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Potentiation of antitumor effects of tumor necrosis factor α and interferon γ by macrophage-colony-stimulating factor in a MmB16 melanoma model in mice

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Abstract The efficacy of systemic infusion of recombinant human macrophage-colony-stimulating factor (M-CSF) in combination with local treatment with human recombinant tumor necrosis factor (TNF) α and mouse recombinant interferon (IFN) γ was studied in vivo on a subclone of B16 melanoma (MmB16) in mice. Short-term intravenous administration of M-CSF at a dose of 10^6 units daily had no antitumor effect in vivo. Similarly, local treatment of tumor with TNFα (5 μg daily) did not produce any therapeutic effect. However, simultaneous administration of the same dose of TNFα with IFNγ (1000 units daily) resulted in a synergistic effects manifested by the retardation of tumor growth. Addition of systemic infusion of M-CSF to the local therapy with TNFα and IFNγ induced further augmentation of antitumor efficacy and delayed progression of MmB16 melanoma. The strengthened antitumor effect of combination therapy including M-CSF, TNFα and IFNγ was most probably due to the increased release of monocytes from the bone marrow, their recruitment into the site of tumor growth and subsequent local stimulation of their antitumor activity.

Key words Tumor necrosis factor α · Interferon γ · Macrophage-colony-stimulating factor · Tumor immunotherapy

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Introduction

When activated, cells of the monocyte/macrophage lineage release soluble factors toxic to tumor cells and directly kill tumor cells, thus representing an important component of the host defense against malignancy [8, 12, 24]. The activation of monocytes and macrophages is achieved by their interactions with cytokines, which are released during immune reactions, or results from a direct response of these cells to microorganisms or their products [11, 12, 18, 27]. The list of cytokines activating monocytes and/or macrophages includes, among others, tumor necrosis factors (TNF) α and β, interferons, chemokines, and growth factors for bone marrow cells (colony-stimulating factors) [4, 8, 26, 27]. While the synergistic antitumor effect of interferon (IFN) γ and TNFα is well known [8, 14, 18], the role of macrophage-colony-stimulating factor (M-CSF) and its interaction with other cytokines participating in defense against neoplasia has been less explored (for review see [16]). This growth factor, originally described by its ability to induce the proliferation and differentiation of progenitor cells of the monocyte/macrophage lineage [10], acts also on mature cells and stimulates or costimulates them to produce and release a number of soluble factors, including interleukin-1 (IL-1), granulocyte-colony-stimulating factor (G-CSF), TNF, IFNγ, plasminogen activator, and oxygen metabolites [21, 33, 34, 35]. Furthermore, M-CSF has been shown to be a potent chemoatractant for monocytes [13, 32], and pretreatment of monocytes with M-CSF increases their tumoricidal activity under stimulation by various endo- and exogenous activators in vitro [26].

The systemic administration of M-CSF alone, even at high doses, had either weak or no effect on established tumors and metastasis formation [3, 22, 23]. However, by its ability to increase greatly the blood monocyte number [19, 30] and by increasing

susceptibility of these cells to other activation signals [26], M-CSF is an agent of potential use in combination therapy.

In our study we attempted to use M-CSF in conjunction with two cytokines of proven antitumor effect, namely TNFα and IFNγ, in the hope of obtaining an increase in antitumor efficacy of these cytokines in the treatment of locally growing MmB16 melanoma in mice.

Materials and methods

Mice

Male (C57BL/6 × DBA/2) F_1 mice, hereafter called B6D2F1, at 10–12 weeks of age, were used in experiments. Mice were obtained from a local animal facility and were kept in conventional conditions with full access to food and water during experiments.

Tumor

MmB16 melanoma, a metastasizing subclone of B16 melanoma adapted to growth in vitro, was kindly provided by Prof. C. Radzikowski from the Institute of Immunology and Experimental Medicine, Wroclaw, Poland. The original B16 melanoma was obtained from the National Cancer Institute, Bethesda, Maryland. Cells were maintained in RPMI-1640 medium (Gibco BRL, Paisley, Scotland) supplemented with antibiotics, 2-mercaptoethanol (50 μM), L-glutamine (2 mM) and 10% fetal calf serum (all from Gibco BRL) (culture medium) and passaged every 3–4 days after a short trypsinization with trypsin/EDTA (Gibco BRL). For in vivo experiments, MmB16 cells were washed twice in RPMI-1640 medium and adjusted to a concentration of 5×10^7 cells/ml medium. Mice were inoculated with 10^6 melanoma cells in 20 μl medium into the footpad of the right hind limb.

Cytokines

Recombinant human macrophage-colony-stimulating factor was kindly provided by Cetus Corp. (Emeryville, Calif.). This highly purified protein was produced in *Escherichia coli* as described [15] and had a specific activity of 1.12×10^7 units/mg protein. Murine recombinant IFNγ (specific activity 1×10^7 units/mg) was purchased from Genzyme Corporation. Recombinant human TNFα (specific activity 5×10^7 units/mg) was kindly provided by Knoll AG/BASF, Ludwigshafen, Germany.

MTT assay

The cytotoxic effect of TNFα and IFNγ on melanoma cells in vitro was tested in a standard MTT assay. Tumor cells were dispensed in a 96-well flat-bottomed microtiter plate (Costar) at a concentration of 2.5×10^3 cells $(100\ \mu l)^{-1}$ well^{-1}. Plates were incubated overnight at 37° C in 5% CO_2 and then serial dilutions of TNFα and IFNγ were added in triplicate (50 μl) to a final volume of 200 μl. After an incubation period of 68 h, 25 μl 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) (Sigma Chemicals, St. Louis, Mo.) solution (2.5 mg/ml) was added to each well. The plates were centrifuged 4 h later (350 *g*/10 min) and 200 μl supernatant was carefully removed from wells and replaced with 200 μl acid dimethylsulfoxide. Complete solubilization of formazan crystals was achieved by repeated (three or four times) pipetting of the solution. The plates were read on an enzyme-linked immunosorbent assay (ELISA) reader (Organon Technika reader 510), using a 540-nm filter. The means and standard errors of the mean were determined for triplicate samples. The cytotoxic effect of TNFα and/or IFNγ was expressed as the relative viability (% of control) and was calculated as follows:

$$\text{relative viability (\%)} = \frac{\text{experimental absorbance} - \text{background absorbance}}{\text{absorbance of untreated controls} - \text{background absorbance}} \times 100$$

In vitro assay for macrophage cytotoxic activity

Peritoneal exudate cells were obtained by peritoneal lavage from B6D2F1 mice 4 days after an intraperitoneal injection of 2 ml 2.4% thioglycolate broth (Difco). Lavages were performed with chilled Dulbecco's phosphate-buffered saline (PBS) (Gibco BRL) containing 10 U/ml heparin. Cells were washed twice in Dulbecco's PBS, counted and resuspended in culture medium at the concentration of 10^6 cells/ml. Macrophage monolayers were prepared by dispensing 2×10^5 thioglycolate-elicited peritoneal cells in 0.2 ml into wells of a 96-well flat-bottomed microtiter plate (Costar). After a 2-h incubation at 37° C (5% CO_2 environment), nonadherent cells were removed by gentle washing (three times) with warm RPMI-1640 medium. Each well was then filled with 100 μl culture medium and aliquots of serial dilutions of M-CSF, TNFα and/or IFNγ were added to a final volume of 200 μl. After the incubation period of 24 h (37° C, 5% CO_2 in humidified atmosphere) macrophage cultures were thoroughly washed in warm RPMI-1640 medium and 5×10^3 MmB16 cells were added to each well. The cultures were incubated for 72 h. Four hours before the end of the test, MTT solution was added to each well and the next steps were performed as in the MTT assay described above. Cytocidal/cytostatic effects of macrophages on melanoma cells were expressed as the relative viability of tumor cells (% of control), calculated as follows: relative viability = $[(A_{exp} - A_{min})/(A_{max} - A_{min})] \times 100$, where A_{exp} is the absorbance in treated macrophage cultures with MmB16 melanoma cells, A_{min} the absorbance in cultures of macrophages alone, and A_{max} the absorbance in untreated macrophage cultures with MmB16 melanoma cells.

Treatment and monitoring

In the experiment in which effects of TNFα and/or IFNγ were studied in vivo, melanoma-bearing mice were treated intratumorally (i.t.) with either agent alone or in combination for 10 consecutive days, starting from day 7 following inoculation of 10^6 melanoma cells. Cytokines were given at doses 5 μg (TNFα) and/or 1000 U (IFNγ) in 20 μl 0.1% bovine serum albumin (BSA)/saline. Therapeutic efficacy of systemic administration of M-CSF either alone or in combination with local i.t. injections of TNFα and IFNγ was studied in an experiment in which M-CSF was given intravenously (via plexus retroorbitalis) at a daily dose of 10^6 U/0.1 ml 0.1% BSA/saline on days 6–9 and TNFα/IFNγ treatment (daily doses 5 μg and 1000 U respectively) was initiated on day 7 and continued for 1 week. Each injection of M-CSF and of cytokines was paralleled by the injection of 0.1% BSA/saline into mice from the remaining groups. Local tumor growth was determined by measuring footpad diameter with calipers every 2 days, starting on day 5 or 7 following inoculation of tumor cells. Tumor size was calculated according to the formula: tumor diameter = (diameter of footpad with growing tumor) − (diameter of non-treated contralateral footpad).

Statistical analysis

Data are expressed as means ± SE. Differences in tumor diameter in experiments in vivo and differences between samples in tests in vitro were analyzed for significance by Student's *t*-test (two-tailed). The differences in survival time were calculated by analysis of variance.

Results

Effects of TNFα and IFNγ on MmB16 melanoma cells in vitro

The response of MmB16 melanoma cells to different concentrations of TNFα and IFNγ in cultures in vitro is shown in Fig. 1. Continuous exposure of melanoma cells for 72 h to TNFα resulted in a dose-dependent cytotoxic effect. Treatment of melanoma cells with TNFα in combination with IFNγ resulted in an enhanced cytotoxic effect. Since the dose/response curves of TNFα plotted against different doses of IFNγ were parallel, this effect seemed to result from additive rather than the synergistic interactions between TNFα and IFNγ.

Activation of macrophages by TNFα, IFNγ and M-CSF in vitro

Pretreatment of macrophages for 24 h with TNFα alone did not influence the ability of these cells to kill melanoma cells when TNFα was used at a very high concentration (1 μg/ml) (Fig. 2). In contrast, incubation of macrophages over the same period with IFNγ induced strong antitumor cytotoxic activity. The most effective, synergistic activation of macrophages was demonstrated when both cytokines were added together to the cultures, causing, for instance, 47% relative viability of melanoma cells when macrophages were activated with 10 U/ml IFNγ and 1000 ng/ml TNFα ($P < 0.05$ versus IFNγ alone: 75% relative viability; $P < 0.01$ versus TNFα alone: 122% relative viability; $P < 0.01$ versus control) or 14% relative viability when macrophages were precultured with 50 U/ml IFNγ and 1000 ng/ml TNFα ($P < 0.05$ versus IFNγ alone: 33% relative viability; $P < 0.001$ versus TNFα alone: 122% relative viability; $P < 0.001$ versus control). M-CSF was neither able to activate cytotoxic activity of macrophages nor influence the activation state of macrophages stimulated with TNFα and/or IFNγ (Fig. 2).

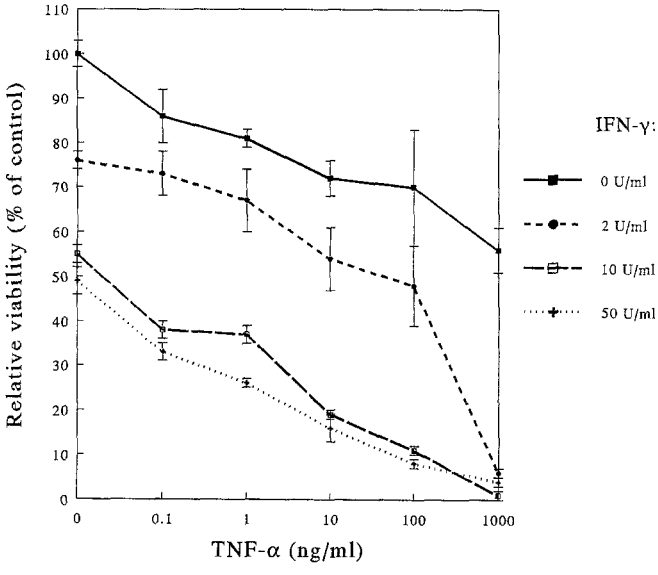

Fig. 1 Effects of tumor necrosis factor(TNF)α and interferon(IFN)γ on MmB16 melanoma cells in vitro. One-day-old monolayers of melanoma cells were exposed for 72 h to various concentrations of TNFα (0.1–1000 ng/ml) and IFNγ (2–50 U/ml). Cytotoxic effect, expressed as relative viability (% of untreated control), was tested in a standard MTT assay (see Materials and methods)

Effects of TNFα, IFNγ and M-CSF on growth of MmB16 melanoma in mice

In the initial in vivo experiment we examined the effect of TNFα and IFNγ, used either alone or in combination, on MmB16 melanoma development. Tumor immunotherapy was started on day 7 following inoculation of 10^6 melanoma cells and was continued for 10 consecutive days. As shown in Fig. 3, repeated i.t. injections of 5 μg TNFα did not produce any therapeutic effects and tumor progression was comparable to that in controls. Intratumoral injections of IFNγ (1000 U daily) caused a moderate therapeutic effect, which was manifested in a delay of tumor growth. The best result was obtained in the group of mice treated with both cytokines. Combination treatment with TNFα and IFNγ, as also reported previously by others [29] (for review see [17]), produced synergistic antitumor effects and retardation of the tumor growth (Fig. 3).

In the next step, systemic therapy with M-CSF was added to the local treatment of MmB16 melanoma with TNFα and IFNγ. In this protocol, intravenous infusion of 10^6 U M-CSF for 4 days was combined with seven daily injections of TNFα and IFNγ (5 μg and 1000 U respectively). Administration of M-CSF was started on day 6 and the therapy with TNFα and IFNγ was initiated 1 day later, when a significant response in monocyte number in blood was expected [30]. As presented in Fig. 4, the inhibitory effect of TNFα and IFNγ on MmB16 melanoma growth was potentiated by a short-term course of M-CSF therapy. For example, combination treatment with M-CSF, TNFα and IFNγ reduced tumor diameter on day 19 to

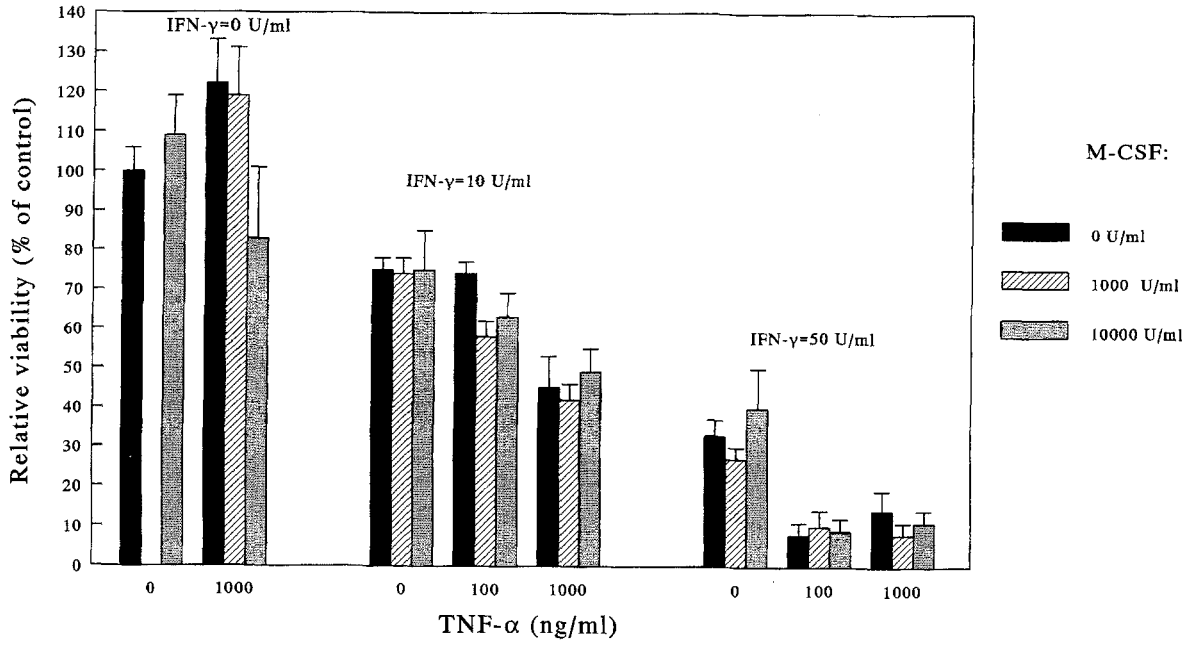

Fig. 2 Effects of pretreatment with macrophage-colony-stimulating factor (M-CSF), TNFα and IFNγ on antitumor activation of macrophages in vitro. Thioglycolate-elicited peritoneal macrophages were cultured, alone or in combination, with M-CSF (10^3 or 10^4 U/ml), TNFα (100 or 1000 ng/ml) and/or IFNγ (10 or 50 U/ml) for 24 h followed by washing and addition of 5×10^3 MmB16 melanoma cells/well. After 3 days, cytocidal/cytostatic effect of macrophages, expressed as relative viability (% of untreated control), was tested in an MTT assay

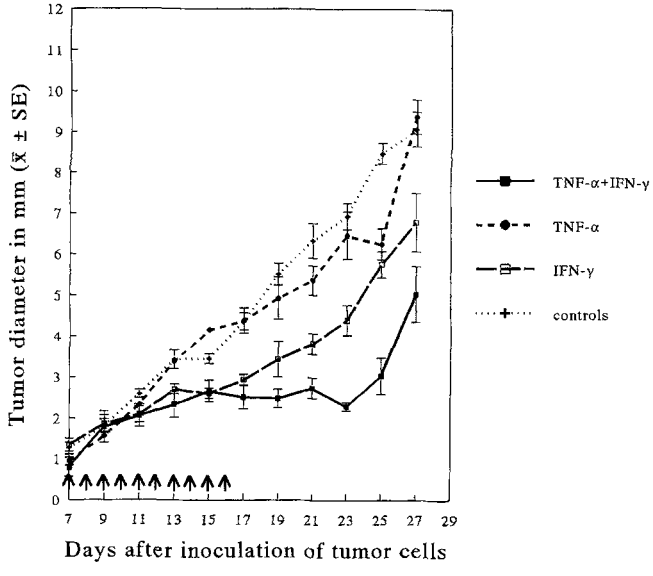

Fig. 3 The effect of treatment wth TNFα and/or IFNγ on MmB16 melanoma growth in B6D2F1 mice. Mice were inoculated with 10^6 melanoma cells into the footpad of the right hind limb and treated with ten intratumoral injections (days 7–16, *arrows*) of TNFα (5 μg/injection), IFNγ (1000 U/injection) or TNFα in combination with IFNγ (the same dosages). Groups contained between three and seven mice. TNFα + IFNγ group versus controls: significant difference in tumor diameter on days 13, 15 ($P < 0.05$) and 17–27 ($P < 0.01$); TNFα group versus controls: significant difference on days 15 and 25 ($P < 0.01$); IFNγ group versus controls: significant difference on days 13 ($P < 0.05$), 15–25 ($P < 0.01$) and 27 ($P < 0.05$); TNFα + IFNγ group versus the IFNγ group: significant difference on days 21 ($P < 0.05$), 23 and 25 ($P < 0.01$)

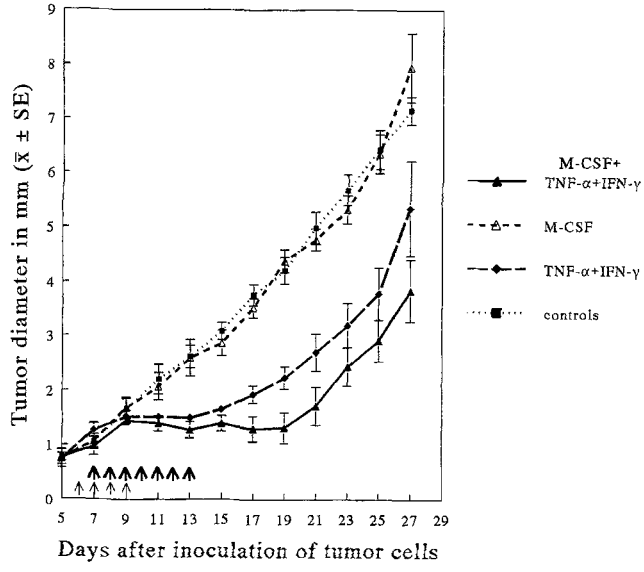

Fig. 4 The effect of treatment with M-CSF, alone or in combination with TNFα and IFNγ on MmB16 melanoma growth in B6D2F1 mice. Mice were inoculated with 10^6 melanoma cells into the footpad of right hind limb and treated with four i.v. injections (on days 6, 7, 8, 9, *thin arrows*) of M-CSF (10^6 U/injection), with seven intratumoral injections of TNFα and IFNγ (5 μg and 1000 U respectively, from day 7 to day 13, *thick arrows*) or with a combination of M-CSF and TNFα and IFNγ at the same dosage and timing as in the previous groups. Groups contained five to nine mice. M-CSF + TNFα + IFNγ group versus controls: significant difference in tumor diameter from day 11 to 27 ($P < 0.01$); TNFα + IFNγ group versus controls: significant difference from day 11 to 25 ($P < 0.01$); M-CSF + TNFα + IFNγ group versus the TNFα + IFNγ group: significant difference on day 19 ($P < 0.05$)

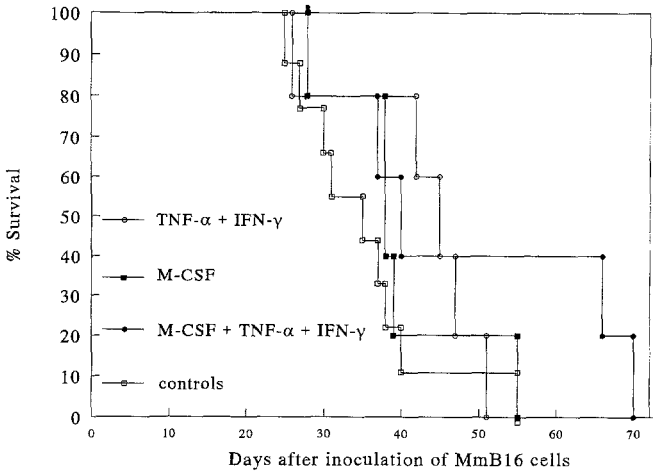

Fig. 5 The effect of treatment with M-CSF, alone or in combination with TNFα and IFNγ, on the survival time of MmB16-melanoma-bearing B6D2F1 mice. For details see Fig. 4. Mean survival time of mice ± SE for the TNFα + IFNγ group was 42 ± 4 days, for the M-CSF group 40 ± 4 days, for the M-CSF + TNFα + IFNγ group 48 ± 8 days, and for the controls 35 ± 3 days

1.3 ± 0.3 mm (mean ± SE) in comparison to 2.2 ± 0.2 mm in the TNFα + IFNγ group ($P < 0.05$), 4.4 ± 0.2 mm in the M-CSF group ($P < 0.001$), and 4.2 ± 0.2 mm in controls ($P < 0.001$). Survival of mice treated with M-CSF, TNFα and IFNγ was slightly prolonged in comparison to mice in other groups (mean survival ± SE: 48 ± 8 days in the M-CSF + TNF-α + IFN-γ group compared to 42 ± 4 days in the TNF-α + IFN-γ group, 40 ± 4 days in the M-CSF group and 35 ± 3 days in the controls) but by analysis of variance no significant differences between groups were found (Fig. 5).

Discussion

This study has shown that systemic M-CSF infusion can potentiate the therapeutic effect of local administration of TNFα and IFNγ in the treatment of MmB16 melanoma in mice. While M-CSF alone produced no antitumor effect, the concomitant use of this growth factor with TNFα and IFNγ, which were used in our study in ineffective (5 μg/injection for TNFα) and weakly effective (1000 U/injection for IFNγ) doses, resulted in a significant retardation of tumor growth (Figs. 3, 4). The delay in tumor growth was not accompanied by significant prolongation of survival in the mice, although the mean survival time in the M-CSF + TNFα + IFNγ group seemed slightly longer than those observed in mice treated with TNFα + IFNγ, M-CSF alone, or especially, in controls (48 compared to 42, 40 and 35 days respectively). It is possible that to achieve significant prolongation of the survival time, administration of M-CSF for a longer period and perhaps in continuous infusion would be required. Such a treatment, routinely used in humans [9, 34], is, however, difficult to perform on small animals including mice.

As shown by Hume [19], intravenous injections of recombinant human M-CSF for 4 days were able to increase the blood monocyte count in mice several times, and in vitro studies demonstrated that the plateau response to M-CSF occurred after 3–5 days [1]. This was the rationale for adopting a similar timing scheme our treatment protocol. The following scenario could be proposed to explain the synergy in antitumor effects of M-CSF when used in conjunction with TNFα and IFNγ. (a) Short-term M-CSF treatment resulted in an increase of the number of monocytes circulating in the blood [19, 30]. (b) This was followed by higher sequestration rate of these cells at the site of tumor development because of chemotactic effects of locally injected TNFα and IFNγ [20]. (c) Finally, in situ activation of macrophages and direct cytotoxic effect of TNFα and IFNγ on melanoma cells brought about effective killing of tumor cells [8, 14, 18] on a simple quantitative basis, and a delay in tumor progression. Such a scenario seems highly probable in the light of results reported recently by Sodhi et al. [27]. They found in the in vitro model that macrophages derived from bone marrow cultures treated for 4 days with M-CSF became highly effective in tumor cell killing under stimulation with various cytokines, among others, TNFα and IFNγ.

The above proposed course of events does not exclude, however, the possibility that other mechanisms contributed to augmentation of the antitumor effect of TNFα/IFNγ by M-CSF. As demonstrated by Sampson-Johannes and Carlino [26], pretreatment of human monocytes for 3 days with recombinant human (rh) M-CSF rendered these cells susceptible to activating signals exerted by a number of agents, including IFNγ. One can therefore consider the possibility that a similar mechanism could have occurred in our model and that enhanced local antitumor efficacy of macrophages could have been due to their preactivation by M-CSF while they were circulating as monocytes in the blood.

The synergistic antitumor effects of TNFα and IFNγ combination treatment have been well known since the mid 1980s [5, 8, 14]. The role of IFNγ seems critical in this combination. With respect to the antitumor activity against B16 melanoma, rhTNFα was either ineffective or produced marginal effects when administered as a single agent in vivo [6, 36]. Some authors, however, did find rhTNFα efficacious in the treatment of this melanoma [25]. The results of our studies are consistent with the former observations – although we observed some anti-melanoma effect of our recombinant human TNFα in vitro, this cytokine was ineffective in in vivo experiments. Unlike the situation in humans, rhTNFα can trigger p55 TNFα receptor (TNF-R55) but not p75 (TNF-R75) in mice [6]. Since recent

reports suggest that TNF-R75 contributes to the cytocidal, TNF-R55-mediated effect of TNFα [2], failure to trigger signalling via TNF-R75 in mice could be the reason for a weak antitumor activity of human recombinant TNFα in a mouse model. Nevertheless, rhTNFα retains in mice the ability to synergize with and to potentiate the antitumor activity of IFNγ, which was confirmed in our in vitro and in vivo experiments (Figs. 2–4).

In our studies we were able to augment the synergistic antitumor effects of TNFα and IFNγ by a short-term intravenous administration of M-CSF. Injection of M-CSF alone did not influence MmB16 melanoma progression. This is consistent with the study of Bock et al. [3], who found no impact of systemic administration of M-CSF on pulmonary and hepatic metastases of B16 melanoma or on growth of several lines of sarcoma. What is interesting is that they found some antimetastatic effects when M-CSF was used in conjunction with specific monoclonal antibodies. In some studies, M-CSF treatment resulted in antitumor activity with a concomitant decrease in the size of the solid tumors as well as a decrease in the number and size of metastases [22, 23]. But again, as observed by Lu et al. [22] in experiments in which they used M-CSF and local irradiation, combination therapy including M-CSF was much more effective than using M-CSF as a single agent. Our study constitutes another variant of combination therapy with M-CSF, which could prove to be useful in tumor treatment.

Activation of immune antitumor effector mechanisms, both specific and nonspecific, is dependent on mutual interaction between various cytokines. Because the role of macrophages, both as targets and effectors, seems to be critical in this interplay [7, 28], we designed a protocol directed at stimulating these cells. The combination of a growth factor specific for monocyte/macrophage lineage with local treatment by TNFα and IFNγ proved effective in this schedule. Recent studies in man have demonstrated that M-CSF has been generally well tolerated in a continuous infusion for 7 days but, despite biological effects manifesting themselves as an increase in monocyte number in blood, no objective antitumor responses were observed [9]. A phase I clinical trial has also been reported in which combination of M-CSF and IFNγ was used to treat cancer patients [34]. We hope that, in an appropriate therapeutic regimen, M-CSF could be useful by augmenting antitumor efficacy of other cytokines and/or chemotherapeutic drugs, especially in the therapy of locally growing tumors [31].

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