Prevention of Hepatic Tumor Metastases in Rats with Herpes Viral Vaccines and γ-Interferon

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Abstract

Previous studies showed that γIFN decreases metastatic hepatic tumor growth by stimulating Kupffer cells (KC). The present studies examine whether lymphocyte stimulation via cells engineered to secrete GM-CSF or IL-2 decreases hepatic tumor growth, and whether stimulation of both macrophages and lymphocytes is more effective than either individually. Rats were immunized with irradiated hepatoma cells transduced by herpes viral ampiclon vectors containing the genes for GM-CSF, IL-2 or LacZ. On day 18, half of each group was treated with 5 × 10⁴ U γIFN, or saline intraperitoneally for 3 d. On day 21, all rats received 5 × 10⁴ hepatoma cells intrasplenically. On day 41, rats were killed and tumor nodules were counted. Separate rats underwent splenocyte and KC harvest for assessment of lymphocyte- and macrophage-mediated tumor cell kill in vitro. GM-CSF or IL-2 vaccines or γIFN decreased tumor nodules significantly (GM-CSF 13±4, IL-2 14±6 vs. control 75±24, P < 0.001). Combination therapy was more effective, and completely eliminated tumor in 4 of 12 IFN-GM-CSF and 8 of 11 IFN-IL-2 animals. Additional rats underwent partial hepatectomy, an immunosuppressive procedure known to accelerate the growth of hepatic tumor, following tumor challenge. Therapy was equally effective in this immunosuppressive setting. Vaccination is associated with enhancement of splenocyte-mediated tumoricidal activity, whereas the effect of γIFN is mediated by KC. GM-CSF and IL-2 vaccine therapy and pretreatment with γIFN represent effective strategies in reducing hepatic tumor. Combination therapy targets both lymphocytes and macrophages, and is more effective in reducing tumor than either therapy alone. (J. Clin. Invest. 1997. 99:799–804.) Key words: hepatectomy • IL-2 • GM-CSF • liver • HSV

Introduction

Hepatic tumors are the most common gastrointestinal tract malignancy worldwide (1). Surgical resection is the most effective treatment for hepatic tumors; however, recurrence is frequent and usually occurs in the liver (2). In addition, resection, a procedure commonly used as therapy for liver cancer, may accelerate the growth of local residual cancer (3, 4). Previous studies in our laboratory demonstrated that enhancement of Kupffer cell (KC) tumoricidal activity by systemic administration of the immunomodulator γIFN significantly reduces the uptake and growth of hepatic tumor found in a metastatic model (4). The current study attempts to determine if stimulation of the lymphocyte antitumor mechanisms is complementary to the macrophage tumoricidal mechanisms.

One of the most successful strategies directed at stimulating lymphocyte antitumor immunity is the use of cytokines, such as IL-2 and GM-CSF. IL-2 is a protein (synthesized and secreted primarily by T helper cells) which activates lymphocytes (5); GM-CSF is produced by a wide range of cell types including B and T cells, macrophages, and endothelial cells, and it functions to stimulate proliferation, maturation, and function of hematopoietic cells (6). The high systemic levels of cytokines required to produce an antitumor response in humans have led researchers to examine whether local cytokine production in the presence of tumor antigens could be effective without the toxicity associated with high systemic levels (7). Several gene therapy models utilizing IL-2- and GM-CSF-secreting tumor vaccines have demonstrated these cytokines’ ability to stimulate the immune system, and to produce protective immunity in both rat and murine systems (8–12).

Herpes Simplex virus ampiclon (HSV) vectors are efficient vehicles for introducing foreign genes into cells. HSV vectors rapidly infect cells, transducing them in a matter of minutes with high local expression of the foreign gene for a limited duration (13–15). In the current experiments, this transient expression allows us to distinguish immunologic responses from effects due to ongoing cytokine production. We constructed replication-defective HSV ampiclon vectors containing the genes for IL-2, GM-CSF, or LacZ in order to examine whether gene799 therapy with tumor cells tailored by HSV vectors to produce cytokines could induce immunity and decrease metastatic tumor uptake and growth. We sought to determine if targeting both lymphocyte function and macrophage function is more effective than either alone, and to examine the relative roles of each in decreasing tumor growth. Furthermore, since partial hepatectomy (PH) has been shown to be immunosuppressive and to accelerate the growth of hepatic tumor, we examined the effectiveness of these treatment strategies in the clinically relevant model of hepatic resection in the face of microscopic residual disease.

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Materials and Methods

Animals. Male Buffalo rats (National Cancer Institute, Bethesda, MD) were housed in individual cages in a temperature- (22°C) and humidity-controlled room with a 12-h day/night cycle. Animals were provided food (PMI Mills, St. Louis, MO) and water ad libitum. Food consumption and body weight were measured daily. All animals received care under approved protocols in compliance with Memorial Sloan-Kettering Cancer Center’s Institutional Animal Care and Use Committee guidelines.

Tumors. A syngeneic tumor, Morris Hepatoma MA-RH7777 (ATCC No. CRL 1601; Rockville, MD), was maintained in culture under membrane and periodically implanted in flanks to ensure tumorigenicity. Recombinant murine γIFN. Recombinant murine γIFN supplied by Genentech Inc. (South San Francisco, CA) was diluted with sterile water to 5 × 10^6 IU/ml before injection. This dosage has previously been shown to produce significant biological responses including enhanced expression of TNFα receptors (16, 17).

Operative procedures. All operative procedures were performed under pentobarbital anesthesia (25 mg/kg of body weight i.p.) via midline abdominal incision. 70% PH was performed according to the methods of Higgins and Anderson and include resection of the left and median lobes (2). Animals received 3 ml i.p. of normal saline prior to skin closure.

Splenocyte isolation. Animals underwent splenectomy under sterile conditions. Each spleen was placed in a petri dish with 10 ml of media (RPMI + 10% FCS + 50 µg/ml gentamicin). Splenocytes were washed from the spleen by repeated resuspension with media, spun (1,500 rpm, 5 min) and subsequently resuspended in 5 ml of red cell lysis solution (8.8 g NaCl, 50 mM Tris, 10 ml NP-40, 1.68 µg Fe per liter) for 1 min, the solution was neutralized by the addition of 5 ml of media. Cells were spun (1,500 rpm, 10 min), washed 2× with media, and kept in culture in media supplemented with 30 U/ml IL-2 (Chiron Corp., Emeryville, CA) for 2 d prior to use.

Kupffer cell isolation. KC were obtained with standard collagenase perfusion and differential ultracentrifugation (18, 19). Briefly, the portal vein was cannulated and the liver perfused in vivo with three different solutions: Leffert’s solution with 0.5 mM/liter EGTA (38°C, 10 min), Leffert’s solution without EGTA (38°C, 5 min), and Leffert’s solution with 5 mM/liter CaCl₂, 7 mg/ml bovine serum albumin and 0.2 mg/ml collagenase D (Boehringer Mannheim Co., Indianapolis, IN) (38°C, 15 min). The liver was then removed, minced, passed through a 50-µm nylon mesh into RPMI and centrifuged (50 g, 2 min). The supernatant was centrifuged (300 g, 10 min), and the pellet containing KC was resuspended in 20 ml of Media, layered onto an isosmotic Percoll (Pharmacia Biotech Inc., Piscataway, NJ) gradient, and spun (800 g, 15 min). The band containing KC was harvested, diluted with equal volume PBS, and spun (800 g, 10 min). The pellet was resuspended in media + 1% penicillin/streptomycin. Cells were counted, plated in 96-well plates (Costar Corp., Cambridge, MA), and incubated for 4 h. Non-adherent cells were washed off with media. This procedure yields cells which adhere, take up India ink uniformly, and stain positive with Wright-Giemsa stain (Baxter, McGaw Park, IL), confirming this procedure produced KC.

HSV vectors. The murine GM-CSF, human IL-2 and LacZ genes were cloned directionally into HSVprPUC which contains the HSV immediate early 4/5 promoter, a multiple cloning site, and an SV40 A part of the β-lactamase gene from pBR322 (nucleotides 3733–4168). After stringent washing (0.1× SSC 2× for 15 min), blots were exposed to x-ray film, and various timed exposures taken and densitometrically scanned (KLUB Ultroscan; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Band densities and the titers of HSVII2 and HSV GM-CSF (expressed as particles/ml) calculated from the density relative to HSVI2 given that this latter amplicon was titrated by an expression assay, were compared. HSVI2 titers were between 1–2 × 10^9 particles/ml at titers of 1:1–5:1. HSVGM-CSF titers were between 1–2 × 10^10 particles/ml. The ratio of D30 EBA helper virus to ampiclon varied from 2:1 to 5:1. moi refers to the ampiclon. Recombination for wild-type revertants was monitored by plaque assay on Vero cells and occurred at a frequency of 1 × 10–5.

In vitro production of cytokines. To assess in vitro production of cytokines, 10^6 hepatoma cells per 2 ml were plated in six-well plates (Costar), irradiated with 10,000 rad, and rested for 1 h. Cells were then exposed to HSVI2, HSVGM-CSF, HSVlac, or Media for 20 min at moi’s of one and two and washed 2× with media. Cell culture supernatants were harvested on days 1, 2, 4, and 7 post-exposure, and cytokine levels were measured by ELISA (IL-2, R & D Systems, Minneapolis, MN; GM-CSF, Genzyme Corp., Cambridge, MA).

Effect of vaccination and γIFN on hepatic tumor. Hepatoma cells in culture were irradiated with 10,000 rad, allowed to rest for 1 h, then exposed to HSVI2, HSVGM-CSF, HSVlac or media for 20 min at moi of one. Cells were then washed 2× with media, and 10^6 cells/200 µl were injected intrasplenically. Preliminary experiments in our laboratory have demonstrated intrasplenic vaccination to be more effective than either intraperitoneal or subcutaneous vaccination. An additional control group underwent injection of media alone. On day 18, half the animals in each group received either 5 × 10^6 U of γIFN i.p. or normal saline for 3 d. On day 21, all animals received a challenge of 5 × 10^6 hepatoma cells/200 µl intrasplenically followed by splenectomy 10 min later, allowing sufficient time for the hepatoma cells to migrate to the liver (23). Animals were killed 20 d later, and tumor nodules were counted. Additional animals were vaccinated, killed on d 2 and 18 post-vaccination, and heart, lung, liver, kidney and serum harvested for assessment of in vivo production of cytokines by ELISA.

Effect of immunization and γIFN on hepatome-teenched tumor growth. To assess the effects of vaccination on tumor growth in the setting of PH, animals were immunized intrasplennially with hepatoma vaccines (HSVII2, HSVGM-CSF, HSVlac) produced as above. On day 18, half the animals in each group received either 5 × 10^6 U of γIFN intraperitoneally, or normal saline for 3 d. On day 21, all animals received a challenge of 5 × 10^6 hepatoma cells/200 µl in-
were undetectable in heart, lung, serum, and kidney. with the in vivo data: cytokines levels are seen only in the liver day 1 and decreasing thereafter. The in vitro data correlate (Morris hepatoma cells were exposed to HSVGM-CSF (mean number of liver nodules and percent specific lysis, expressed as displacement. shown to correlate directly with tumor volume as measured by water displacement.

Effect of vaccination and γIFN on splenocyte and KC function. Animals in each treatment group underwent vaccination and γIFN treatment as above, and splenocytes and KC were harvested on day 21 post-vaccination. Tumorcidal activity was assessed by mixing effectors with Europium-labeled tumor cells in an in vitro assay. Labeled cells were plated at a concentration of 5 × 10^5 cells/100 μl per well in a 96-well U-Bottom plate (Costar) containing effector cells in wells at varying effector to target ratios. The plate was spun (200 rpm, 5 min), incubated (4 h, 37°C), and respun (500 rpm, 5 min). 20 μl of supernatant were transferred to a 96-well Flat Bottom plate (Costar) already containing 180 μl/well of Delfia Enhancement Solution (Wallac Oy, Turku, Finland). The plate was read in a 1232 Delfia Fluorometer (Wallac Oy). Maximum lysis was measured by lysis of cells with 1% Triton X. Percent specific lysis is equal to experimental – spontaneous release/max. release – spontaneous release × 100. Spontaneous release varied between 5 and 15% of max. Assays were performed in triplicate.

Statistical analysis. Statistical significance of differences in the number of liver nodules and percent specific lysis, expressed as mean±SEM, were analyzed using ANOVA or Student’s t-test (SPSS Inc., Chicago, IL) as appropriate.

Results

In vitro and in vivo production of cytokines. Control cells not exposed to cytokine gene-containing vectors do not produce cytokines including IL-2, GM-CSF, γIFN, IL-12, or TNFα. No cytokines are seen immediately after transduction with HSVIL2 and HSVGM-CSF and washing, indicating that proteins were not injected along with tumor cells. Cells exposed to HSVIL2 or HSVGM-CSF produce nanogram quantities of these cytokines per 10^6 cells after vaccination (Figs. 1, A and B), peaking on day 1 and decreasing thereafter. The in vitro data correlate with the in vivo data: cytokines levels are seen only in the liver (IL-2, 461 pg/organ) and spleen (IL-2, 12 pg/organ) on day 2, but levels are below detectability on day 18. Cytokine levels were undetectable in heart, lung, serum, and kidney.

![Figure 1](image1.png)

**Figure 1.** In vitro production of GM-CSF and IL-2. 10^6 irradiated Morris hepatoma cells were exposed to HSVGM-CSF (A) or HSVIL2 (B) for 20 min at multiplicities of infection of two (□) and one (○). Supernatants were evaluated for cytokine production by ELISA.

![Figure 2](image2.png)

**Figure 2.** HSV tumor vaccines and γIFN reduce tumor burden. Animals underwent vaccination with irradiated cells transduced by either HSVGM-CSF (GM-CSF) or HSVIL2 (IL-2). Controls (CTRL) consisted of vaccination with cells transduced by HSVlac, non-transduced cells, or media. On day 18 after vaccination, half of each group was treated preoperatively with either 50,000 U γIFN or saline for 3 d. On day 21, all animals were challenged intrasplenically. Animals were killed 21 d after challenge, and tumor nodules were counted. There were no significant differences among any of the three control groups: to simplify the illustration, the three control groups have been pooled. *P < 0.01 vs. CTRL/IFN; *P < 0.01 vs. CTRL/Sal; n = 11 to 13 per group. GM-CSF/IFN, 4/12 without tumor; IL-2/IFN, 8/11 without tumor.

Effect of vaccination and γIFN on hepatic tumor. There was no significant effect of vaccination with irradiated cells or vaccination with irradiated cells transduced by HSVlac compared to injection of media alone. Animals immunized with IL-2- or GM-CSF-secreting cells or pretreated with γIFN had significantly fewer tumor nodules than all three control groups (Fig. 2, P < 0.001). Combination treatment with IL-2- or GM-CSF-secreting tumor cell vaccines and pretreatment with γIFN was more effective than any single treatment; complete responses were seen in 8 of 11 IL-2 animals and 4 of 12 GM-CSF animals (Fig. 2). No animal treated with γIFN alone was without tumor.

Effect of immunization and γIFN on hepatectomy-enhanced tumor growth. Hepatectomy significantly increased the number of tumor nodules compared to controls (Fig. 3, NoHep vs. CTRL/Sal, P < 0.001). Animals immunized with IL-2- or GM-CSF-secreting cells or pretreated with γIFN had significantly fewer tumor nodules than control (Fig. 3, CTRL/Sal vs. all others P < 0.001). Combination treatment with cytokine-secreting vaccines and pretreatment with γIFN was more effective than any single treatment (Fig. 3, P < 0.01). Thus, combination therapy of cytokine vaccines and γIFN is effective in decreasing tumor growth in the setting of hepatectomy.

Effect of vaccination and γIFN on splenocyte and KC function. Vaccination with HSVlac or irradiated cells had no significant effect on either KC or splenocyte activity. Splenocytes from animals vaccinated by either HSVIL2 or HSVGM-CSF had significantly greater killing of targets than splenocytes from control or γIFN-treated animals (Figs. 4, A and B). γIFN did not appear to affect splenocyte lytic activity.
KC from rats pretreated with γIFN had significantly greater killing of targets than KC from controls (Figs. 5, A and B). KC from rats vaccinated with HSVii2 also had significantly greater killing of targets than KC from controls ($P < 0.05$), but not as great as γIFN. Vaccines secreting GM-CSF did not seem to affect KC activity.

Discussion

Recent studies have focused on the use of cytokines to enhance the immune system in an attempt to decrease tumor growth (8, 9, 24). Systemic administration of cytokines is accompanied by unwanted serious side effects (5, 7). Transfer of cytokine genes into tumor cells gives the advantage of being able to produce high concentrations of cytokines of interest in the area of putative tumor antigens. Also, as identification of specific cytokine genes increases, we are able to transfer specific isolated genes to determine their role in immunomodulation.

The fundamental principles of immunotherapy for solid tumors are the suppositions that tumor antigens can be recognized, and that animals can elicit a tumor-specific immune response (5, 25). In this experiment, we vaccinated animals with irradiated tumor cells that had been infected by HSV vectors, and were producing IL-2 or GM-CSF (cytokines which have been shown previously to stimulate antitumor immune responses) (8–11, 26).

HSV vectors are efficient and effective in producing high levels of cytokines locally and generating an immune response (13, 14, 27). In this experiment, cytokine production was seen only in the target organs, the liver and the spleen. Furthermore, production was transient, with levels returning to below detectability prior to the subsequent tumor challenge 3 wk later. Thus, any effects on cellular immunity and tumor growth were not dependent upon ongoing cytokine production during challenge.

Vaccination with HSVii2 or HSVGM-CSF reduced the amount of tumor in the liver after subsequent challenge with highly tumorigenic doses of unmodified tumor cells. The results are not simply due to the prior presence of tumor antigen, since irradiated cells alone did not reduce tumor. Nor was the
effect of vaccination due to the presence of HSV, since treat-
ment with HSVl Vac did not cause a decrease in tumor. Even in
the setting of PH, which has been shown to be immunosup-
pressive and to accelerate the growth of hepatic tumor, vacci-
nation was ineffective. Combination therapy abrogated the ef-
fect of PH, for animals so treated had no more tumor than the
non-hepatectomized control.

Production of cytokines in the presence of tumor antigens
induces a lymphocyte immune response, as evidenced by the
increased tumoricidal activity of splenocytes from vaccinated
animals. Generating an immune response requires a simple
vaccination protocol utilizing fresh tumor cells, a 20-min tran-
sduction, and a single injection into the spleen and portal sys-
tem to result in finite cytokine production. Such a protocol is
clinically feasible.

Perioperative immunostimulation has been examined as a
means of decreasing tumor (28, 29). In the current study, we
employed γIFN, a multifunctional protein with a wide range of
immunostimulatory effects, for immunoaactication (26, 30).
In this experiment, pretreatment with γIFN resulted in decreased
hepatic tumor after splenic injection of tumor. This effect is
due to stimulation of KC causing increased tumoricidal activity
of these tissue-based macrophages (4, 26).

The combination of IL-2 and γIFN has begun to be exam-
ined in clinical trials (31). In the current study, the combina-
tion of γIFN and cytokine vaccines was more effective than
either method alone, and, in many cases, prevented the estab-
lishment and growth of tumor in the liver after subsequent
challenge with highly tumorigenic doses of unmodified tumor
cells. Whether these effects were synergistic or simply additive is
difficult to distinguish. γIFN did not appear to increase the
tumoricidal activity of splenocytes; the cytokine vaccines ap-
peared to have little direct effect on macrophages. However, in
addition to augmenting macrophage kill, γIFN may also im-
prove antigen processing, thereby increasing the efficiency of
T-cell mediated immunity.

Liver cancer effects over 2 million people worldwide per
year. Surgical resection is the most effective treatment, yet the
most common site for recurrence after resection is within the
liver itself, indicating that microscopic residual disease is left
behind after “curative” surgery. Experimental evidence indi-
cates that hepatic resection is immunosuppressive and en-
hances the growth of residual tumor (19, 32). The current ex-
periment confirms that tumor growth in the liver is enhanced
by PH. Moreover, the current study demonstrates that even in
the immunosuppressive setting of liver resection, macrophage,
and lymphocyte anti-tumor function can be restored by γIFN
administration and tumor immunization.

Clinical studies have already shown the feasibility of ex-
tracting tumor, transducing cells with genes, and returning the
transduced cells to the host (33); HSV vectors are potentially
useful tools for this clinical application because they can trans-
fect irradiated, non-replicating cells rapidly and efficiently. In
experiments using human tumors isolated from patients in the
operating room, the entire isolation, transduction with HSV
vectors, and irradiation process was uniformly less than 240
min, and was free of the technical problems of primary tissue
culture (34). γIFN has been used in clinical trials for several
years with varying effectiveness as a therapeutic tool for large
tumor burdens. In our clinically relevant model of hepatocel-
lar and local recurrence, these two treatments retarded
growth of microscopic residual metastases. These data encour-
age future studies of the use of an immunomodulator, such as
γIFN, coupled with directed vaccine gene therapy in the adju-
vant treatment of the large numbers of patients undergoing
liver resection for cancers.

References

1. Lotze, M.T., J.C. Flickinger, and B.L. Car. 1993. Hepatobiliary neo-
plasms. In Principles of Oncology. V.T. Devita, S. Hellman, and S.A. Rosen-
3. Loizidou, M.C., R.J. Lawrence, S. Holt, N.J. Carty, A.J. Cooper, P.
growth within the rat liver following intraportal injection of syngeneic tumor cells. Clin.
Exp. Metastasis. 9:35–49.
against hepatic tumor growth in rats by increasing Kupffer cell tumoricidal activ-
34–39.
8. Bubenkiv, J.N., V. Voitenkov, J. Kieler, V.S. Prassolov, P.M. Chumakov,
containing an inserted IL-2 gene and producing IL-2 inhibits growth of human
9. Gansbacher, B., L. Zier, B. Daniels, K. Cronin, R. Rannerji, and E. Gil-
bo. 1990. Interleukin-2 gene transfer into tumor cells abrogates tumorigenic-
Gansbacher, and E. Gilbo. 1994. Immunotherapy of prostate cancer in the
54:1760–1765.
11. Dranoff, G., E. Jaffe, A. Lazeyn, P. Golumbek, H. Levitsky, B. Brose,
irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulat-
ing factor stimulates potent, specific, and long-lasting anti-
12. Abe, J., H. Wakimoto, Y. Yoshida, M. Aoyagi, K. Hirakawa, and H. Ha-
manda. 1995. Antitumor effect induced by granulocyte/macrophage colony-stimulat-
ing factor gene-modified tumor vaccination: comparison of adenovirus- and
592.
cient deletion mutant packaging system for defective herpes simplex virus vec-
tors: Potential applications to human gene therapy and neuronal physiology.
vectors with glucocorticoid-inducible gene expression. Hum. Gene Ther. 6:419–
428.
lar endothelial growth factor from a defective herpes simplex virus type 1 am-
gamma correlates with enhanced immune function of organ-associated, but not
17. Kawada, N., Y. Mizoguchi, K. Kobayashi, S. Morisawa, T. Monna, and
S. Yamamoto. 1991. Interferon gamma modulates production of Interleukin 1
and tumor necrosis factor by murine Kupffer cells. Liver. 11:42–45.
613.
Escherichia coli stimulates macrophage mediated alterations in hepatocellular
function during in vitro coculture: a mechanism of altered liver function in sep-
21. Patterson, T., and R. Everett. 1990. A prominent serine-rich region in
Vmw175, the major transcription regulator protein of herpes simplex virus type
22. Geschwind, M., B. Lu, and H. Fedoroff. 1994. Expression of neu-


