Antimetastatic Intraoperative Chemotherapy of Human Colon Tumors in the Livers of Nude Mice

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ABSTRACT

We have developed a new antimetastatic chemotherapeutic strategy for combination with hepatic resection of human colon cancers in a high-metastasis nude mouse model. The new procedure involves i.p. administration of 5-fluorouracil (5-FU) 2 h before hepatic resection of the human colon tumors, with therapy continued postoperatively for 4 consecutive days. We termed this strategy neo-neoadjuvant chemotherapy. The regime significantly prolonged animal survival compared with preoperative 5-FU neoadjuvant therapy, 5-FU postoperative adjuvant therapy, surgery alone, 5-FU without surgery, or the untreated control. The median survival of neo-neoadjuvant i.p. 5-FU-treated group was 81 days, compared with 27 days for the control group ($P < 0.009$). The median survival of animals in the neoadjuvant group was 37 days ($P < 0.021$ compared with the control group). There was also a significant difference between the median survival of neo-neoadjuvant, and the neoadjuvant group ($P < 0.031$). When all animals in the control group had died, 70% of animals with neo-neoadjuvant and 60% of animals with neoadjuvant 5-FU were still alive ($P < 0.003$ and $P < 0.011$, respectively). When all animals with neoadjuvant 5-FU treatment had died, 70% of animals with neo-neoadjuvant treatment were still alive ($P < 0.003$). Survival of all other treatment groups, including 5-FU without surgery, surgery alone, and adjuvant postoperative chemotherapy, was not significantly different from the untreated control group. Two animals in the neo-neoadjuvant group were free of tumors when sacrificed at days 154 and 165 post surgery. Whereas 100% of animals in the control, 90% in the 5-FU alone, 70% in the surgery alone, 60% in the 5-FU adjuvant, and 40% in the neoadjuvant groups had metastases in the lymph nodes draining the liver, only 10% of animals in the neo-neoadjuvant group had metastases. These data suggest that the neo-neoadjuvant therapy increased survival by preventing metastasis of cancer cells not removed in the liver resection procedure. The results of this study indicate that the neo-neoadjuvant treatment strategy for resection of colon cancer liver metastasis should be explored clinically.

INTRODUCTION

Each year, 150,000 new colon cancer cases are diagnosed in the United States. Approximately 40–50% of these patients have liver metastases either at the time of first diagnosis or after radical resection of the primary tumor (1). Hepatic resection has been widely accepted as the only curative treatment for colon cancer liver metastasis (1, 2). The survival of these patients is directly related to tumor involvement of lymph nodes draining the liver (1). However, up to 60% of patients with colon cancer liver metastasis have recurrences after a median of only 9–12 months (3–6). In more than half of these cases, the liver is the first site of recurrence (7–12). The results of systemic chemotherapy for metastatic colon cancer have not shown significant survival benefits (13). It was thought that delivery of a higher concentration of chemotherapeutic agents to liver metastases via the hepatic artery after a curative resection could improve the survival of patients with colon cancer liver metastases. Postoperative adjuvant intra-arterial chemotherapy has not significantly prolonged survival (14, 15) in some cases but has in others (16, 17). Intra-arterial chemotherapy also is associated with considerable complications and high cost (14, 18, 19).

The portal vein supplies blood to hepatic micrometastases smaller than 0.5 mm (20). In an attempt to eradicate micrometastases after curative liver resection, postoperative adjuvant portal infusion of chemotherapeutic agents was used. These studies were discontinued because of high rates of complications (6, 21).

Adjuvant, postoperative i.p. chemotherapy also has not significantly improved the survival of patients with colon cancer liver metastases (9, 22).

5-FU[2] remains the most frequently used chemotherapeutic agent for treatment of colon cancer. Unfortunately, systemic adjuvant postoperative 5-FU chemotherapy does not significantly improve the survival of patients with colon cancer liver metastases (9, 13–15, 18, 19, 21–26). New therapeutic strategies are, therefore, urgently needed.

The search for new anticancer agents and treatment modalities has been impeded by the limited availability of clinically accurate mouse models, specifically, highly metastatic models. Toward this goal, over the past 12 years in our laboratory, we have established clinically representative metastatic mouse models of human cancer with a novel method of SOI of intact tumor tissue fragments (27–32). We recently developed the

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[2] The abbreviations used are: 5-FU, 5-fluorouracil; SOI, surgical orthotopic implantation.

Table 1 Antimetastatic intraperitoneal 5-FU chemotherapy in nude mice for human hepatic colon tumors

Group	No. of animals	Therapeutic regimens listed in order of administration of treatment modalities
Control	10	Saline, 0.5 ml, once a day for 5 days; beginning 3 days after tumor implantation.
5-FU alone	10	20 mg/kg in 0.5 ml once a day for 5 days; beginning 3 days after tumor implantation.
Liver resection alone	10	Curative liver resection 3 days after tumor implantation.
Adjuvant	10	Curative liver resection 3 days after tumor implantation + 2 days rest, then first dose of 5-FU (20 mg/kg in 0.5 ml) once a day for 5 days.
Neoadjuvant	10	Three days after tumor implantation, 5-FU (20 mg/kg in 0.5 ml) once a day for 5 days. Then 2 days rest and subsequent curative liver resection.
Neo-neoadjuvant	10	5-FU (20 mg/kg in 0.5 ml) beginning 2 h prior to curative liver resection, 3 days after tumor implantation. Then, four more daily doses beginning from first postoperative day.

AC3488 model of highly metastatic human colon cancer, which metastasizes to the liver (32) and to lymph nodes draining the liver (33). The neoplastic involvement of the lymph nodes draining the liver, the portal, celiac, and mediastinal lymph nodes, is of considerable importance for the outcome of patients with colon cancer metastatic to the liver (1, 5).

The aim of this study was to evaluate the efficacy of a new strategy of intraoperative chemotherapy, termed neo-neoadjuvant therapy, for the treatment of resectable highly malignant human colon cancer liver metastases in the AC3488 metastatic model in nude mice. With the neo-neoadjuvant chemotherapy method that we developed, we demonstrate in this model the prevention of metastasis of liver-implanted tumors to the lymph nodes draining the liver and significant prolongation of survival of the treated animals.

MATERIALS AND METHODS

Animals. Athymic *nu/nu* BALB/c mice (Charles River Laboratories, Wilmington, MA) of both sexes, 6–7 weeks of age, were used in the study. The animals were maintained in a specific pathogen-free environment in compliance with United States Public Health Service guidelines governing the care and maintenance of experimental animals. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals under assurance number A3873-1. Mice were fed with an autoclaved laboratory rodent diet (Tecklad LM-485; Western Research Products, Orange, CA).

Human Colon Cancer. We previously established the highly metastatic AC3488 human colon cancer SOI model in nude mice (32). The tumor results in the death of all of the transplanted animals from metastases within 30–35 days. The original fresh specimen was obtained from a resected liver metastasis of a patient with colon cancer at the Department of Surgery, School of Medicine, University of California, San Diego, CA (32).

Tumor Implantation on Nude Mice Livers. In the present study, AC3488 human colon tumor tissue fragments were implanted directly on the left lobe of the liver of 60 animals to simulate liver metastasis. Prior to intrahepatic transplantation, liver metastases of AC3488 (~2 cm in size) that originated from SOI to the colons of nude mice were harvested and carefully inspected under a dissecting microscope (×5) to remove necrotic tissue. The harvested tumor tissues were then divided equally into small pieces of 1 mm^3 each. Tumor tissue fragments were mixed thoroughly before the implantation procedure to ensure that each mouse received equally viable tissues. The left lobe of the liver of each mouse was isolated via a left subcostal incision under isoflurane anesthesia. A small cut was then made on the glissonian capsule. Two pieces of the above tumor tissue fragments were inserted into the incision on the left lobe and then fixed in place using an 8-0 nylon suture. The abdomen was closed with a 6-0 silk suture using different instruments than those used for tumor implantation to prevent any spreading of the tumor in the incision site.

Curative Partial Hepatectomy. Hepatectomy was performed 3 days post transplantation. Mice were anesthetized with isoflurane inhalation and put in a supine position. The abdomen was sterilized with iodine and alcohol swabs. To prevent any residual tumor growth, the left subcostal incision site, which was used for tumor implantation, was completely excised. Through this abdominal wall opening, the tumor-bearing left lobe of the liver was identified, and its bilio-vascular bundle was ligated with a 6-0 nylon suture. The entire left lobe was then resected. The incision line was at least 1 cm distant from the liver metastasis. The resected lobe corresponded to ~30% of the total liver. The abdomen was then closed with a 6-0 silk suture using different instruments than those used for the resection to prevent any spreading of the tumor at the incision site.

Chemotherapeutic Agent. One cycle of i.p. 5-FU at 20 mg/kg, given in 0.5 ml once a day for 5 days, was used in this study. 5-FU was obtained from the Calbiochem-Novabiochem Corporation (La Jolla, CA).

Study Design. Sixty mice, all surgically implanted with AC3488 on the left lobe of the liver, simulating liver metastases, were divided into six groups. Each group contained 10 mice (Table 1).

Statistical Analysis. The incidence of survival at defined time points and the incidence of metastasis was analyzed using Fisher's exact test. The median survival was analyzed using Wilcoxon's rank-sum test.

RESULTS

Survival of Animals. Animals with hepatic colon tumors treated with intraoperative neo-neoadjuvant i.p. 5-FU during curative hepatectomy survived much longer than all other treatment or control groups (Fig. 1). The median survival of the neo-neoadjuvant i.p. 5-FU group was 81 days, compared with 27 days for the control group ($P < 0.009$). The median survival of animals in the neoadjuvant group was 37 days ($P < 0.021$ compared with the control group). There also was a significant difference between the median survival of 81 days for neo-

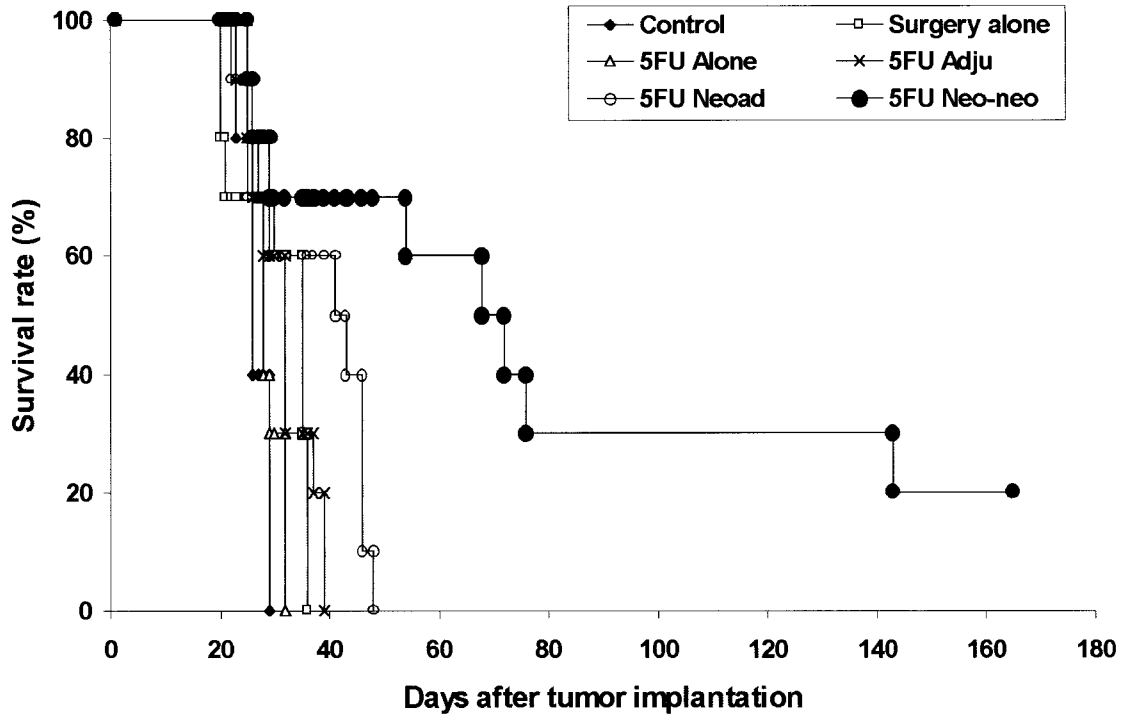

Fig. 1 Survival efficacy of antimetastatic intraoperative chemotherapy of human colon tumors in the livers of nude mice. Comparison of survival between animals in the untreated control group and animals in different treatment groups. The median survival of the neo-neoadjuvant i.p. 5-FU-treated group (●) was 81 days, compared with 27 days for the control group (♦; $P < 0.009$). The median survival of animals in the neoadjuvant group (∗) was 37 days ($P < 0.021$ compared with the control group). There was also a significant difference between the median survival of the neo-neoadjuvant and the neoadjuvant group ($P < 0.031$). The median survival was analyzed using Wilcoxon's rank-sum test. See text for experimental details.

neoadjuvant therapy and 37 days for neoadjuvant therapy ($P < 0.031$). Although survival of the surgery-alone, 5-FU-alone, and adjuvant postoperative 5-FU groups was longer than the control group, this was not statistically significant (Fig. 1). When all animals in the control group had died, 70% of the animals receiving neo-neoadjuvant 5-FU and 60% of the animals with receiving neoadjuvant 5-FU were still alive ($P < 0.003$ and $P < 0.011$, respectively). When all animals receiving neoadjuvant 5-FU treatment had died, 70% of the animals receiving neo-neoadjuvant treatment were still alive ($P < 0.003$). Two animals receiving neo-neoadjuvant treatment did not show any sign of neoplastic disease when they were sacrificed at days 154 and 165 post tumor implantation.

The results of a second study confirmed the first study. In this study, when all animals in the control group had died, 100% of the animals in the neo-neoadjuvant group were still alive. Three animals in the neo-neoadjuvant group were disease-free at sacrifice, 80 days after tumor implantation.

Metastasis from the Liver. In the control group, 100% of the animals had portal, celiac, and mediastinal tumor-involved lymph nodes as a result of metastasis from the liver (33). In animals treated with 5-FU alone, 90% of the animals had lymph node metastases. In animals treated only with surgical resection without chemotherapy, 70% of the animals had lymph node metastases. In animals treated with surgery and subsequent adjuvant chemotherapy, 60% of the animals had lymph node metastases. In the neoadjuvant chemotherapy group, 40% of the animals had lymph node metastases. In contrast, in the neo-neoadjuvant group, only 1 animal had lymph node metastases (10%) despite recurrences of liver tumors in 8 of 10 animals. Two mice treated with neo-neoadjuvant therapy were sacrificed at days 154 and 165 post tumor implantation and were found to be free of neoplastic disease, as mentioned above. All other animals in all groups had recurrences of tumor in the liver. The liver and lymph node metastatic rates in the different treatment groups are shown in Table 2.

DISCUSSION

Adjuvant, postoperative chemotherapeutic regimes have not significantly improved survival of patients with metastatic colon cancer (4, 6, 13–15, 18, 19, 21). Up to 60% of patients with resected colon cancer liver metastases have recurrences, with a median time before recurrence of only 9–12 months (3–6). Recurrences are mostly intra-abdominal, with the liver as the first relapsing site (3–10). The recurrences in the residual liver after a curative liver resection are believed to be due to microscopic residual foci of tumor or the spreading of malignant cells by surgical manipulation or both (7).

It is known that the portal vein supplies microscopic liver metastases of <0.5 mm in diameter (20). Pharmacokinetic and phase 1 clinical studies have indicated that i.p. administration of

Table 2 Antimetastatic efficacy of intraperitoneal 5-FU regimens in nude mice with hepatic human colon tumors

Colon tumors were transplanted on the left lobe of the liver and resected 3 days after implantation as described in "Materials and Methods." The various treatment modalities were performed as described in "Materials and Methods." The animals were analyzed for metastases both grossly and microscopically when they were sacrificed at the time they were moribund.

Group	Liver metastases	Lymph node metastases
Control	10/10[a]	10/10[a]
5-FU alone	10/10	9/10
Liver resection alone	10/10	7/10
Adjuvant	10/10	6/10
Neoadjuvant	10/10	4/10
Neo-neoadjuvant	8/10	1/10

[a] Number of animals involved with metastases/total number of animals in each group.

5-FU achieves drug concentrations in the peritoneal cavity between 300- and 2200-fold higher than in the systemic circulation (23, 34, 35). Anatomical considerations and experimental data suggest that a major mechanism of clearance of compounds placed into the peritoneal cavity is by way of the portal circulation (36). i.p. infusion of 5-FU results in a portal vein concentration ~4-fold higher than that in the systemic circulation (34, 37). The delivery of the drug to the liver by way of the portal circulation essentially equals the amount of drug entering the liver during intrahepatic artery infusion (34, 37).

The two sites most frequently involved with recurrences after a curative liver resection are the residual liver and i.p. organs (1–5). i.p. 5-FU was effective in one study for patients with three or less liver metastases (9). However, i.p. 5-FU did not significantly improve the survival of patients with liver metastases in another study (22).

It is also known that liver metastases >0.5 mm in diameter are supplied by the arterial circulation (38, 39). Intra-arterial chemotherapy as an adjuvant regime has not significantly improved the survival of patients after curative resection of colon cancer liver metastases in some studies (14, 15, 18, 19). However, recent reports have indicated that the combination of adjuvant hepatic artery infusion and systemic chemotherapy increased the disease-free and overall survival of patients compared with surgery alone or adjuvant systemic chemotherapy alone (16, 17). However, the adjuvant regime can have serious complications and high cost (14, 18, 19).

Intraportal injection of chemotherapeutic agents has been used as an adjuvant, postoperative regime to attempt to improve survival after curative resection of colon cancer liver metastases. Unfortunately, these studies were discontinued because of high rates of complications (6, 21). Standard postoperative systemic adjuvant chemotherapy also did not improve the survival of patients with resectable colon cancer liver metastases (9, 13). However, nonresectable colon cancer liver metastases can become operable after long-term preoperative, neoadjuvant chemotherapy (40).

The timing of the initiation of chemotherapy in this study is important for two reasons: (*a*) the effect of growth factors, which stimulate tumor growth, released early after liver resection (41, 42); and (*b*) surgical manipulation can spread malignant cells (7). The highest concentration of 5-FU after i.p. injection in liver metastases is reached after 2 h (43). In our study, in the neo-neoadjuvant group, the first dose of 5-FU was injected i.p. 2 h before the start of the operation. The subsequent four doses were administrated daily from the first postoperative day through day 4 after surgery. We chose the i.p. route because high concentrations of the chemotherapeutic agent can then reach the portal circulation, which supplies liver micrometastases.

The neo-neoadjuvant strategy significantly prolonged survival and prevented recurrence to lymph nodes draining the liver in 9 of 10 animals; in addition, 2 animals were disease free (Table 2). Thus, despite the recurrences of tumor in the liver in 8 of 10 animals treated with neo-neoadjuvant 5-FU, metastasis from the liver metastasis to lymph nodes draining the liver occurred in only 1 animal. The neo-neoadjuvant therapy thus seemingly reduced the malignancy of the tumor as well as eliminated at least part of the population of residual cancer cells. Future experiments will use longer treatment periods as well as multicycle therapy in the neo-neoadjuvant setting to reduce the recurrence rates.

The data in this study demonstrate that intraoperative neo-neoadjuvant therapy for resectable colon cancer liver metastases is an effective and convenient procedure. Intraoperative neo-neoadjuvant chemotherapy for colon cancer liver metastases can now be evaluated in a pilot clinical study.

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