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Eradication of Hepatic Metastases of Carcinoma H-59 by Combination Chemoimmunotherapy with Liposomal Muramyl Tripeptide, 5-Fluorouracil, and Leucovorin[1]

Takayuki Asao,[2] Henry R. Shibata, Gerald Batist, and Pnina Brodt[2]

Department of Surgery, Division of Surgical Research, McGill University, and the Royal Victoria Hospital, Montréal, Québec, Canada H3A 1A3 [H. R. S., P. B.]; Department of Oncology, The Jewish General Hospital, Montréal, Québec, Canada H3T 1E2 [G. B.]; and Gunma University, Maebashi, Japan 371 [T. A.]

ABSTRACT

We have investigated the effect of a combined chemoimmunotherapy protocol with liposomal muramyl tripeptide phosphatidylethanolamine (MTP-PE), 5-fluorouracil (5-FU), and 5-formyltetrahydrofolate (leucovorin) on the growth of hepatic metastases using carcinoma H-59, a liver-homing subline of the Lewis lung carcinoma (P. Brodt, Cancer Res., *46:* 2442–2448, 1986). C57BL/6 mice inoculated with the tumor cells via the intrasplenic route received three i.v. injections of liposomal MTP-PE, the first of which was administered 3 days prior to tumor cell inoculation. Chemotherapy with 5-FU and leucovorin at the maximal tolerated doses (30 mg/kg per injection) was initiated immediately after tumor inoculation and continued on alternate days for a total of 4 injections. The incidence of liver metastases in animals which received the combined therapy was compared to that in animals treated with chemotherapy or immunotherapy alone. We found that while the number of liver metastases was reduced in all of the treatment groups as compared to control untreated or placebo-treated animals, the combined effect of 5-FU leucovorin and liposomal MTP-PE was significantly better than that of chemotherapy or immunotherapy alone. This was reflected in a reduced incidence (70% as compared to 100% in all other groups) and in a significant reduction in the number and size of the liver nodules. Our results suggest that the efficacy of 5-FU and leucovorin in the treatment of hepatic metastases could be significantly augmented by the addition of the liposome-encapsulated immunoadjuvant MTP-PE.

INTRODUCTION

The liver is the major site of metastasis for some of the most malignant human neoplasms, including carcinoma of the gastrointestinal tract and melanoma. Hepatic metastases are frequently the cause of death following the curative resection of a primary colonic cancer, and in fact 51% of patients who die of colorectal carcinoma have been found at autopsy to have liver metastases (1). The development of drugs for the treatment of hepatic metastases is therefore critical to the successful clinical management of gastrointestinal malignancies and of colorectal carcinoma in particular.

Although 5-FU[3] has been used for many years as the drug of choice in the therapy of colorectal carcinoma patients, the efficacy of this drug has been uncertain (2). Recently the modulation of 5-FU efficacy by leucovorin has been reported to increase the response rate in metastatic disease from 16% to 45%, resulting in the improvement of patient survival rates (3–5). However, a malignant tumor contains a heterogeneous cell population, and a single chemotherapeutic drug is unlikely to affect all cell types. Furthermore, this combination treatment can be very toxic and can be associated with some lethal side effects (4). Further antitumor activity, therefore, may best be achieved by the addition of nonchemical biological agents.

A synthetic molecule, MDP, which resembles the smallest structural unit of peptidoglycan of the bacterial cell wall, has been shown to activate the tumoricidal potential of hepatic (6–10), pulmonary (11, 12), and peritoneal macrophages (10, 13). Kleinerman *et al.* (14, 15) have shown that this immunoadjuvant can also activate blood monocytes of cancer patients. A lipophilic derivative of MDP, known as muramyl tripeptide phosphatidylethanolamine (CGP 19835A; Ciba-Geigy), was developed that incorporates more efficiently into liposomes and is approximately twice as potent as MDP in activating the tumoricidal potential of macrophages (14).

In a previous study we have shown that liver metastases of tumor H-59, a liver-homing subline of the murine carcinoma 3LL, can be significantly reduced by the administration of liposomal MDP alone (7). Here this carcinoma model of hepatic metastasis was used to investigate the efficacy of a combined protocol with liposomal MTP-PE, 5-FU, and leucovorin in the treatment of experimental liver metastases.

MATERIALS AND METHODS

Animals and Cells. Female C57BL/6 mice, 8–12 weeks old, were obtained from Charles River, Ltd. (St. Foy, Québec, Canada). Animals were regularly screened for the presence of common laboratory pathogens, including murine hepatitis virus.

The origin and metastatic properties of carcinoma H-59 were previously described (16). The tumor was maintained *in vivo* by subcutaneous implantation of hepatic nodules isolated from tumor-bearing mice. Single cell suspensions were prepared from enzymatically dispersed tumor tissue, and the tumor cells were maintained *in vitro* for no longer than 2 weeks prior to use in the experiments (7).

Single cell suspensions were prepared from cultured cells by a brief incubation of the cell monolayer with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 0.2% disodium EDTA, and the cells were washed in Hanks' balanced salt solution and adjusted to a concentration of 2×10^5 cells/ml in Hanks' balanced salt solution prior to injection.

Reagents. Liposome-encapsulated MTP-PE (CGP 19835A) and empty liposomes (placebo) were supplied by Ciba-Geigy, Ltd. (Basel, Switzerland). Leucovorin was obtained from Cyanamid Canada, Inc. (Montréal), and 5-FU was obtained from Hoffmann-LaRoche, Ltd. (Mississauga, Ontario, Canada). The concentrations of these drugs were adjusted by dilution with saline.

Assay of Antiproliferative Activity. Cultured H-59 cells were harvested by a brief exposure to Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 0.2% disodium EDTA and seeded onto 38-mm^2 wells of flat-bottomed 96-well culture plates (Falcon). The cells were exposed to different concentrations of the drugs for 48 h. The medium containing the antineoplastic drug was discarded, and the monolayer was washed and replenished with RPMI 1640 containing 10% fetal calf serum. The cells were then incubated at 37°C for 48 h, and the number

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[2] To whom requests for reprints should be addressed, at Division of Surgical Research, McGill University, 740 Dr. Penfield Ave., Montréal, Québec, Canada H3A 1A3 (Dr. Pnina Brodt), or Department of Surgery, Gunma University Maebashi, 371, Japan (Dr. Takayuki Asao).

[3] The abbreviations used are: 5-FU, 5-fluorouracil; leucovorin, 5-formyltetrahydrofolate; MDP, muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine); MTP-PE, muramyl tripeptide phosphatidylethanolamine.

of viable cells was counted using the trypan blue exclusion dye. The relative growth inhibition as compared to controls (T:C ratio) was calculated as follows:

$$\text{T:C ratio (\%)} = \frac{A}{B} \times 100$$

where A is the number of cells cultured with agent and B is the number of cells cultured with control medium.

Liver Metastasis Assay. The intrasplenic inoculation of tumor cells was performed as previously described (7). Mice were anesthetized with 65 mg/kg sodium pentobarbital and inoculated with 2×10^5 H-59 cells suspended in 1 ml Hanks' balanced salt solution. Mice were sacrificed 2 weeks after tumor inoculation, and a complete autopsy was performed. The livers were removed, their wet weights were determined, and the liver lobes were separated for counting of the metastatic nodules. Nodule size was determined using the scale described by Wexler (17).

Treatment Protocol. All drugs were injected into the lateral tail vein in a volume of 100 μl. The maximum tolerated doses for 5-FU and leucovorin were determined with normal, non-tumor-bearing mice according to the guidelines set by the European Multicenter Study (18). The animals in the control group received the same volume (100 μl) of physiologic saline. For treatment of tumor-inoculated mice, leucovorin and 5-FU were administered at concentrations of 30 mg/kg on days 1, 3, 5, and 7 (tumor inoculation was on day 0). In the immunotherapy group, liposomal MTP-PE was administered at a concentration of 4 μg/mg lipid/ml. Each animal received 1 mg MTP-PE in 250 mg lipid/kg body weight, 3 days prior to tumor inoculation and on days 0 and 3. Empty liposomes were used as a placebo. The lipid composition was 175 mg phosphatidylcholine and 75 mg phosphatidylserine for each mg of MTP-PE.

Statistical Analysis. The Mann-Whitney U test was used to analyze differences in survival rates and in the numbers of metastases. The Student's t test was used to analyze differences in liver weights and T:C rates and the χ^2 test to assess differences in the incidence of liver metastasis.

RESULTS

To determine the sensitivity of H-59 cells to 5-FU and leucovorin, the antiproliferative effect of these drugs on the tumor cells was first tested *in vitro*. The results are shown in Table 1. The growth of H-59 cells was inhibited by 5-FU in a dose-dependent manner at concentrations of 0.05 μg/ml or higher ($P < 0.001$). While leucovorin alone had no effect on the growth of H-59 cells even at a concentration as high as 125 μg/ml, it could significantly enhance the antiproliferative effect of 5-FU when added at a concentration of 0.5 μg/ml ($P < 0.05$). These results are similar to findings described for colorectal carcinoma (3, 4) and confirmed the suitability of carcinoma H-59 as a model system for the subsequent studies.

To determine the maximum tolerated dose of 5-FU when administered to C57Bl/6 mice alone or in combination with leucovorin various doses of the drugs were inoculated i.v. Results in Table 2 show that when injected together with leucovorin (30 mg/kg) the highest dose of 5-FU which resulted in 100% survival was 30 mg/kg. The mean weight loss of animals in this treatment group was 9.1%. The drugs were therefore used at these doses in all subsequent experiments.

To determine the effect of 5-FU and leucovorin on experimental hepatic metastases of H-59, the drugs were inoculated into C57BL/6 mice immediately following the intrasplenic injection of 2×10^5 tumor cells. Results shown in Table 3 demonstrate that 5-FU when administered in combination with leucovorin was significantly more effective in inhibiting the growth of liver metastases than either of these drugs alone.

To determine whether treatment with liposomal MTP-PE could further reduce the number of liver metastases, the additive effect of 5-FU, leucovorin, and liposomal MTP-PE was investigated. As shown in Table 4, 30% of animals treated with the combined chemoimmunotherapy protocol had no visible liver metastases at a time when the incidence in each of the other experimental groups (MTP-PE alone or 5-FU plus leucovorin) was 100%. Positive animals in the combined treatment group had a significantly reduced number of liver metastases ($P < 0.001$), and the mean diameter of the individual metastases was significantly decreased ($P < 0.02$). Interestingly, treatment with either chemotherapy or immunotherapy alone, while it resulted in a reduction in the number of liver metastases, had no significant effect on the size of the nodules.

DISCUSSION

MDP was identified as the minimum structure of mycobacterial peptidoglycan having immunoadjuvant activity. This biological response modifier and its analogues can potentiate host immunity to tumors (12) as well as viruses (19) and bacteria (20). However, the long period of exposure required to induce the tumoricidal activity of macrophages by this compound and its rapid excretion from the circulation after i.v. administration limit their effectiveness *in vivo* (12, 21, 22). Liposomes inoculated i.v. accumulate in the reticuloendothelial system and are taken up by phagocytic cells. They have therefore been utilized successfully as carriers of immunoadjuvant drugs (23). Liposomal encapsulation of MDP has been shown to enhance significantly the efficacy of this drug *in vivo* and its ability to activate the tumoricidal potential of macrophages *in vitro* (6, 7, 12, 24, 25).

Table 1 *Growth inhibition of H59 cells* in vitro *by 5-FU and leucovorin*

H59 cells at a concentration of 1.5×10^4 cells/0.2 ml medium were seeded in 96-well culture plates and incubated for 48 h with various concentrations of 5-FU and leucovorin. The drugs were removed by washing, and the cells were maintained in drug-free medium for an additional 48 h. The number of surviving cells was determined microscopically. Results are expressed as the relative growth in comparison with the control (T:C ratio %) and represent means ± SD of triplicate wells.

5-FU concentration (μg/ml)	Concentration of leucovorin (μg/ml)				
		0.1	0.5	2.5	125
	100 ± 10	106 ± 7	127 ± 18	118 ± 5	101 ± 1
0.01	101 ± 19	105 ± 17	105 ± 25	96 ± 2	ND[a]
0.05	79 ± 4	50 ± 4 [b,c]	55 ± 4 [b,d]	65 ± 11	ND
0.25	21 ± 4 [e]	17 ± 4 [e]	10 ± 0.3 [d,e]	9 ± 2 [d,e]	ND
1.25	2 ± 0.1 [e]	1.5 ± 0.1 [e]	1.6 ± 0.2 [e]	1 ± 0.2 [e]	ND

[a] ND, not done.
[b] $P < 0.02$ *versus* cells cultured with control medium.
[c] $P < 0.001$ *versus* cells cultured with the same concentration of 5-FU but without leucovorin.
[d] $P < 0.05$ *versus* cells cultured with the same concentration of 5-FU but without leucovorin.
[e] $P < 0.001$ *versus* cells cultured with control medium.

Table 2 *The maximum tolerated dose of 5-FU in combination with leucovorin*

5-FU and leucovorin were administered via the lateral tail vein on alternate days for a total of 4 injections. Body weights were determined 2 days after the last injection and survival rates 6 days later.

Dose of drug (mg/kg/injection)				
5-FU	Leucovorin	*n*	% weight loss	Survival rate
0	0	10	0.0	100
20	0	7	3.6	100
20	10	7	6.4	100
20	20	7	2.7	100
20	30	5	3.4	100
30	0	8	4.5	100
30	10	8	6.4	100
30	20	8	8.1	100
30	30	8	9.5	100
40	0	8	8.2	87.5
50	0	10	14.8	30

Table 3 *Effect of combination therapy with 5-FU and leucovorin on hepatic metastases of tumor H-59*

Animals were inoculated intrasplenically with 2×10^5 H-59 cells. The treatment was as described in "Materials and Methods." In all groups the incidence of liver metastases was 100%.

Treatment	*n*	No. of nodules/liver Median (range)	Net weight of liver (g)[a]
Saline	11	149 (35–350)	3.7 ± 1.4
Leucovorin	12	89 (22–350)	2.7 ± 1.3
5-FU	15	67 (6–350)	2.4 ± 1
5-FU + leucovorin	11	17 (5–112)[b,c]	1.9 ± 0.9

[a] Mean ± SD.
[b] $P < 0.001$ *versus* saline-treated group.
[c] $P < 0.002$ *versus* treatment with 5-FU or leucovorin only.

A lipophilic derivative of MDP, muramyl tripeptide conjugated to phosphatidylethanolamine (MTP-PE) was developed and reported to have an increased efficacy for macrophage activation (14, 26). Its beneficial effect *in vivo* in the growth inhibition of lung (27–29), liver (8), and peritoneal (30) tumors has been demonstrated.

Recently it has been shown (7) that a significant proportion of liposomes injected i.v. are retained in the liver (59% of injected liposomes as compared to 1% in the lung at 24 h following injection). These liposomes localize exclusively in the fixed macrophages of the liver (Kupffer cells).

Studies in several laboratories including our own have shown that liposome-encapsulated MDP can activate the tumoricidal potency of Kupffer cells and significantly reduce the growth of experimental hepatic metastases of liver metastatic tumors such as H-59 (7), M5076 (10), and B16-F1 melanoma (31). Liposome-encapsulated MDP appear, therefore, to provide a promising therapeutic agent for the treatment of liver metastases.

The objective of the present study was to investigate the therapeutic efficacy of a combination therapy based on the immunoadjuvant effect of liposomal MTP-PE on one hand and the cytotoxic effect of 5-FU and leucovorin on the other. These studies were prompted by recent reports on the beneficial effects of 5-FU and leucovorin in the management of advanced colorectal carcinoma for which the liver is the primary site of metastases (1). Since carcinoma H-59 can disseminate via lymphatic channels and/or the portal vein to form multiple metastases in the liver, it mimics the natural disease course of colorectal carcinoma. This pattern of metastasis together with our *in vitro* growth inhibition studies showing a biochemical modulation of the cytotoxic effect of 5-FU on H-59 cells by leucovorin provided a rationale for the use of this tumor line as a model system. Previous studies have already demonstrated that hepatic metastases of H-59 are sensitive to treatment with liposomal MDP (7).

The present results show that while treatment with either liposomal MTP-PE or with 5-FU and leucovorin was effective in reducing the number of hepatic metastases of carcinoma H-59, there was an additive effect when the two treatments were combined, resulting in a complete response in 30% of the animals and in significant reductions in the number and size of hepatic metastases in the remaining 70%.

Kleinerman *et al.* (32) administered liposomal MTP-PE with Adriamycin, cisplatin, or methotrexate to patients with osteosarcoma and demonstrated that these chemotherapeutic drugs when used as single agents did not suppress the ability of liposomal MTP-PE to activate the tumoricidal potential of macrophages, as tested in *in vitro* cytotoxicity assays. Our results suggest that when combined with 5-FU and leucovorin, the ability of liposomal MTP-PE to inhibit the growth of liver metastases is not diminished. Instead the chemotherapeutic drugs appear to supplement the tumoricidal effect of MTP-PE, resulting in a significantly improved outcome. While the precise mechanisms involved in this apparent synergistic effect remain to be elucidated, our findings are consistent with the postulate that tumor H-59 consists of subpopulations of cells with different sensitivities to chemotherapy with 5-FU and leucovorin. Liposomal MTP-PE-activated Kupffer cells may be effective in eradicating the drug-resistant subpopulations. Alternatively, it is conceivable that treatment with liposomal MTP-PE may also have a direct effect on the tumor cells, which can result in their increased sensitivity to 5-FU and leucovorin.

In phase I trials, liposomal MTP-PE has been administered i.v. to patients with advanced malignancies (26, 33, 34). Limited toxicity and a partial response in one case have been reported (26). Animal studies including our present results suggest that this drug may be most effective when treatment is

Table 4 *The effect of combination therapy with liposomal MTP-PE, 5-FU, and leucovorin on hepatic metastases of H-59*

Mice were inoculated with 2×10^5 H-59 cells using the intrasplenic route (7). Treatment was as described in "Materials and Methods." Mice were sacrificed 14 days following tumor cell inoculation.

Treatment	*n*	Incidence of metastasis (%)	No. of nodules/liver	Average size of nodules[a]	Net weight of liver (g)
Placebo + saline	12	100	162 (29–339)	1.45 ± 0.36	1.95 ± 0.87
MTP-PE + saline	11	100	34 (3–102)[b]	1.65 ± 0.25	2.40 ± 0.94
Placebo + 5-FU + leucovorin	10	100	57 (8–156)[c]	1.55 ± 0.28	1.64 ± 0.81
MTP-PE + 5-FU + leucovorin	10	70[d]	6 (0–63)[e]	0.87 ± 0.74[f,g]	1.46 ± 0.47[g]

[a] Mean ± SD (mm).
[b] $P < 0.005$ *versus* placebo + saline.
[c] $P < 0.05$ *versus* placebo + saline.
[d] $P < 0.05$ *versus* all other groups.
[e] $P < 0.001$ *versus* placebo + saline or placebo + 5-FU + leucovorin and $P < 0.025$ *versus* MTP-PE + saline.
[f] $P < 0.05$ *versus* placebo + saline or placebo + 5-FU + leucovorin.
[g] $P < 0.02$ *versus* MTP-PE + saline.

initiated prior to the establishment of hepatic micrometastases in the liver (7, 10). It appears therefore that neoadjuvant therapy followed by postsurgical treatment with chemotherapeutic agents may be required for an optimal response. Because treatment in the present study was administered in the absence of a growing local tumor, whereas in the clinical setting it would always be initiated only subsequent to the growth of a primary tumor, the efficacy of the combination chemoimmunotherapy protocol in the treatment of postsurgical hepatic metastases requires further confirmation.

In conclusion, the present results suggest that liposomal MTP-PE may be a useful addition to 5-FU and leucovorin in the treatment of colorectal carcinoma. Based on the success of the treatment schedule, it appears that liposomal MTP-PE may have an optimal effect when added to 5-FU and leucovorin in the neo- or postoperative adjuvant setting when hepatic micrometastases are likely to be present or forming. The present tumor model provides an experimental system for further optimization of the treatment protocol with the aim of achieving high and sustained response rates.

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