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RESEARCH PAPER

# Improved Efficacy of a Microencapsulated Macrophage Colony Stimulating Factor and Methotrexate in Melanoma

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**ABSTRACT**

*This study examines the effects of a combination therapy of both methotrexate (MTX) and albumin microspheres containing recombinant human macrophage colony-stimulating factor (rhM-CSF) in melanoma tumors. Melanoma tumors were induced in C57BL/6 male mice with subcutaneous injection of B-16 tumor cells. Therapy started once the tumor size reached 0.5 cm in diameter. Mice were divided into several groups, and dosing was carried out daily until death. Group I received MTX solution (2 mg/kg or 15 mg/kg), group II received rhM-CSF solution (100 μg), group III received albumin rhM-CSF microspheres (100 μg), and groups V–XV received different combinations of both agents daily. The weight, tumor size, and survival time (in days) were recorded. From the results, the control (no rhM-CSF administered) group survived for 11.8 ± 1.92 days, and the group that received MTX solution survived for 19.4 ± 5.03 days. However, the group that received both the MTX solution (15 mg/kg) and albumin rhM-CSF microspheres (100 μg/kg) demonstrated a significant increase ($p < .05$) in the survival time (30.4 ± 3.27 days). The concentrations of cytokines (tumor necrosis factor alpha [TNF-α] and interleukin-1 beta [IL-1β]) in the different treatment groups were monitored to determine the effect of rhM-CSF on the immune system. The TNF-α concentration was significantly higher in the group that received the combination therapy (204 ± 54.6 pg/ml) versus the control group (31.5 ± 7.02 pg/ml). The IL-1β concentration was significantly higher ($p < .05$) in the rhM-CSF microsphere (100 μg/kg)*

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*treated group (62 ± 17.2 pg/ml) versus the rhM-CSF solution (29.1 ± 8.7 pg/cc).*
***Key Words:*** *B-16 malignant melanoma; IL-1β; Macrophages; Methotrexate; Poly-lactic-*co*-glycolic acid; Recombinant human macrophage colony-stimulating factor; TNF-α.*

## INTRODUCTION

Actively proliferating tissues such as malignant cells, bone marrow, fetal cells, and cells of the urinary bladder are in general more sensitive to methotrexate (MTX) (1), which is an antimetabolite used in the treatment of certain neoplastic diseases (2). Although MTX is a potent anticancer agent, it tends to cause very serious side effects (3). Furthermore, the major side effect observed with MTX therapy is a suppressed immune system in cancer patients taking it (4). As a result of the downregulation of the immune system, cancer patients are constantly in danger of life-threatening infections. In fact, a major cause of treatment-related deaths in cancer patients is infection. Myelosuppression is a common and serious complication observed with most chemotherapeutic agents (5).

Recombinant human macrophage colony-stimulating factor (rhM-CSF) stimulates the macrophage proliferation and colony formation by activating the stem cells (6,7). The recent molecular cloning of colony-stimulating factor may make it possible to accelerate hematopoietic recovery during chemotherapy and thereby reduce therapy-related toxicity (8). In addition to its macrophage colony inducing activity, other effects of rhM-CSF include stimulating the proliferation of isolated macrophages, enhancing macrophage antibody-dependent cell-mediated cytotoxicity, priming and enhancing macrophages in their ability to kill tumor cells and microorganisms, regulating the release of cytokines and other inflammatory modulators from macrophages, stimulating pinocytosis, and supporting osteoclast differentiation (9–12). Unfortunately, exogenous M-CSF has a short half-life (14 to 18 min) and is eliminated rapidly (13). Hence, in an attempt to enhance the immune system in cancer patients with rhM-CSF, a high concentration of this agent is required, which can be toxic to the patient. To decrease toxicity and increase drug concentration and increase the half-life, the study of drug delivery systems via a carrier system has attracted great interest in recent years (14,15).

A number of efforts to develop more rational approaches to specific cancer therapy are based on the concept of drug carrier targeting. To determine the efficacy of microencapsulated rhM-CSF, B-16 malignant melanoma mice were treated with a combination chemotherapy of MTX and rhM-CSF albumin microspheres. The survival rates of the treated animals were monitored. The plasma concentrations of tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) were also monitored to determine the effect of rhM-CSF on cytokine concentrations.

## MATERIALS AND METHODS

### Chemicals and Reagents

The MTX was a gift from Lederle (Pearl River, NY). The rhM-CSF was a gift from Dr. S. Clark of the Genetics Institute (Cambridge, MA). The TNF-α, IL-1β, and antibodies for the enzyme-linked immunosorbent assay (ELISA) were purchased from R&D Systems (Minneapolis, MN). Polystyrene micro-ELISA plates were purchased form Fisher Scientific (Pittsburgh, PA). All other chemicals and reagents for the ELISA were purchased from Sigma Chemical Company (St. Louis, MO).

### Animals

Male C57BL/6 mice (18–22 grams, 6 weeks old) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and used in all experiments. They were housed in individual cages and allowed free access to food and water.

### Formulation of Albumin Microspheres Containing rhM-CSF

Albumin microspheres containing rhM-CSF were prepared using a method reported earlier (16) by polymerization from a water-in-oil emulsion. The microspheres were collected by centrifugation at 10,000 rpm for 20 min and washed with ethanol. The size of the albumin microspheres was determined under light microscope and electron microscope (size = 2–4 μm).

### Combination Therapy of MTX and rhM CSF Microspheres in the Treatment of Malignant Melanoma

Male C57BL/6 mice weighing approximately 18–20 g were anesthetized with ether and were injected sub-

cutaneously with B-16 tumor cells ($1 \times 10^6$ cells) into the right shoulder. Their weight, tumor size, and survival time (days) were measured daily. The tumor growth was determined by measuring the long axis of the tumor daily. The dosing began once the tumor size reached 0.5 cm in diameter. The animals were divided into control and treatment groups with similar weights and tumor sizes ($n = 6$). The treated animals received the agents as described below; the controls received phosphate buffered saline (PBS) (pH 7.4) or blank albumin microspheres. The drugs, methods of administration, and dosing schedules were as follows: the MTX solution (2 mg/kg or 15 mg/kg) was administered subcutaneously daily or every 3 days, the rhM-CSF solution (10 μg/kg or 100 μg/kg) was administered i.p. daily; the MTX (2 mg/kg or 15 mg/kg) and the rhM-CSF microspheres (10 μg/kg or 100 μg/kg) were administered in a combination therapy.

### Determination of TNF-α and IL-1β Concentration in Mice with Malignant Melanoma

Male C57BL/6 mice weighing approximately 18 g were anesthetized with ether and were injected with B-16 tumor cells. Their weights and tumor sizes were measured daily. The dosing began once the tumor size reached 0.5 cm in diameter. The animals were divided into control and treatment groups of similar weight and tumor size ($n = 6$). The treated animals received the agents as described in the above experiment. The study was terminated 10 days posttherapy, and plasma from each group was obtained. The concentrations of TNF-α and IL-1β were analyzed by ELISA as described next.

### Enzyme-Linked Immunosorbent Assay for TNF-α and IL-1β

All assays were carried out in duplicate in a 96-well polystyrene microtiter plate.

#### TNF-α Assay

The assay for TNF-α was carried out as described before (17). Briefly, ELISA microtiter plates were coated with 100 μl of TNF-α standards (0, 0.1, 1, 10, 25, 100, 500, and 1000 ng/ml TNF-α) or plasma samples in binding buffer (100 mM carbonate buffer, pH 9.6). The plates were immediately treated with 100 μL of coating buffer (0.5% albumin in PBS) and incubated overnight at 4°C. The plates were washed three times with washing buffer (PBS pH 7.4 containing 0.05% Tween), and 100 μl of the first antibody to TNF-α (goat antimurine immunoglobulin G [IgG] TNF-α antibody; dilution 1:4000, R&D Systems, Minneapolis, MN) were added into each well and incubated for 1 hr at 37°C. After washing the plates with washing buffer, 100 μl of the second antibody (rabbit antigoat IgG antibody; dilution 1:1000, Bio-Rad, Melville, NY), covalently linked to horseradish peroxidase (HRP), were added to each well and incubated for 1 hr at 37°C. The plates were washed, and bound TNF-α was detected by adding 100 μl of *o*-phenylenediamine dihydroxy-hydrochloride (1 mg/ml in phosphate citrate buffer, pH 5) and hydrogen peroxide (0.06%). The reaction was stopped with 50 μL of $H_2SO_4$ (4 N), and the absorbance of each well was read at 450 nM in a double-beam microplate reader (Bio-Rad). The concentration of TNF-α in the samples was calculated using the standard curve.

The ELISA method for IL-1β was a direct assay similar to the one mentioned above; however, the first antibody was goat antimurine IL-1β antibody (R&D Systems), and the second antibody was rabbit antigoat IgG covalently linked to HRP (18).

## RESULTS AND DISCUSSION

The rhM-CSF albumin microspheres, prepared by the modified polymerization method from a water-in-oil emulsion, were smooth and spherical in shape. An electron micrograph of the microspheres containing rhM-CSF is presented in Fig. 1. The size distribution measurements by electron micrographs showed that more than

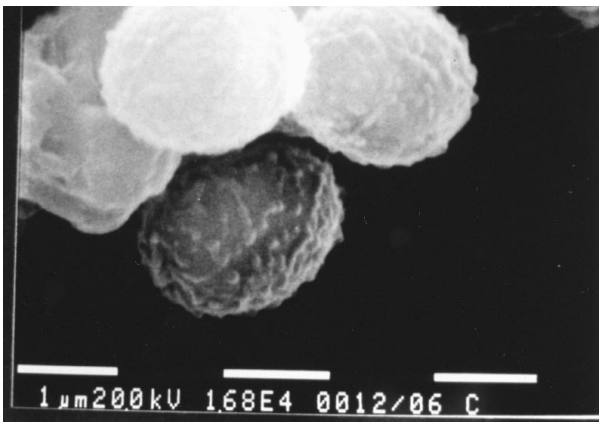


**Figure 1.** Scanning electron micrographs (×18,000) of rhM-CSF albumin microspheres showing spherical and porous surface characteristics.

90% of the microspheres had diameters in the range of 2–4 μm.

### Combination Therapy of MTX and rhM-CSF Microspheres in the Treatment of Malignant Melanoma

The mean survival time (in days) and the mean tumor size of each treated group ($N = 6$) of malignant melanoma mice are listed in Table 1. The mean survival time for mice treated with 2 mg/kg of MTX solution was 19.4 ± 5.02 days, and the mean survival time for mice treated with 15 mg/kg of MTX solution was 19.6 ± 3.84 days. No significant differences ($p > .05$) were observed in groups that received subcutaneous injections of 2 mg/kg or 15 mg/kg of MTX solution daily. The survival time for the group treated with 15 mg/kg of MTX solution every 3 days was 14.2 ± 1.8 days versus 12.8 ± 1.8 days for the groups treated with 2 mg/kg/day of the MTX solution.

Figure 2 represents a dose response treatment of rhM-CSF solution versus rhM-CSF microspheres at doses of 10 and 100 μg/kg in malignant melanoma mice. A significant difference ($p < .05$) was observed in the survival time between the groups treated with rhM-CSF microspheres and rhM-CSF solution at 10 μg/kg. The mean survival time in groups treated with rhM-CSF microsphere (10 μg/kg) was 20.8 ± 4 days versus 14.2 ± 3.7 days for groups treated with rhM-CSF solution (10 μg/kg). No significant difference was observed in the group treated with 100 μg/kg ($p > 0.05$).

Figure 3 illustrates the effects of microencapsulated versus free solutions of rhM-CSF with MTX in combination therapy of malignant melanoma in mice. The combination therapy with the microencapsulated rhM-CSF (100 μg/kg) with MTX (15 mg/kg) showed a significant increase in the survival time (30.4 ± 3.27 days) compared to that of the free solution of rhM-CSF with MTX (21.0 ± 2.2 days) at the same dose ($p < .05$).

### Determination of TNF-α and IL-1β Concentration in Mice with Malignant Melanoma

Table 2 lists the concentration of IL-1β in mice with malignant melanoma. The IL-1β concentration in the

***Table 1***

*Combined Effect of rhM-CSF and MTX in Mice with Melanoma*

| Treatment groups ($N = 6$) | Survival Time (Days) | SD | Tumor Size (cm) | SD |
|---|---|---|---|---|
| Control | 11.8 | 1.9 | 2.56 | 0.27 |
| Albumin blank MC | 12.4 | 2.30 | 2.34 | 0.18 |
| MTX solution (2 mg/kg daily) | 19.4* | 5.03 | 2.62 | 0.18 |
| MTX solution (15 mg/kg daily) | 19.6* | 3.84 | 2.52 | 0.19 |
| MTX solution (2 mg/kg, Q 3 days) | 12.8 | 1.6 | 2.56 | 0.19 |
| MTX solution (15 mg/kg, Q 3 days) | 14.2 | 1.8 | 2.66 | 0.13 |
| MCSF solution (10 μg/kg) | 14.2 | 3.7 | 2.58 | 0.19 |
| MCSF solution (100 μg/kg) | 18.8* | 2.5 | 2.66 | 0.14 |
| MCSF MC (10 μg/kg) | 20.8* | 4.0 | 2.64 | 0.19 |
| MCSF MC (100 μg/kg) | 22.0* | 5.7 | 2.74 | 0.13 |
| Blank albumin MC (MCSF MC 100 μg/kg) | 19.8* | 1.9 | 2.48 | 0.35 |
| MCSF solution (100 μg/kg), MTX solution (15 mg/kg) | 21.0* | 2.20 | 2.72 | 0.04 |
| MCSF solution (10 μg/kg), MTX solution (15 mg/kg) | 17.4* | 1.14 | 2.60 | 0.21 |
| MCSF MC (100 μg/kg) MTX solution (15 mg/kg) | 30.4* | 3.27 | 2.76 | 0.16 |
| MCSF MC (10 μg/kg), MTX solution (15 mg/kg Q3 days) | 23.4* | 2.40 | 2.74 | 0.11 |

Data represented as mean (± standard deviation) for tumor size and number of days animals survived.
Q 3 days = every three days injection; MCSF MC = rhM-CSF microspheres.
*$p < .05$ from control.

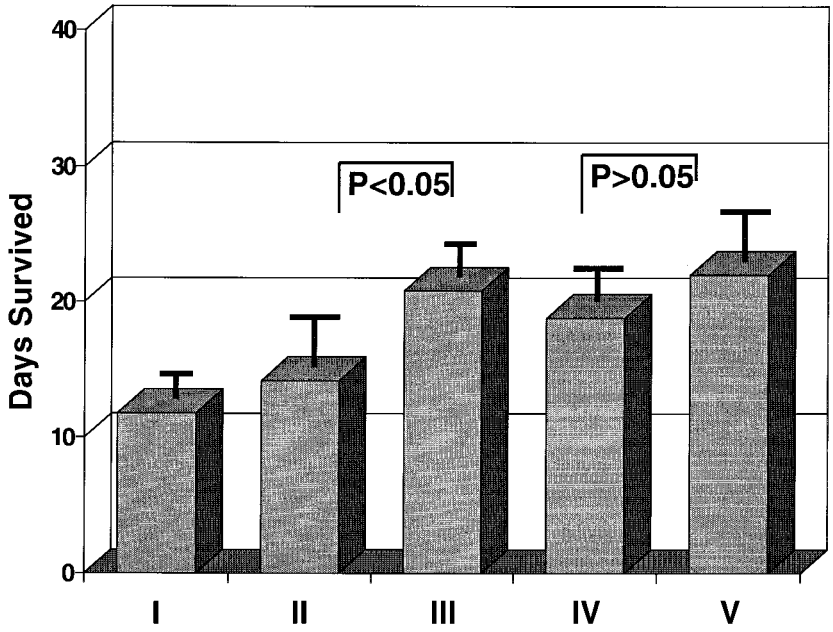


**Figure 2.** Comparison of the effect of rhM-CSF solution versus rhM-CSF microspheres (intraperitoneal daily) in the treatment of malignant melanoma. Data represented as mean days survived (± standard deviation). (Statistical significance $p < .05$, analysis of variance with multiple comparison Tukey test). Group I, control; group II, MCSF solution, 10 μg/kg; group III, MCSF microspheres, 10 μg/kg; group IV, MCSF solution, 100 μg/kg; group V, MCSF microspheres, 100 μg/kg).

control group (no treatment ) was 18.5 ± 4.8 pg/ml. The concentration in the group treated with blank microspheres was 22.0 ± 1.1 pg/ml. The IL-1β concentration was significantly higher ($p < .05$) in the group treated with rhM-CSF microspheres (100 μg/kg) (62 ± 17.2 pg/ml) versus the group treated with rhM-CSF solution (29.1 ± 8.7).

Table 2 also lists the concentrations of TNF-α in mice with malignant melanoma. The TNF-α concentration in the control group was 31.5 ± 7.02 pg/ml. The TNF-α concentration in the group treated with rhM-CSF (100 μg/kg) solution was 60.0 ± 20.1 pg/ml. The TNF-α concentration in the group treated with rhM-CSF (100 μg/ml) microspheres was 105 ± 28 pg/ml. There was a significant increase ($p < .05$) in the TNF-α concentration (152 ± 41 pg/ml) when the animals were treated with the combination of methotrexate (15 mg/kg) and rhM-CSF microspheres (100 μg/kg) versus the control (18.5 ± 4.8 pg/ml).

## CONCLUSION

The current study was performed to determine the effectiveness of microencapsulation therapy on the inhibition of tumor growth in animals with malignant melanoma. The combination therapy of MTX with rhM-CSF in the microencapsulated form enhanced the survival rate of tumor-bearing mice significantly compared to the group receiving treatment without microencapsulation ($p < .05$). These results suggested that combination therapy of microencapsulated rhM-CSF and MTX solution was more effective in regard to survival rate than the solution of rhM-CSF and MTX. The present data suggest that the microencapsulated rhM-CSF was probably protected from degradation, with an increased half-life and a sustained release profile. This could account for the longer survival time in the tumor-bearing animals. On the other hand, the short half-life and quick elimination of free solutions of rhM-CSF ($T_{1/2}$ = 14 to 18 min) could account for the shorter survival time.

Our results are similar to those of Sampson-Johannes and Carlino (19), who also observed that the tumoricidal activity of macrophages was enhanced on pretreatment with rhM-CSF solution. Treatment with rhM-CSF stimulated murine macrophages to secrete a variety of agents and cytokines and resulted in an enhanced expression of Fc receptors on the macrophages (20,21).

Tabata and Ikada (22) have extensively studied the

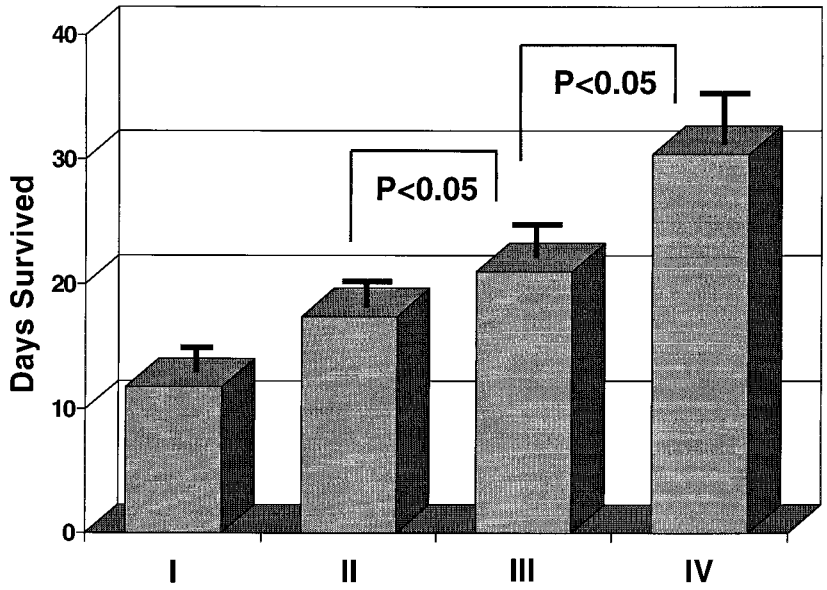


**Figure 3.** Comparison of the effect of rhM-CSF (intraperitoneal daily) and MTX (subcutaneous daily) in the treatment of malignant melanoma. Data represented as mean days survived (± standard deviation). (Statistical significance $p < .05$, analysis of variance with multiple comparison Tukey test). Group I, control; group II, MCSF solution, 10 μg/kg and methotrexate 15 mg/kg; group III, MCSF solution 100 μg/kg and methotrexate 15 mg/kg; group IV, MCSF microspheres, 100 μg/kg and methotrexate 15 mg/kg.

***Table 2***

*IL-1β and TNF-α Concentrations (pg/ml) in Mice with Malignant Melanoma Treated with Different Regimens (*N = *6)*

| Groups | Mean IL-1β (pg/ml) | SD | Mean TNF-α (pg/ml) | SD |
|---|---|---|---|---|
| Control | 18.5 | 4.8 | 31.5 | 7.02 |
| Blank albumin | 22.0 | 2.1 | 38.8 | 10.6 |
| MTX solution (15 mg/kg) | 28.2 | 11.1 | 37.6 | 6.02 |
| MCSF solution (100 μg/kg, Q 3 days) | 64.3* | 16.5 | 89.2* | 29.2 |
| MCSF albumin MC (100 μg/kg, Q 3 days) | 30.4 | 8.1 | 173* | 71.1 |
| MCSF solution (10 μg/kg) | 37.1 | 16.6 | 64.7* | 19.7 |
| MCSF solution (100 μg/kg) | 29.1 | 8.7 | 60.6* | 20.1 |
| MCSF albumin MC (10 μg/kg) | 28.1 | 4.7 | 50.6* | 7.5 |
| MCSF albumin MC (100 μg/kg) | 62* | 17.2 | 105* | 28 |
| MCSF solution (100 μg/kg), MTX solution (15 mg/kg) | 37.3 | 14.1 | 115* | 42 |
| MCSF solution (10 μg/kg), MTX solution (15 mg/kg) | 24.1 | 1.38 | 39.6* | 3.1 |
| MCSF MC (100 μg/kg), MTX solution (15 mg/kg) | 37.3 | 15.5 | 152* | 41 |

Q 3 days = every 3 days injection; MCSF MC = M-CSF microspheres.
*$p < .05$ from control.

mechanisms of activation by various microsphere-encapsulated agents such as macrophage activating factor and interferon encapsulated with gelatin and liposomes. They concluded that immunomodulators are not efficient in vivo because they are cleared rapidly. However, encapsulated agents were more efficient in activating macrophages in our studies because they were selectively taken up rapidly by the phagocytic cells (23–29). In addition, Tabata and Ikada (30) suggested that participation of macrophage surface receptors is not required, and macrophage activation results from the interaction of macrophage activating factor with intracellular targets.

Both the TNF-α and IL-1β are good indicators of macrophage activation (31,32). By administering microencapsulated rhM-CSF along with MTX, the survival time of the tumor-bearing animals increased along with the TNF-α levels and IL-1β levels. The increase in survival rate could be explained by the fact that rhM-CSF may be activating the immune system and allowing the activated macrophages to prevent the growth of tumor cells. These studies clearly indicate that microencapsulation of agents may be the route of choice during chemotherapy.

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