Therapy of Autochthonous Skin Cancers in Mice with Intravenously Injected Liposomes Containing Muramyltripeptide


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ABSTRACT

Liposomes containing the immunomodulator muramyltripeptide phosphatidylethanolamine (MTP-PE) or free saline were injected i.v. into BALB/c mice with autochthonous skin cancers induced by chronic exposure to ultraviolet irradiation. Treatment with liposomes containing MTP-PE produced significant retardation in the growth of the primary autochthonous skin cancers and significantly prolonged survival of the treated mice as compared with the saline-treated controls. Since ultraviolet-irradiated mice have a defect in immunological recognition of their autochthonous tumors, the therapeutic effects of liposomes containing MTP-PE are very encouraging. These results suggest that liposomes containing MTP-PE could be used for the treatment of patients with skin cancer or other malignant diseases.

INTRODUCTION

Rodent macrophages and human monocytes can be rendered tumoricidal subsequent to their interaction with liposomes containing various immunomodulators (1). The systemic administration of liposomes containing a wide variety of immune modulators, including MTP-PE, into rodents has been shown to activate macrophage tumoricidal activity and to treat established visceral metastases produced by transplantable tumors (1–13). Since the systemic activation of macrophages by agents encapsulated within liposomes is direct and independent of functional T-lymphocytes (14), these data raise the possibility that systemic macrophage activation could be beneficial for the treatment of cancer.

To date, the treatment of experimental tumors by liposomes or other biological response modifiers has been demonstrated only in normal rodents inoculated with transplantable tumors. Since the immunological and other characteristics of normal animals are not comparable to those of animals bearing induced or spontaneous autochthonous neoplasms (15, 16), we wished to determine whether similar therapeutic results could be obtained in primary tumor-bearing hosts. Because the treatment of disseminated autochthonous neoplasms is the major challenge facing the clinical oncologist, we examined whether the systemic administration of liposomes containing MTP-PE could have therapeutic benefits in mice with primary skin cancers induced by UV radiation, a carcinogen ubiquitous to humans (17). Chronic exposure of mice to UV radiation results in the development of single or multiple skin neoplasms (15, 16). These tumors are antigenic, and most are rejected when transplanted into normal syngeneic recipients. However, the tumors grow progressively in immunologically deficient recipients (18, 19). In addition, UV-induced neoplasms grow progressively in syngeneic mice that have been exposed to low-dose, nontumorigenic UV radiation (20). Studies of this phenomenon revealed that the inability of UV-irradiated mice to reject challenges with syngeneic, UV-induced tumors is due, at least in part, to the presence of tumor-specific suppressor T-cells in their lymphoid organs (21–25). The reactivity of the UV-induced suppressor cells is antigen restricted; these T-cells do not suppress the rejection of either allogeneic, UV-induced tumors, or syngeneic, chemically induced tumors (26). Furthermore, the immune response of UV-irradiated mice to a variety of exogenous antigens is normal, suggesting that the suppressor cells show selectivity for antigens expressed on syngeneic UV-induced tumors (21–26). The UV carcinogenesis model, therefore, presents a unique system for testing immunotherapeutic agents for treatment of autochthonous tumors (27, 28).

MATERIALS AND METHODS

Tumor Induction and Treatment Protocol. Specific pathogen-free female BALB/c mice, 5 weeks of age, were obtained from the Animal Production Area of the NCI-Frederick Cancer Research Facility. The UV radiation source was a bank of Westinghouse FS40 sunlamps that delivered an average dose rate of 2.8 J/m²/s over the wavelength range of 280–340 nm. Five mice were housed per cage, 20 cm below the sunlamps. Unshaved mice were irradiated for 2 h, 3 times per week. This UV radiation induced squamous cell carcinomas, fibrosarcomas, hemangiosarcomas, rhabdomyosarcomas, and other less well-differentiated sarcomas on the ears beginning at 22 weeks of the 30 weeks of radiation. When a palpable, primary skin tumor (2–3 mm in diameter) was detected, the animal was randomly assigned to either the control (HBSS) or liposome-MTP-PE treatment groups. Treatment consisted of biweekly i.v. injections of multimellar liposomes. Each treatment dose of liposomes consisted of 2.5 μmol of phospholipids and 12.5 μg of entrapped MTP-PE (27, 29). The liposomes were composed of phosphatidycholine and phosphatidylserine at a 7:3 ratio. These liposomes are efficiently engulfed by macrophages (30, 31) and have been shown to be nontoxic (32). Liposome therapy was continued for 4 weeks (total of 8 i.v. injections).

Phospholipids and Preparations of Liposomes. Chromatographically pure egg phosphatidycholine and brain phosphatidylserine were purchased from Avanti Polar Lipids, Birmingham, AL. The phospholipids were dissolved in chloroform and MTP-PE was dissolved in methanol:chloroform (1:2) and added to the phospholipids. After evapo-
ration of the solvents and appropriate drying procedures, HBSS was added and liposomes were produced by mechanical agitation. The concentration of MTP-PE in the liposomes was 5 μg MTP-PE/μmol phospholipids. The incorporation of MTP-PE into the phospholipid bilayer membrane was confirmed by the ability of antimonialylpeptide-phosphatidylethanolamine antibodies to specifically precipitate liposomes containing MTP-PE. Practically all (>98%) of the MTP-PE was incorporated into the liposomes as determined from studies with radiolabeled MTP-PE.

**RESULTS**

The therapeutic efficacy of the liposomal MTP-PE was determined by several parameters which included the growth kinetics of the primary skin tumor, the development of metastatic cancer, and the survival of mice. The combined survival data of 2 independent experiments are shown in Fig. 1. Mice treated with 8 i.v. injections of MLV containing MTP-PE had a significantly prolonged survival (33) as compared with the saline-treated controls (P = 0.017). For the saline-treated group, there were 15 mice per experiment with homogeneous survival patterns, as determined by the generalized Kruskal-Wallis test (P = 0.5). There were 10 mice in each experimental liposome-MTP-PE group, and their survival pattern was also homogeneous (P = 0.8). The median survival time for the HBSS-treated group was 17 weeks, which was extended to 30 weeks in the MLV-MTP-PE treatment group, nearly a 50% prolongation of the median survival. The injection of saline was utilized as the negative control. Data from two studies were pooled, one study had 10 mice while the second had 15 mice. Individually, the 2 studies were nearly significant 0.071 and 0.053, respectively; however, the number of animals per group was insufficient to achieve significance. A third experiment with 20 animals per group did itself achieve significance (P = 0.008). Thus, we pooled the data shown in Fig. 1.

Treatment of mice with liposomes containing MTP-PE was also significant in retarding the growth of the primary skin tumor.

The median tumor volume in liposome-MTP-PE-treated versus saline-treated mice is shown in Fig. 2 for the 10-week period following the detection of the tumor and the onset of treatment for the same animals shown in Fig. 1. Although the treatment (8 i.v. injections) was carried out for only 4 weeks, it is apparent that the highly significant decrease in tumor volume in liposome-MTP-PE-treated mice was maintained for a considerably longer period of time. Although we monitored the primary tumor size throughout the survival analysis, after 10 weeks this measurement became variable due to the death of some mice. Nonetheless, a clear and significant reduction in tumor burden was maintained as determined by the multivariate distribution free Wilcoxon test as proposed by Kozol et al. (33, 34). Thus, when tumor volumes were ranked at weeks 5, 9, and 17 (at 17 weeks 40% of animals in the saline group had died and subsequent analysis was deemed inappropriate), there was a significant reduction in tumor burden in the liposome-MTP-PE-treated group as compared to the saline-treated controls (P = 0.04, 0.03, and 0.02, respectively). Although the tumor burden in the treated animals was reduced as compared to the saline-treated animals, many of the animals treated with liposomes incorporating MTP-PE (4 weeks) did ultimately die of their primary tumor burden, although it took 23 weeks for the median tumor size in these animals to reach a size equal to that observed at 8 weeks in the saline-treated animals. By gross and histological analysis, all of the saline-treated animals died of either primary or metastatic tumor burden. In contrast, 4 of the 20 (20%) of the animals treated with liposomes incorporating MTP-PE were both grossly and histologically tumor free at necropsy. Presumably, these animals died of age. The Wilcoxon rank test demonstrated that the initial tumor burdens in the randomly assigned groups were statistically identical. Although there was no correlation between
survival and final tumor burden (presumably due to metastatic disease), there was a significant correlation between initial tumor volume and survival for both the saline ($P = 0.017$) and MLV-MTP-PE ($P = 0.021$) treatment groups, respectively. Nonetheless, there was a 2-fold prolongation of the median survival time in the MLV-MTP-PE group compared to the saline treatment group (Fig. 1). The i.v. injection of liposome-MTP-PE also reduced the incidence of metastasis. All moribund or dead mice were necropsied, and the organs were examined histologically for evidence of cancer spread. At necropsy, 30% of the saline-treated mice had histological evidence of pulmonary metastases, whereas pulmonary metastases were evident in only 10% of mice treated with liposome containing MTP-PE. Therapeutic effect within this limited sampling (20 animals) of animals treated with liposomes incorporating MTP-PE appeared to occur independent of the type of tumor present, although insufficient animals have been examined to date to undertake statistical analyses.

**DISCUSSION**

The lethality of most cancers can be attributed to their ability to spread to distant organs where they produce metastases. The outcome of the metastatic process is dependent upon many properties of the host and intrinsic properties of the tumor cells (35). To control metastasis by immunological means, the metastatic cells must be antigenic and the primary host must be capable of recognizing and destroying cancer cells. The fact that malignant neoplasms are heterogeneous and contain subpopulations of cells differing immunogenic and metastatic properties (34) calls for the reappraisal of the adequacy of many of the approaches to this problem. Moreover, the importance of the antigenic phenotype of tumor cells in the primary host can be questioned (15, 16, 21). Clearly, in the case of UV-irradiated mice, which are incapable of immunological rejection of UV-induced syngeneic tumors (18, 19, 20), the degree of tumor cell immunogenicity may be irrelevant. We have shown previously that the systemic activation of macrophages can be accomplished by the i.v. injection of liposome-MTP-PE in UV-irradiated mice (14), and that tumoricidal macrophages lyse tumorigenic cells regardless of their immunogenic or antigenic properties (1).

In previous studies, we have shown that the injection of liposomes incorporating MTP-PE results not only in the augmentation of alveolar macrophages but also in macrophages within pulmonary metastases (36). In other unpublished studies we have shown that the i.v. injection of liposomes incorporating MTP-PE resulted in the activation of macrophages within autochthonous UV-induced tumors. Although these results do not prove that macrophages are the effector cell involved in the therapeutic activity, they do suggest that they may indeed be one of the effector cells involved in the therapeutic activity associated with such treatment protocols. Primary skin cancers induced by UV light are more analogous to the clinical situation than are transplantable tumors. For this reason, the positive results we obtained by the systemic administration of liposome-MTP-PE are encouraging and suggest appropriate clinical testing in patients with skin cancer.

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**REFERENCES**


TREATMENT OF MURINE SKIN CANCER WITH MTP-PE


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