

An Animal Model for Chemotherapy-Associated Steatohepatitis and Its Prevention by the Oral Administration of Fatty Acid Bile Acid Conjugate

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BACKGROUND: Preoperative chemotherapy for hepatic resection of colorectal liver metastases is associated with the development of chemotherapy-associated steatohepatitis (CASH). This increases the risk of perioperative morbidity and mortality. To the authors' knowledge, an animal model for CASH has not been described previously. It has been established that fatty acid bile acid conjugates (FABACs) prevent the formation of diet-induced fatty liver. The current study was designed to establish an animal model of CASH and to use that model to study the effect of FABACs on its occurrence. **METHODS:** C57BL/6 mice were given different doses of oxaliplatin and irinotecan. Oxaliplatin administered once weekly at a dose of 6 mg/kg for a total dose of 24 mg/kg was tolerated best and was associated most consistently with CASH. Thus, that dose was chosen as the induction model for CASH. Subsequently, mice were divided into a control group (no treatment), an oxaliplatin group, and a CASH-prevention group, which received oxaliplatin and C20-FABAC at a dose of 150 mg/kg daily. The animals were killed after 28 days. **RESULTS:** Liver fat content was significantly lower ($P < .0001$) in the control group (51.63 mg/g) and the prevention group (62.13 mg/g) compared with the oxaliplatin group (95.35 mg/g). This difference was mainly because of the accumulation of liver triglycerides in the oxaliplatin group. **CONCLUSIONS:** The current results indicated that C57BL/6 mice receiving weekly oxaliplatin can be used as a model for CASH. Oral FABAC therapy reduced the development of CASH in animals that received oxaliplatin. To the authors' knowledge, this report is the first description of a model and a potential preventive treatment for CASH. *Cancer* 2010;116:251-5. © 2010 American Cancer Society.

KEYWORDS: liver metastasis, chemotherapy-associated steatohepatitis, animal model, prevention, fatty acid bile acid conjugate.

Preoperative chemotherapy (irinotecan and oxaliplatin) is used increasingly for the treatment of patients with colorectal liver metastases,¹ for which liver resection is currently the best chance for cure. Among these patients, the 5-year survival rate currently ranges from 50% to 60%, which is a significant improvement on the median rate of 30% achieved with previous treatments.²

However, the administration of irinotecan or oxaliplatin is associated with the development of chemotherapy-associated steatohepatitis (CASH).³ This hepatic injury increases the risk of perioperative morbidity and mortality⁴ and also

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may affect the ability of patients to undergo extended liver resection, particularly those patients who have large tumors or tumors in an unfavorable locations.³ To our knowledge, an animal model for CASH has not been described previously.

Fatty acid bile acid conjugates (FABACs) are novel synthetic lipid molecules that are prepared by conjugation of cholic acid at position 3 with saturated fatty acids of variable chain lengths using an amide bond.⁵ It has been demonstrated that FABACs prevent the formation of diet-induced fatty liver in animal models of nonalcoholic fatty liver disease (NAFLD).^{6,7} Among the different FABACs, the conjugate with arachidic acid (C20-FABAC) (Aramcol) was identified as the most potent in vitro and is used the most frequently in vivo.⁸ It is given orally at a dose of 150 mg/kg daily.⁵

The current study had 2 objectives. The first was to establish an animal model of CASH, and the second was to use that model to study the effect of FABAC on the occurrence of CASH.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice, aged 4 weeks and weighting approximately 20 g, were used in this study. All animals were held at room temperature (22°C) in standard cages in the animal facility at our institution under a 12-hours light/12-hours dark cycle. They were fed a regular rodent chow diet that contained a maximum of 4 g % fat.

Establishment of an Animal Model of CASH

We conducted a literature search for the regimens and doses of irinotecan and oxaliplatin that were described in previous in vivo studies that involved mice. The mice in the current study were injected intraperitoneally, and they were killed at the end of treatment. The liver were excised, weighed, and homogenized with saline at a ratio of 1:3 or 1:5 (weight/volume) in cryo vials on ice. Lipids were extracted from an aliquot of the liver homogenate according to the procedure described by Folch et al.⁹ The total amount was calculated after aliquot evaporation to a constant weight. Among the different regimens that we tested, the regimen that was tolerated best and associated most consistently with CASH was chosen for the prevention experiments.

Study of the Effect of FABAC on the Occurrence of CASH

Mice were divided into 3 groups: a control group without any treatment, a chemotherapy group treated with the

CASH induction regimen, and a FABAC group that received both CASH induction chemotherapy regimen and C20-FABAC (arachidyl-amido-cholanoic acid) at a dose of 150 mg/kg daily administered by gavage. The animals were killed at the end of the chemotherapy course. Their livers were homogenized and analyzed for fat content (measured as mg lipid/g liver tissue) as described previously. In addition, lipids were separated by thin-layer chromatography on silica gel plates (Merck, Rehovot, Israel). They were developed with a solvent system that consisted of hexane, ethyl ether, and acetic acid at a volume of 70:30:31.¹⁰ The lipids were identified by the distance relative to the front according to known standards. They were detected by iodine vapors or phosphomolibdate reagent (Supelco, Rehovot, Israel) and quantified by densitometry (BIS 202D; Rhenium, Jerusalem, Israel), and compared with calibration curves of the appropriate standards. Formalin-fixed liver samples were stained with hematoxylin and eosin. Moreover, coded histology slides of animal livers were analyzed for fat infiltration under a light microscope by an experienced pathologist who was blinded to the treatment.

Statistical Analysis

Each sample was tested in duplicate. Values are expressed as the mean \pm standard deviation. The differences between groups were analyzed using an analysis of variance and post-hoc analysis using a least significant difference test with the SPSS software package (version 11; SSPS Inc., Chicago, Ill). *P* values $<.05$ were considered statistically significant. The study was approved by the animal experimentation committee of our institution.

RESULTS

Establishment of an Animal Model of CASH

A literature search regarding suggested doses and regimens of irinotecan and oxaliplatin in mice was conducted.¹¹⁻¹⁵ Accordingly, our 21 mice were divided into 7 groups with 3 mice per group. The first group was a control group without treatment. Three groups received intraperitoneal injections of oxaliplatin at increasing doses of 6 mg/kg, 10 mg/kg, or 14 mg/kg on Days 1, 8, 15, and 22 of a 28-day cycle. Three groups received intraperitoneal injections of irinotecan at increasing doses of 25 mg/kg, 50 mg/kg, or 100 mg/kg on Days 1, 5, and 9 of a 28-day cycle. Each group was kept in a separate cage. Two mice in the control group died. All 6 mice that received oxaliplatin at doses of 10 mg/kg and 14 mg/kg suffered clinically from severe

neuropathy (unstable and wide base gait) and eventually died. In all 3 irinotecan treatment groups, the mice experienced severe diarrhea. The groups of control mice and mice that received oxaliplatin 6 mg/kg were clinically uneventful. Animals were killed on Day 29. On liver analysis, total fat content was 65.3 mg/g, 73.6 ± 0.1 mg/g, 73.2 ± 0.1 mg/g, and 73.5 ± 0.2 mg/g in the control group and in the irinotecan treatment groups at doses of 25 mg/kg, 50 mg/kg, and 100 mg/kg, respectively. In the group that received oxaliplatin at a dose of 6 mg/kg once weekly up to a total dose of 24 mg/kg, the liver fat content was 126.3 ± 0.2 mg/g, which was comparable to the level achieved in previous *in vivo* studies on diet-induced fatty liver.⁶ This difference between the oxaliplatin group and the irinotecan or control groups was significant ($P = .006$), but the difference was not significant between the control group and the irinotecan groups. Thus, because this oxaliplatin regimen was clinically tolerated the best and was associated most consistently with CASH compared with higher doses of oxaliplatin or different irinotecan regimens, it was chosen as the induction model for CASH to study the effect of FABAC on its occurrence.

Study of the Effect of FABAC on the Occurrence of CASH

In total, 42 mice were divided into 3 groups: a control group ($n = 6$) with no treatment, a chemotherapy group ($n = 18$) that received the CASH induction regimen (oxaliplatin 6 mg/kg once weekly up to a total dose of 24 mg/kg), and a FABAC group ($n = 18$) that received both the CASH induction chemotherapy regimen and C20-FABAC at a dose of 150 mg/kg daily administered by gavage. Four mice in the oxaliplatin group and 6 mice in the FABAC group died during the treatment period. The remaining animals (6 in the control group, 14 in the oxaliplatin group, and 12 in the FABAC group) were killed on Day 29. There were no significant differences noted with regard to animal or liver weights between the groups (Fig. 1). Liver fat content was found to be significantly ($P < .0001$) higher in the oxaliplatin group (95.35 ± 22.2 mg/g) compared with the FABAC group (62.1 ± 15 mg/g) and the control group (51.6 ± 9.5 mg/g). The difference between the control group and the FABAC group was not significant (Fig. 1). Lipid analysis by thin-layer chromatography revealed that the differences between the groups was caused mainly by a significantly ($P = .002$) higher content of triglycerides in the oxaliplatin group (59.4 ± 32.5 mg/g) compared with the FABAC group (31.6 ± 11.2 mg/g) and the control group (14.5 ± 8.2 mg/g)

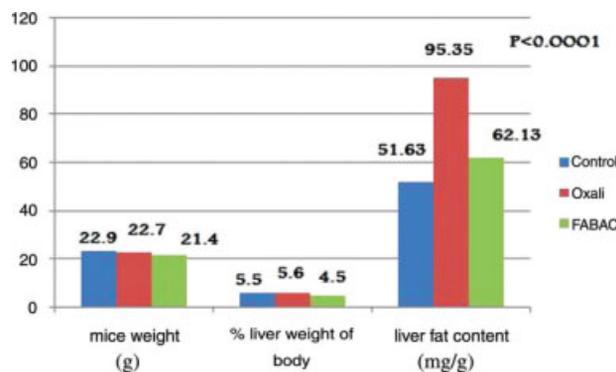


Figure 1. This chart illustrates a comparison of the weight, relative liver weight, and liver fat content between the groups. The P value was determined using an analysis of variance (ANOVA). Oxali indicates oxaliplatin; FABAC, fatty acid bile acid conjugate.

(Fig. 2). The difference in triglyceride levels between the control group and the FABAC group was not found to be significant. The levels of other lipids, including cholesteryl esters, cholesterol, and free fatty acid, were similar in the 3 groups (Fig. 2). The histopathologic examination suggested microvesicular fat accumulation in the oxaliplatin group. The general appearance of formalin-fixed liver samples stained with hematoxylin and eosin was similar in the 3 groups, with normal-appearing liver tissue. However, 10 frozen liver samples that were available for fat staining revealed the presence of microvesicular fat in oxaliplatin-treated mice. Unfortunately, for most mice, frozen liver samples were not available.

DISCUSSION

The current study revealed that C57BL/6 mice receiving intraperitoneal injections of weekly oxaliplatin at a dose of 6 mg/kg up to a total of 24 mg/kg, can be used as a model for CASH. In addition, oral FABAC therapy reduces the development of CASH in this animal model. To the best of our knowledge, this is the first description of a model and a potential preventive treatment for CASH.

Current preoperative chemotherapy used to treat patients who have colorectal liver metastasis usually involves the administration of irinotecan or oxaliplatin and significantly improves patient survival.^{1,2} However, such treatment is associated with the development of CASH,³ which increases the risk of perioperative morbidity and mortality.⁴ In addition, CASH may affect the ability of these patients to undergo liver resection.³ Thus, studies on CASH induction and prevention should lead to significant clinical benefits.

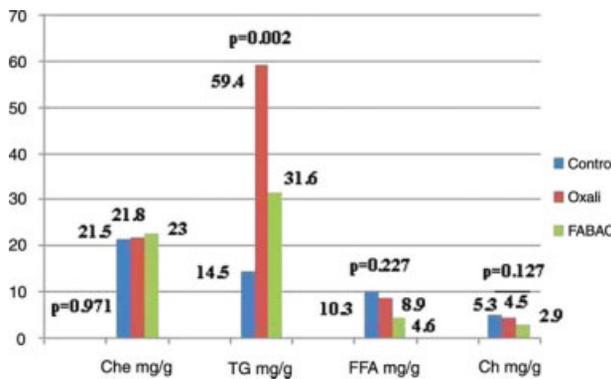


Figure 2. This chart illustrates a comparison of different lipid types of liver content as analyzed by thin-layer chromatography. *P* values were determined using post hoc analyses, least significant difference (LSD) tests, and analyses of variance (ANOVA). Oxali indicates oxaliplatin; FABAC, fatty acid bile acid conjugate; Che, cholesteryl esters; TG, triglycerides; FFA, free fatty acid; Ch, cholesterol.

Because an animal model for CASH has not been described previously, first, we needed to establish such a model. We chose C57BL/6 mice, which serve as a good animal model for another, more common form of steatohepatitis, NAFLD.⁶ To identify the appropriate CASH induction chemotherapeutic regimen, we tested different doses of irinotecan and oxaliplatin that were described in the literature as part of studies in mice.¹¹⁻¹⁵ Irinotecan, regardless of the dose, was associated with severe diarrhea and a nonsignificant increase of liver fat content (range, 73.2-73.6 mg/g). Intermediate (10 mg/kg) and high (14 mg/kg) doses of oxaliplatin were associated clinically with significant neuropathy and eventually were fatal. However, the lowest dose of oxaliplatin (6 mg/kg) was well tolerated clinically and was associated with a significant accumulation of liver fat (126.3 mg/g) compared with what was observed in NAFLD induction studies. Thus, in C57BL/6 mice, intraperitoneal injection of weekly oxaliplatin at a dose of 6 mg/kg up to a total of 24 mg/kg can be used as a model for CASH.

In animal models of NAFLD, FABACs reportedly prevented the formation of diet-induced fatty liver.^{6,7} Among the different FABACs, C20-FABAC (Aramchol), given at a dose of 150 mg/kg daily, was identified as the most potent and, thus, the most frequently used *in vivo*.^{5,8} Consequently, we used C20-FABAC in the second part of the current study to test its ability to prevent CASH occurrence.

In the current study, we observed that C20-FABAC significantly decreased the occurrence of CASH in oxali-

platin-treated mice (Fig. 1). A 35% reduction of liver fat content was noted in the FABAC group. This was caused mainly by a significant reduction (47%) in liver triglyceride content (Fig. 2). Triglycerides also are the main fats that accumulate in diet-induced NAFLD. The general appearance of formalin-fixed liver samples stained with hematoxylin and eosin was similar in the 3 groups and revealed normal liver morphology. Ten available frozen liver samples that were stained for fat revealed the presence of microvesicular fat in oxaliplatin-treated mice. Unfortunately, for most mice, frozen liver samples were not available. Thus, although these findings suggest that oxaliplatin may result in microvesicular fat accumulation, definitive conclusions regarding fat distribution cannot be drawn. It should be noted that the evaluation of liver fat by light microscopy can be unreliable, even by experienced pathologists, whereas a quantitative chemical analysis (expressed as mg lipid/g liver tissue) provides precise and reproducible measurements.⁶

The mechanisms of the hepatic effects of FABACs in CASH prevention remain to be elucidated. However, data from previous studies can suggest hypotheses for further testing. Regarding the prevention of NAFLD, 3 main hepatic effects of FABACs were described^{6,16}: reduction of *de novo* fatty acid synthesis, reduced stearoyl-coenzyme A desaturase 1 activity, and increased fatty acid degradation (β oxidation). In the current study, hepatic changes in the mice with CASH were similar to those described in previous NAFLD animal experiments. Thus, all of these hepatic effects of FABAC also may be relevant to the prevention of CASH.

On the basis of currently available data, FABACs are a potentially nontoxic therapy. In hundreds of animals that have been tested to date, no evidence of significant toxicity has been noted. Rarely, minor aminotransferase elevations were reported^{6,17} and, in the current study, no evidence of FABAC-related toxicity was observed.

In conclusion, C57BL/6 mice that are treated by intraperitoneal injections of weekly oxaliplatin at a dose of 6 mg/kg for a total of 24 mg/kg can be used as a model for CASH. Oral FABAC therapy was found to reduce the development of CASH in animals that received oxaliplatin. To the best of our knowledge, this report is the first description of a model and a potential preventive treatment for CASH. Future studies are warranted to confirm our results and to elucidate the underlying mechanisms.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures

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