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Treament of Liver Metastases from Colon Carcinoma with Autologous Tumor Vaccine Expressing Granulocyte-Macrophage Colony-Stimulating Factor

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Background and Objectives: In preclinical studies, tumor cells genetically altered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) can generate systemic antitumor immunity. Clinically relevant immunotherapeutic approaches for the treatment of colorectal cancer should address efficacy within the liver, a common site of metastatic disease. We investigated the effect of irradiated colon cancer cells engineered to produce GM-CSF on protecting from and treating established liver metastases.

Methods: Using a model of liver metastasis by intrahepatic injection of CT-26 murine colon carcinoma cells in syngeneic BALB/c mice, GM-CSF-producing irradiated cells were given as an intradermal vaccine either 14 days prior to hepatic challenge or in animals with early established tumor (days 5 and 10). The presence of tumor, tumor volume, and survival were endpoint determinants.

Results: Animals receiving GM-CSF-producing vaccination demonstrated significant protection from subsequent hepatic challenge of viable tumor cells, even at the highest challenge doses. In animals with early established tumors, a significant response was seen with prolongation in survival.

Conclusions: We conclude that GM-CSF autologous tumor vaccination was effective for the treatment of hepatic colorectal metastases in this murine model. These findings provide support for immunotherapeutic approaches for metastatic liver cancer.

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KEY WORDS: autologous cancer vaccine; GM-CSF; liver metastases; cytokines

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INTRODUCTION

Recent efforts at developing successful tumor vaccines have demonstrated that approaches that modify autologous tumor cells to express immunostimulatory molecules can result in significant antitumor effects [1–7] . When studied in a variety of tumor models, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to be the most potent cytokine after it was transduced into tumor cells and used as vaccine, even in poorly immunogenic tumors [8]. This effect has been shown to depend on the recruitment of antigen-presenting cells (APC), such as dendritic cells, to the immunization site [9]. Vaccinated animals generate T-cell-dependent tumor-specific immunity to subsequent challenge of viable tumor cells at a distant site.

For developing clinical applications for treatment of gastrointestinal malignancies, demonstrating efficacy for disease within visceral sites such as the liver is important [10]. In colorectal cancer patients, the liver is the most common, and often the only site of clinical metastases. Previous reports demonstrating efficacy of immunotherapeutic strategies in animal models have examined extrahepatic sites of tumor challenge, particularly in the subcutaneous tissue. These sites are easier to inoculate and evaluate for an effect, but may be immunologically different from tumor within the liver [11]. Such "clinically irrelevant" challenge sites may partially explain differences seen between animal models and patients with metastatic cancer. In this study, we examined the effect of GM-CSF-transduced autologous vaccine on colorectal tumor implanted within the liver in a murine model.

MATERIALS AND METHODS

Murine Tumor Lines

The murine colorectal cancer cells, CT-26 cells, were transduced with the murine GM-CSF gene by using the replication-defective MFG retroviral vector as previously described [8]. The transduced lines (CT-26/GM-CSF) and parental CT-26 cells from which they were derived were grown on Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum and penicillin/streptomycin. All vaccine cells were tested for in vitro GM-CSF production by a standard enzyme-linked immunoabsorbent assay (ELISA) technique (Endogen, Cambridge, MA). Transduced CT-26 cells secreted GM-CSF levels in the range of 120–150 ng/10^6 cells per 24 h.

Murine Vaccination

Mice used for all vaccination studies were purchased as 6- to 12-week-old BALB/c female mice from Harlan (Indianapolis, IN). For all experiments, 10 mice were allocated to each group. On the day of vaccination, tumor cells were trypsinized from the culture flask, washed once in medium containing serum, and then in Hank's balanced saline solution (HBSS), and counted. Cells were resuspended in HBSS, irradiated with 3,500 cGy from a Cs^{137} source discharging 1,378 cGy/min (Gammacell model no. 62 irradiator, Nordin International, Inc., Kanata, Ontario, Canada). Vaccination was performed by intradermal injection of either 10^5 or 10^6 irradiated CT-26/GM-CSF into the left flank by using a l-cm^3 tuberculin syringe with a 30-gauge needle.

Hepatic Tumor Model

Mice were anesthetized with an intraperitoneal injection of 0.1 ml of a solution containing ketamine hydrochloride (25 mg/ml), xylazine (2.5 mg/ml), and 14.25% ethyl alcohol diluted 1:3 in 0.9% sodium chloride solution. Under sterile technique, laparotomy was performed through a midline incision and the left lobe of the liver was exposed. With use of an operative microscope, wild-type CT-26 cells (CT-26 WT) were injected in 10 μl volume of HBSS beneath the hepatic capsule with the aid of a 30-gauge needle. Blanching of the liver parenchyma confirmed localized injection, and gentle compression applied for 60 sec minimized cellular extravasation and promoted hemostasis. The abdominal incision was closed with 4-0 vicryl sutures. By this method, a solitary intraparenchymal tumor can be identified by the second postoperative day (3 mm in size) without evidence of extrahepatic diseases, such as peritoneal carcinomatoses or pulmonary metastases.

In Vivo Protection Against Subsequent Hepatic Challenge

CT-26/GM-CSF vaccination was evaluated for its ability to protect animals against subsequent intrahepatic challenge with CT-26 WT. One group of animals was vaccinated with 10^5 irradiated CT-26/GM-CSF and compared to two other control groups: one vaccinated with 10^5 irradiated CT-26 WT cells and one vaccinated with culture medium. Mice were challenged 14 days later with 5×10^4 CT-26 WT cells intrahepatically. Twenty-one days later, animals were euthanized and the presence or absence of tumor was determined by gross inspection and histologic analysis. In a parallel set of experiments, animals were followed after vaccination and challenge, and survival time was determined.

To identify whether or not the antitumor effect of CT-26/GM-CSF vaccination was dependent on the challenge dose, animals vaccinated with 10^6 irradiated CT-26/GM-CSF were allocated into three different groups (10 per group) in which 10^4, 10^5, and 10^6 CT-26 WT were injected intrahepatically 14 days following vaccination. The presence or absence of tumor and tumor volume were determined 21 days following challenge, as well as parallel experiments to determine survival.

TABLE I. Protection Against Subsequent Hepatic Challenge: Comparison of Groups by Presence of Tumors and Tumor Volume

Vaccination (day -14)	No. mice with tumor	*P* value[a,b]	Mean tumor volume (mm^3)	*P* value[a,c]
10^5 CT-26/GM-CSF[d]	0/10	<0.0001	0 ± 0	<0.0001
10^5 CT-26 WT[e]	7/9	0.21	67.98 ± 41.5	<0.0003
Control	10/10	—	888.9 ± 291.1	—

[a]Compared with control.
[b]Fisher's exact test.
[c]Wilcoxon test.
[d]GM-CSF-producing CT-26 cells.
[e]Parental CT-26.

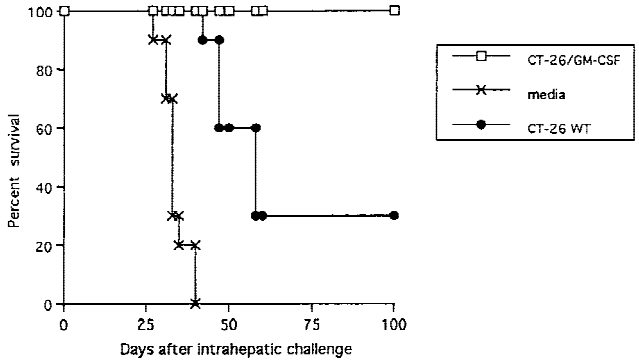

Fig. 1. Protection against subsequent hepatic challenge. Comparison of groups by survival. Groups of mice (10 per group) injected intradermally with 10^5 irradiated CT-26/GM-CSF, 10^5 irradiated CT-26 WT, or media. All mice were challenged 14 days later by intrahepatic injection of 1×10^4 CT-26 WT. Differences between median survival times reached statistical significance when compared to media group, $P < 0.0001$.

Vaccination Against Established Hepatic Tumor

In order to determine the efficacy of CT-26/GM-CSF vaccination on established tumors within the liver, groups of animals underwent tumor implantation prior to vaccination. In these series of experiments, mice were challenged intrahepatically with 5×10^4 CT-26 WT and intradermal vaccinations of CT-26/GM-CSF were given 5 or 10 days later. A third group received intrahepatic challenge on the day of vaccination (day 0). Control groups included those receiving tumor challenge 14 days following vaccination (day -14) and a group vaccinated with culture medium on day -14. Similar endpoints were used for these experiments, including survival and assessment of the liver 21 days following challenge.

Tumor Assessment and Histologic Studies

Mice were euthanized by CO_2 narcosis and autopsy was performed. Livers were harvested and examined in the fresh state for the presence or absence of tumor by visualization and palpation. In animals in which tumor was identified, the size was measured, and tumor volume was calculated (tumor volume $= a \times b \times c \times \pi/6$, where a, b, c are the three dimensional-diameters) and averaged. Specimens were then fixed in 10% formalin, processed for paraffin embedment, and stained with hematoxylin and eosin. Independent pathologic review of all specimens was performed to confirm the presence of viable tumor.

Statistical Analysis

Differences in tumor volume were assessed for statistical significance using Wilcoxon's rank score test [12]. Differences in the number of animals having tumors were assessed using Fisher's exact test [13]. Time to event distributions were estimated using the product-limit method and compared using the log rank test [14]. All *P* values reported are two-sided.

RESULTS

In Vivo Protection Against Subsequent Hepatic Tumor Challenge

Among the group vaccinated with CT-26/GM-CSF (10^5), no mouse had evidence of tumor within the liver. Seventy-eight percent (7 of 9) of mice vaccinated with irradiated CT-26 WT and all (10 of 10) of the nonvaccinated mice developed tumors within the liver. In the animals in which tumors did develop, the average tumor size was significantly smaller in those animals vaccinated with CT-26 WT cells compared to nonvaccinated mice (67.98 ± 41.5 mm^3 vs. 888.9 ± 291.1 mm^3, $P < 0.0003$, Table I). The survival time was also significantly prolonged in animals following CT-26/GM-CSF vaccination. All of the CT-26/GM-CSF-treated mice survived beyond 100 days following challenge, compared to only 30% (3 of 10) survival in wild type cell-treated animals (55 days median survival). All of the nonvaccinated animals died by 33 days after challenge (27 days median survival). Differences between median survivals were significant ($P < 0.0001$, Fig. 1).

Vaccination Against Established Hepatic Tumor

The efficacy of CT-26/GM-CSF vaccination was then evaluated in animals undergoing prior tumor implantation. Vaccination (10^6 CT-26/GM-CSF) was performed

TABLE II. Established Hepatic Tumors: Comparison of Groups by Presence of Tumor and Tumor Volume

Vaccination	Day from vaccination	No. of mice with tumor	*P* value[a,b]	Mean tumor volume (mm^3)	*P* value[a,c]
10^6 CT-26/GM-CSF[d]	−14	0/10	<0.0001	0 ± 0	<0.0001
10^6 CT-26/GM-CSF	0	6/10	0.09	13.86 ± 13.1	<0.0002
10^6 CT-26/GM-CSF	+5	6/10	0.09	15.30 ± 15.1	<0.0002
10^6 CT-26/GM-CSF	+10	8/10	0.47	148.9 ± 83.1	<0.0002
Control	—	10/10	—	2,258.2 ± 885.9	—

[a]Compared with control.
[b]Fisher's exact test.
[c]Wilcoxon test.
[d]GM-CSF-producing CT-26 cells.

on day +10, day +5, and day 0 after intrahepatic challenge of CT-26 WT (Table II). By 5 and 10 days following intrahepatic injection with 5×10^4 viable tumor cells, hepatic tumors were already clearly visible, measuring 3 and 7 mm in diameter, respectively (data not shown). In animals vaccinated on day +5 and day 0, 6 of 10 animals in each group developed visible tumors. In animals vaccinated 10 days following intrahepatic challenge, 8 of 10 had developed tumors when euthanized 14 days later. In those animals in which tumors did develop, the average tumor volumes were 13.86 ± 13.1 mm^3 in the day 0 group, 15.30 ± 15.1 mm^3 in the day 5 group, and 148.9 ± 83.1 mm^3 in the day 10 group. All of the tumors in vaccinated animals were significantly smaller than in the nonvaccinated group which had an average tumor volume of 2,258.2 ± 885.9 mm^3 ($P < 0.0001$). Survival was significantly prolonged in animals receiving CT-26/GM-CSF vaccination, even when administered up to 10 days following challenge (Fig. 2). The median survival of animals vaccinated at days 0, 5, and 10 were 42 days, 40 days, and 35 days, respectively, compared to 32 days in nonvaccinated animals ($P < 0.0001$). As in the previous experiments, all animals vaccinated 14 days prior to intrahepatic challenge survived beyond 80 days.

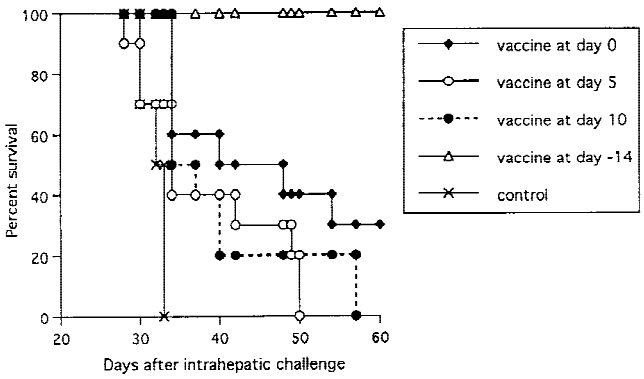

Fig. 2. Vaccination against established tumor. Comparison of groups by survival. Groups of mice (10 per group) were vaccinated with 10^6 irradiated CT-26/GM-CSF, 14 days prior to (day -14), same day (day 0), or 5 days (day 5) and 10 days (day 10) following intrahepatic challenge with 5×10^4 CT-26 WT. Control group received media 14 days prior to the challenge. Differences between groups reached statistical significance when compared to the media-treated group, $P < 0.001$.

Effect of Intrahepatic Challenge Dose Escalation on Antitumor Efficacy

The efficacy of in vivo protection from subsequent challenge of tumor within the liver was tested with increasing challenge cell number. When vaccinated (10^6 CT-26/GM-CSF) 14 days prior to intrahepatic tumor injection, challenge doses of 10^4 and 10^5 cells resulted in only 1 of 10 animals developing tumors. No tumor was seen in any animal in the group receiving the highest (10^6) challenge dose. Incidence of hepatic tumors was lower than that of medium treated animals and tumor volumes were significantly smaller (Table III). When studied for survival, no animals died within any of the vaccinated groups, even in those receiving 10^6 intrahepatic challenge dose (Fig. 3).

Histologic Studies of the Challenge Site

Additional animals that were vaccinated with CT-26/GM-CSF (10^6) or control and subsequently given intrahepatic tumor (5×10^4) were sacrificed on days 2, 7, and 9 following intrahepatic challenge for histologic analysis of the challenge site. In CT-26/GM-CSF-vaccinated animals, a cellular infiltrate was seen, associated with a few viable tumor cells (Fig. 4). In the control (nonvaccinated) group, viable tumors were seen but with fewer inflammatory cells. At 2 days following challenge, the chief component of the cellular infiltrate was polymorphonuclear leukocytes. Eosinophils, monocytes, and lymphocytes were also present. By 7 days following injection, no viable tumor cells were seen, and the infiltrate was one of chronic inflammation composed primarily of lymphocytes. By day 9, the entire lesion was nonviable as an abscess.

DISCUSSION

We have demonstrated in this murine model that autologous tumor vaccine transduced with the GM-CSF

TABLE III. Challenge Dose Escalation: Comparison of Groups by Presence of Tumor and Tumor Volume

Vaccination	No. of challenge cells	No. of mice with tumor	*P* value[a,b]	Mean tumor volume (mm^3)	*P* value[a,c]
10^6 CT-26/GM-CSF[d]	10^4	1/10	<0.0001	2.82 ± 8.9	<0.0001
10^6 CT-26/GM-CSF	10^5	1/10	<0.0001	5.59 ± 17.7	<0.0001
10^6 CT-26/GM-CSF	10^6	0/10	<0.0001	0 ± 0	<0.0001
Control	10^5	10/10	—	2,258.2 ± 885.9	—

[a]Compared with control.
[b]Fisher's exact test.
[c]Wilcoxon test.
[d]GM-CSF-producing CT-26 cells.

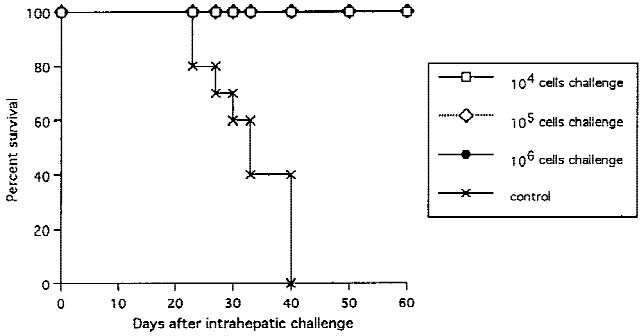

Fig. 3. Escalation of hepatic challenge dose. Comparison of groups by survival rate. Groups of mice (10 per group) were injected intradermally with 10^6 irradiated CT-26/GM-CSF. All mice were challenged 2 weeks later by intrahepatic injection of 10^4, or 10^5, or 10^6 CT-26 WT, respectively. Control group received media and was challenged with 10^5 CT-26 WT. Difference between median survival times reached statistical significance when compared to the control group, $P < 0.0001$. All treated groups had 100% survival for 60 days.

gene is capable of eliciting a significant antitumor effect for colorectal cancer within the liver. Hepatic tumor was effectively eliminated when vaccination was administered prior to implantation, even at high challenge doses. Vaccination was effective even in animals with established liver tumors, although not as effective as when utilized for protection.

In a variety of preclinical models, tumor cells genetically altered to secrete cytokines have been shown to generate systemic tumor immunity [1–7]. These results have led to trials in several types of cancers, including some gastrointestinal malignancies [15]. One difference between previous experimental models and human gastrointestinal metastatic disease is the involvement of predominantly visceral sites, particularly the liver. In colorectal cancer, up to 20% of patients have liver metastases at the time of presentation. Of those whose cancer will subsequently recur, more than 70% will have liver-dominant disease [16]. Based on these clinical observations, we have developed this metastatic liver tumor model to test the efficacy of cytokine-producing autologous tumor vaccine.

GM-CSF is the most powerful immunostimulant of the various cytokines tested [8]. Dranoff et al. [8] showed that the antitumor effect of GM-CSF-producing autologous tumor cells were tumor specific and mediated by CD4+ and CD8+ T lymphocytes. Since the epithelial tumors do not express MHC class II molecules on their cellular surfaces, it was assumed that class I molecules should present antigens to effector cells or there must be an additional route of antigen presentation through host professional APCs. It is now clear that APCs, rather than tumor cells, are a more important pathway through which tumor antigens can be presented to helper T cells. The potency of the effect of GM-CSF locally may relate to its unique ability in promoting differentiation of hematopoietic precursors to dendritic cells, which are the most potent APCs for helper T cells [9,17].

Since Kupffer and pit cells, along with other secondary effector cells, may also play significant roles within the liver [18, 19], it is probable that other subsets of effectors than the usual CD8+ T cells were significantly activated. However, we could not identify any morphologic or quantitative changes of those cells by the conventional hematoxylin-eosin staining. Histologic features of the challenge site revealed nonspecific cellular infiltrates, such as polymorphonuclear leukocytes (PMNs), and macrophages until the sixth day after challenge and lymphocytes thereafter. We could not see a significant eosinophil infiltrate, which was the typical histologic finding previously observed [8]. We do not know exactly why a subcutaneous challenge site recruits many eosinophils, whereas the liver does not. It is still unclear why the chief components of the initial cellular infiltrates were PMNs, even though the animals must have had specific cytotoxic T cells by prior vaccinations. Presumably, the tumor cell itself could attract nonspecific inflammatory cells before lymphocytes arrive or the nonspecific inflammatory reaction is necessary for the activation of the specific T cells. Since nonvaccinated animals showed no significant cellular infiltrates around the tumor cells during the same time period, the latter explanation seems to be more plausible. For clarification

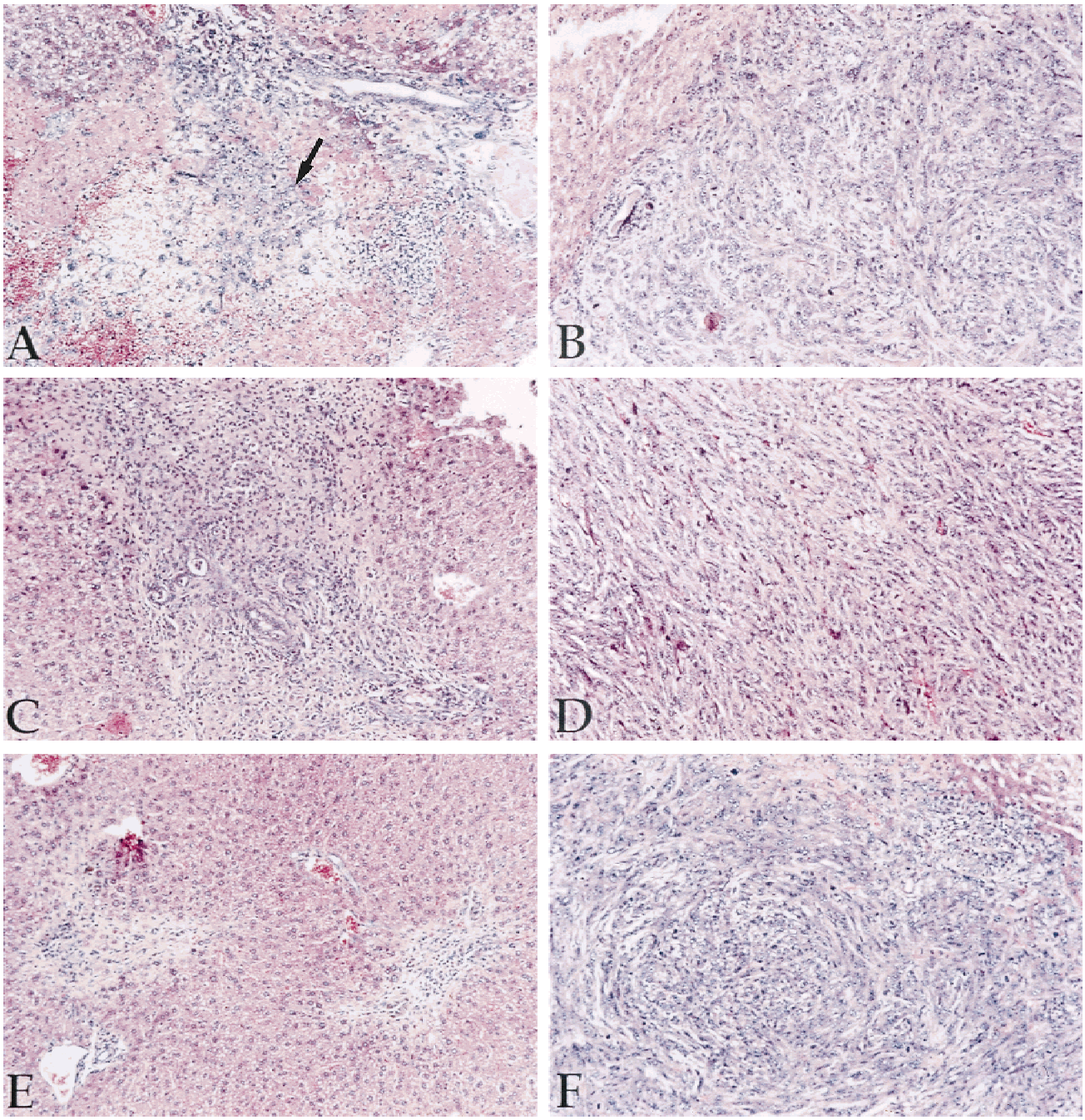

Fig. 4. Photomicrograph of intrahepatic challenge site. Sections were prepared 2 days **(A,B)**, 7 days **(C,D)**, and 9 days **(E,F)** following intraheptaic challenge. Animals vaccinated with CT26/GM-CSF (A,C,E); control animals (B,D,F). A: A few scattered, but viable tumor cells (arrow) are surrounded by PMNs. B, D: No definite inflammatory cell infiltrates with increasing tumor size. C: No viable tumor cells are seen. Chief components of cellular infiltrates are lymphocytes. E: Challenge sites shrunk as microabscesses. F: A well-established tumor. Hematoxylin and eosin stains, ×64.

of these findings, studies on animals depleted of variable effector subsets should be conducted.

We found that the vaccinated host effectively scavenged the large number (10^6) of tumor cells that would be an overwhelming tumor burden to the mouse immune system. There have been few preclinical studies in which mice were challenged with as large a cell number. The effectors of immune system seem to be able to clear the challenged tumor cells independent of their numbers once they are stimulated.

To determine whether this tumor vaccine strategy will be effective, it needs to be evaluated in relevant animal models under conditions that resemble the clinical conditions prevailing in cancer patients [10]. For this reason,

we conducted the second set of experiments in which animals were challenged first and then vaccinated. There were no long-term survivors in the animals vaccinated subsequently. However, the mean tumor volumes and the median survival times of the animals with subsequent vaccinations were significantly smaller and longer respectively than those of the control group. Prolongation of median survival was observed even when the vaccine was administered up to 10 days following tumor challenge. Five to 10 days may be necessary for the full stimulation of tumor-specific T cells following treatment of GM-CSF-producing cells. Probably, GM-CSF-producing autologous tumor vaccine can eradicate the established tumor if smaller numbers of tumor cells are challenged.

We conclude that GM-CSF-producing autologous tumor cells effectively enhance the immunogenicity of their parental forms of the tumor so that the immunized host becomes able to reject the further challenge of parental tumor cells into the liver. The efficacy of the GM-CSF gene-transduced vaccine to treat established tumors warrants further investigation. This study provides support for cell-based immunotherapeutic strategies for the treatment of colorectal cancer. The demonstration that this approach is effective within the liver should form the basis of clinical trials to assess active immunotherapy for liver cancer.

CONCLUSIONS

GM-CSF autologous tumor vaccination was effective for the treatment of hepatic colorectal metastases in this murine model. These findings provide support for immunotherapeutic approaches for the treatment of metastatic liver cancer.

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