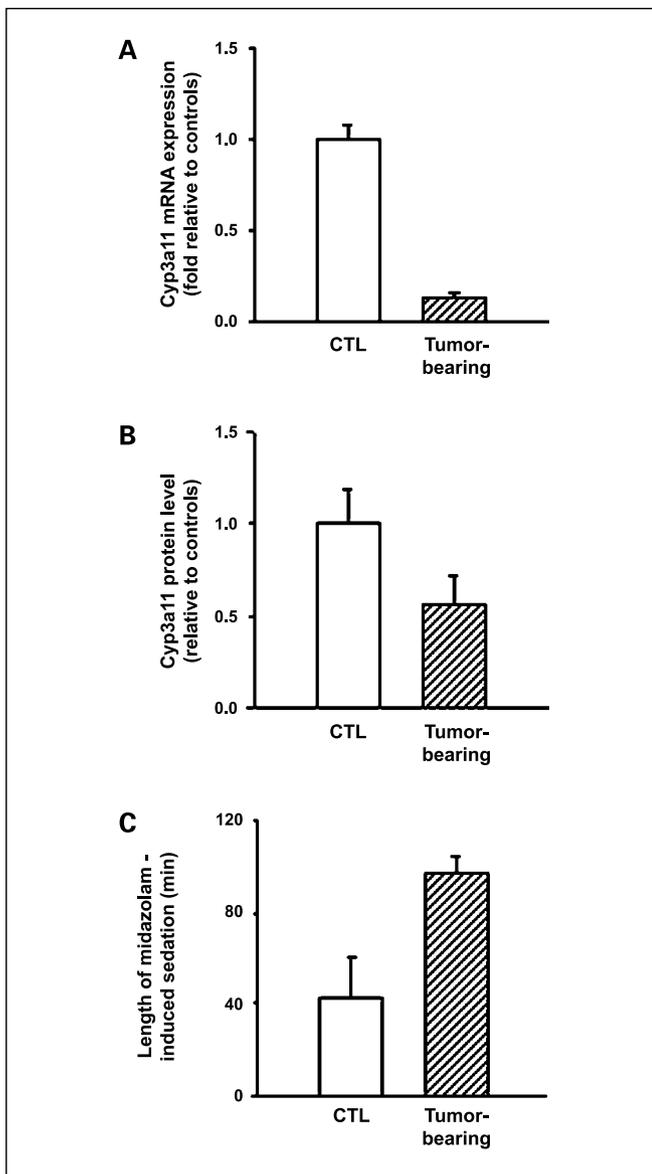
Fig. 2. The effect of EHS sarcoma on the mouse Cyp3a11 mRNA, CYP3A protein, and activity *in vivo. A,* relative quantification of hepatic mouse *Cyp3a11* gene expression in tumor-bearing transgenic and control mice. Hepatic Cyp3a11 mRNA levels were determined by quantitative real-time PCR and normalized to *GAPDH* gene expression. *B,* quantification of mouse CYP3A protein in the liver of tumor-bearing transgenic control mice by Western blot analysis using a rabbit anti-rat CYP3A1 antibody. CYP3A protein expression was quantified by densitometry and then normalized to β-actin protein expression. *C,* measurement of CYP3A-mediated metabolism of midazolam in tumor-bearing FVB mice and control mice. Mice were given 60 mg/kg midazolam (i.p.) and the length of sedation was measured. Columns, average fold inhibition for six mice; bars, SE.

Reduced levels of total CYP protein and CYP-mediated activity have been shown in earlier studies in tumor-bearing rodents (14, 16, 25). However, as this work was conducted before the identification of cytokines or characterization of *CYP* genes, these studies were unable to investigate whether cytokines were involved in the observed modulation of specific CYPs in the presence of cancer. On the other hand, tumor-bearing mice in some studies showed evidence of cytokine-mediated effects, such as hepatomegaly (26), anorexia, and alterations in metabolic conditions consistent with tumor-

mediated cachexia (27). In addition, isolated liver perfusions were used to show that unidentified "toxohormones," as they were termed by the researchers involved, in the blood from tumor-bearing rodents were able to repress CYP enzymes in control livers to the same extent as livers from tumor-bearing animals perfused with their own blood (26, 28). These early studies strongly suggest that the postulated "toxohormones" in the tumor-bearing mice were circulating proinflammatory cytokines.

Cytokines have been implicated in the repression of CYP3A mRNA levels during acute inflammation in rodents (29–32). Administration of individual proinflammatory cytokines, such as IL-1 and IL-6, to isolated human and rat hepatocytes have been shown to directly down-regulate the mRNA expression of *CYP3A* genes (33–36). To further elucidate the role of IL-6 in the down-regulation of CYP3A enzymes, experimental inflammation has been induced with turpentine and lipopolysaccharide. The down-regulation of Cyp3a11 mRNA was abolished in turpentine-treated IL-6 knockout mice but not in lipopolysaccharide-treated IL-6 knockout mice (37). These results confirm that IL-6 is an important mediator in the repression of *CYP3A* genes; however, they also suggest that other factors are involved in the down-regulation of CYP3A expression during lipopolysaccharide-mediated inflammation.

Interestingly, very few of these studies concurrently measured the acute phase response and/or cytokine concentrations. In the absence of such measures of inflammation, it is difficult to determine which proinflammatory cytokines and/or other inflammatory mediators are involved. Only serum concentrations of tumor necrosis factor and IL-6 were measured in the present study, with IL-6 showing significant increases in tumor-bearing mice. However, other cytokines, such as IL-1 (38, 39), IL-2 (40, 41), and IFN-γ (42), have all been shown to down-regulate CYP expression *in vitro* and thus may also be involved in the reduction of CYP3A enzymes in this model of cancer. Other inflammatory mediators, such as nitric oxide, which has been shown to repress CYP expression, may also be involved (32, 43, 44). Further studies are necessary to characterize the inflammatory response in tumor-bearing mice to allow for investigation of the mechanisms involved in the transcriptional regulation of CYPs.

Recent findings have shown that hepatic CYP3A4 mRNA is down-regulated by IL-6 via a pregnane X receptor– and constitutive androstane receptor–dependent mechanism (35, 45). However, there are several signaling pathways that have been characterized for the transcriptional regulation of IL-6-responsive genes. These cytokine-dependent signaling pathways involve numerous downstream transcription factors, including signal transducer and activator of transcriptions (46), nuclear factor-κB (39), activator protein-1 (30), and CAAT/enhancer binding protein (30, 36), which are potentially implicated in the down-regulation of CYPs. As reprogramming of liver gene regulation in response to inflammation involves coordinated induction of acute phase proteins with concomitant repression of CYPs, it is important to characterize the precise role of each factor in these processes. The possibility that repression also involves impaired signaling by nuclear receptors is highlighted by the requirement for hepatocyte nuclear factor 4α in both constitutive and pregnane X receptor–mediated induction of CYP3A4 (36, 47). In addition, inflammatory signaling pathways can inhibit the activity of RXRα, which is the obligate
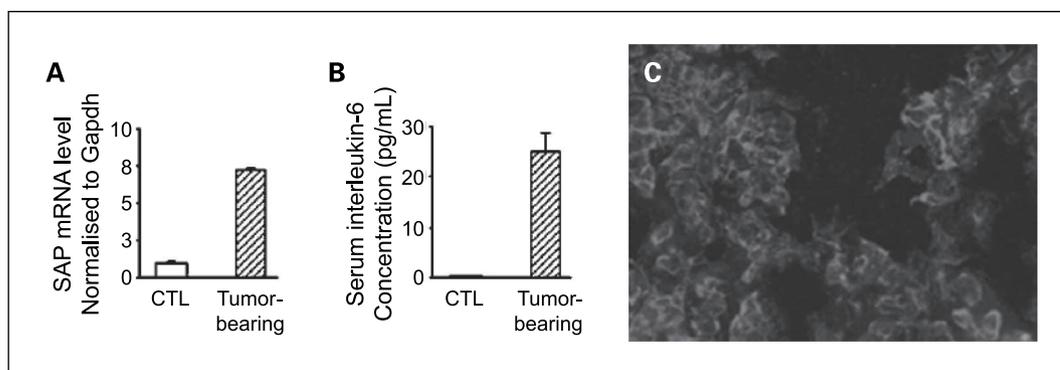
Fig. 3. The acute phase response and source of IL-6 in tumor-bearing mice. *A,* relative quantification of mouse acute phase reactant gene, *SAP*, in the liver of tumor-bearing transgenic mice and control mice. Hepatic *SAP* gene expression was determined using real-time PCR and normalized to GAPDH levels. Columns, mean fold induction of hepatic SAP mRNA levels; bars, SE. *B,* serum concentrations of IL-6 in tumor-bearing transgenic mice ($n = 6$) and controls ($n = 6$). Sera were analyzed for IL-6 concentrations by ELISA. Columns, average IL-6 concentration; bars, SE. *C,* immunolocalization of IL-6 in EHS tumor cells of tumor-bearing transgenic mice. Frozen sections were immunostained with rabbit anti-mouse anti-IL-6 antibody and Alexa Fluor488–tagged goat anti-rabbit IgG secondary antibody. Representative section of EHS tumor tissue exhibiting cytoplasmic localization of IL-6. Preneutralization of antibody by goat serum resulted in no positive immunostaining (control not shown). Bar, 50 μm.

protein partner of pregnane X receptor required to bind regulatory elements in target genes, such as *CYP3A4*. Finally, the similarities between results obtained in the current study and clinical studies in cancer patients support the use of humanized CYP3A4 transgenic mice as models for further studies in the transcriptional regulation of human drug-metabolizing enzymes. Ethical and physical limitations exist with regard to the use of human liver tissue for such studies. Human cell lines, such as HepG2, and primary hepatocytes lack the physical organ structure and thus important interactions between various cell types or factors found circulating in blood and the hepatocytes are absent, making such a model incomparable with the clinical situation (48). Therefore, the use of animal models can be very informative to the study of hepatic drug metabolism *in vivo*. However, marked species differences in the regulation and expression of hepatic *CYP3A* genes make it impossible to ascribe equivalent functions for CYP3A-mediated biotransformations between rodents and man (49). More recently, the development and use of transgenic animals with the human CYP3A4 or human steroid and xenobiotic receptor sequences "knocked in" seem to overcome the species differences in relation to regulation of the *CYP3A* genes (17, 50). As a consequence, humanized mouse models, as shown in the current study, will increasingly be used for investigation of the effect of various disease states, such as cancer, on the regulation of human CYP3A4 as well as for preclinical drug evaluation.

At this stage, it is not clear which anti-inflammatory strategies will prove useful in the clinical setting. Preliminary experiments with the mouse EHS tumor model have shown that CYP3A4 levels in tumor mice are not restored following treatment with either nonsteroidal anti-inflammatory drugs, including cyclooxygenase-1 and cyclooxygenase-2 inhibitors, or corticosteroids, such as dexamethasone (data not shown). Therefore, interventions targeting specific circulating cytokines or their soluble/membrane-bound receptors may be required. Having identified anti-inflammatory drugs with this potential, another important issue will be the degree to which they impact on the natural variability in CYP3A4 due to drug interactions, dietary/herbal components, or other endogenous factors. This will require further *in vivo* pharmacokinetic studies in advanced cancer patients undergoing combined anti-inflammatory treatment in conjunction with chemotherapy.

In summary, our findings support the hypothesis generated from previous clinical studies that IL-6 and the associated acute phase response may be involved in the repression of CYP3A enzymes in patients with cancer. Further work is needed to investigate the interplay of inflammatory signaling pathways with transcription factors, such as hepatocyte nuclear factor 4α (47), which are integral to CYP3A4 regulation. These results provide a preliminary linkage between cancer-associated inflammation and inhibited hepatic drug metabolism. The current studies also provide the rationale and vehicle for assessment of novel treatments targeting inflammatory mediators and their pathways of activation that may improve cytotoxic drug metabolism and tolerance if used before chemotherapy. However, until this hypothesis is tested clinically and extensive studies are carried out in cancer patients to test the efficacy and side effects of anti-inflammatory interventions aimed at normalizing drug clearance, caution should be exercised in applying such combinational therapies.

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