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Effect of Interleukin-3 and Granulocyte–macrophage Colony-stimulating Factor on Growth of Xenotransplanted Human Tumour Cell Lines in Nude Mice

Wolfgang E. Berdel, Monica Zafferani, Reingard Senekowitsch, Ernst D. Kreuser and Eckhard Thiel

The clonal growth of cell lines from some human solid tumours can be stimulated by haematopoietic growth factors such as recombinant human (rh) interleukin-3 (IL-3) and rh granulocyte–macrophage colony-stimulating factor (GM-CSF) *in vitro*. Among these cell lines are the human colorectal adenocarcinoma cell line HTB 38 and the human small-cell lung cancer cell line HTB 119. Here we report on a series of experiments studying the influence of subcutaneously administered rhIL-3 and rhGM-CSF on the *in vivo* growth of HTB 38 and HTB 119 cell lines as xenografts in athymic nu/nu BALB/c mice. Beginning 1 day after transplantation of the tumour the cytokines were administered daily for 20 days as a subcutaneous bolus distant from the tumour lesion at dose levels up to 1 mg/m^2/day. The cytokines caused no significant and reproducible growth modulation of the tumours *in vivo*.
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INTRODUCTION

INTERLEUKIN-3 (IL-3) AND granulocyte–macrophage colony-stimulating factor (GM-CSF) belong to a family of glycoproteins, that control survival, growth and differentiation of haematopoietic progenitor cells and modulate function of mature haematopoietic cells [1]. The genes of these haematopoietins have been molecularly cloned, and recombinant human (rh) factors are available. Some of those factors are currently being studied in clinical trials. Among various other possible indications for their clinical use, rhGM-CSF and rhIL-3 are being studied clinically in patients with malignant tumours to prevent and decrease myelosuppression and accelerate bone marrow recovery after cytotoxic chemotherapy as well as after high-dose chemotherapy followed by bone marrow transplantation [2].

There is increasing interest in the extrahaematopoietic activity of some of these CSF on tumour cells. This area has been reviewed recently [3]. Among non-haematopoietic tumour cell lines responsive for a growth promoting effect of haematopoietic CSF *in vitro* are the human colorectal adenocarcinoma cell line HTB 38 [4] and the human small-cell lung cancer cell line HTB 119 [5]. In order to further study the implications of these findings for the clinical trials with CSF in tumour patients, we have xenotransplanted both cell lines into Balb/c athymic mice and have studied the tumour growth with and without subcutaneous treatment of the mice with rhIL-3 and rh-GM-CSF at daily doses up to 1 mg/m^2.

MATERIALS AND METHODS

HTB 38, a human colon adenocarcinoma cell line and HTB 119, a human small-cell lung cancer cell line were obtained from the American Type Culture Collection (Rockville, Maryland,

Correspondence to W.E. Berdel.
W.E. Berdel, E.D. Kreuser and E. Thiel are at the Department of Hematology and Oncology, Klinikum Steglitz, Freie Universitaet Berlin, Hindenburgdamm 30, 1000 Berlin 45; and M. Zafferani and R. Senekowitsch are at the Klinikum rechts der Isar, Technische Universitaet Muenchen, Ismaninger Street 22, 8000 Munich 80, F.R.G.
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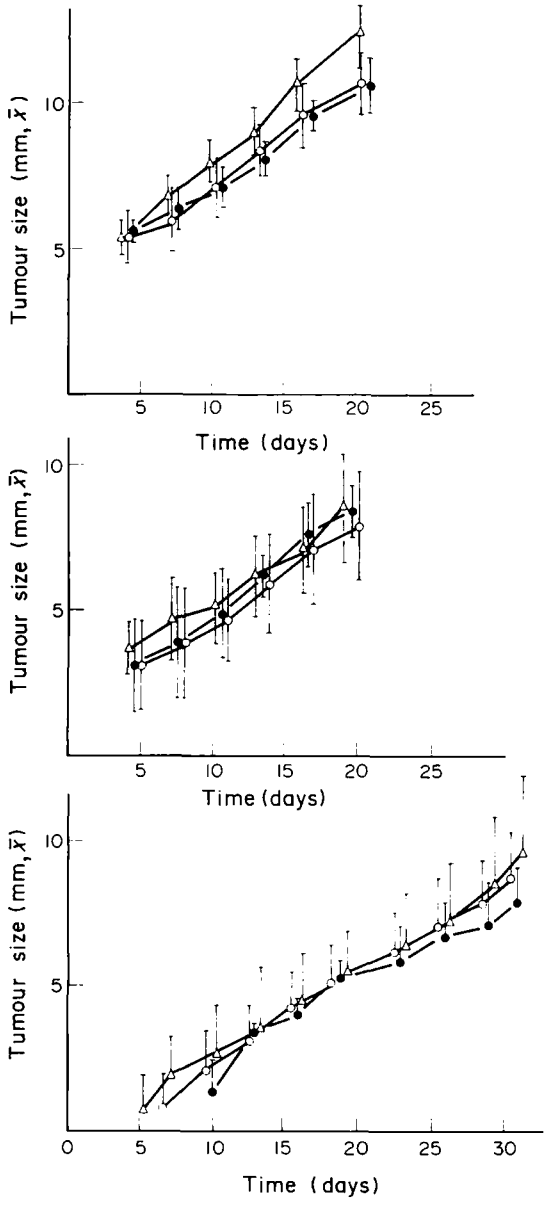

Fig. 1. Growth curves of HTB 38 xenografts in nu/nu BALB/c mice. At day 0 tumour cells were given at the following numbers: 5 × 10^6 (top), 1 × 10^6 (centre), 5 × 10^5 (bottom). Animals were treated daily with 1 mg/m² rhIL-3 (△), 1 mg/m² rhGM-CSF (●) or vehicle (○, controls) for 20 days, beginning on day 1 after tumour transplantation. There were no significant differences between the growth kinetics of the three experimental groups with the exception of $P < 0.05$ when rhIL-3 and control groups were compared at day 20 in 1 experiment with an initial tumour burden of 5 × 10^6 cells (top).

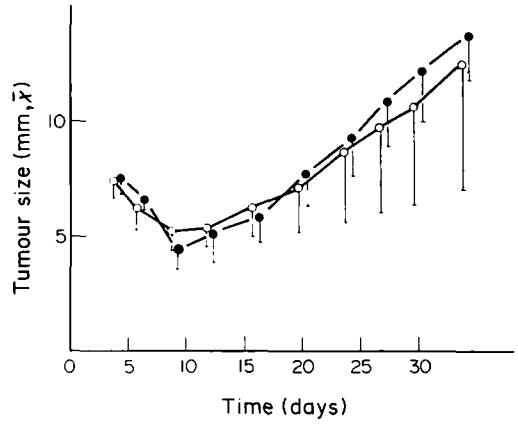

Fig. 2. Growth curves of HTB 119 xenografts in nu/nu BALB/c mice. At day 0, 4 × 10^7 tumour cells were given. Animals were treated daily with 1 mg/m² rhGM-CSF (●) or vehicle (○, controls) for 20 days, beginning on day 1 after tumour transplantation. There were no significant differences between the growth kinetics of the two experimental groups.

USA). Cell culture techniques have been recently described in detail [6].

Athymic (nu/nu) BALB/c mice were obtained from the breeding centre of the Gesellschaft fuer Strahlen- und Umweltforschung (Neuherberg, FRG). Mice were kept under specific pathogen-free conditions, fed on an autoclaved standard diet (Altromin) and sterilised water at low pH *ad libitum*. Tumour cell lines were heterotransplanted intracutaneously into the flank of the mice as single cell suspensions in saline at cell numbers as indicated. Tumour growth was measured at the days indicated with calipers. Mean diameters (length + width/2) are given as tumour size in mm and Figs 1 and 2 show development of mean tumour size for each experimental group (S.D.) over time. Experimental groups contained 6–12 animals.

We have used rhGM-CSF (purified from *E. coli*, batch no. 180587 BI 71018, 68.2 μg/vial) and rhIL-3 (purified from yeast, batch no. 148, 500 μg/ml), provided by Behring Institute (Marburg, FRG) for the *in vivo* experiments. The cytokines were given daily subcutaneously as a bolus distant from the tumour for the time periods indicated in Figs 1 and 2, whereas the control animals obtained vehicle without cytokine on the same schedule.

The HTCA in glass capillaries ($HTCA_{cap}$) was performed as described previously [3, 7].

Plasma levels of both cytokines were measured 0.5 h, 5 h and 24 h after the last injection of either the cytokine or the vehicle by a modified enzyme linked immunosorbant assay (ELISA) as follows: Ready-to-use microtitre plates precoated with polyclonal rabbit anti-IL-3 or anti-GM-CSF antibody were washed with washing solution POD (all reagents for the ELISA were from the Enzygnost POD productline, Behringwerke AG, Marburg, FRG). The ELISA testplates were then washed with washing solution. Serum samples were first prediluted 1:3 in sample buffer and then dilution series were run. Triplicates of samples and standards (100 μl) were incubated for 1 h at room temperature in the coated testplate. The well contents were aspirated and the plate rinsed 3 × with washing solution. 100 μl of the MAB-POD conjugates in conjugate buffer (2 μg/ml) were added and the plate was incubated for 2 h at room temperature. After washing the plates 3 ×, 100 μl of enzyme substrate solution containing *o*-phenylenediamine/H_2O_2 was added. After an incubation period of 30 min in the dark the reaction was stopped by dispensing 100 μl stopping solution (2 N sulphuric acid) into each well. Finally, the absorbance values were read at 492 nm (reference 650 nm) in a Behring ELISA processor II. The IL-3/GM-CSF concentrations were calculated from the calibration curve. A new calibration curve including a control sample was constructed for each assay.

Statistical evaluation of the results was performed using the Mann–Whitney test. *P*-values > 0.05 were interpreted as indicating non-significant differences.

Table 1. Plasma concentrations of rhGM-CSF and rhIL-3 in nu/nu BALB/c mice

	GM-CSF (ng/ml)	IL-3 (ng/ml)
0.5 h *	87.750 †	109.700
5 h	0.821	0.224
24 h	0.067	0.061
Vehicle control ‡	<0.025	<0.025

* Time after last subcutaneous application of 1 mg/m² rhGM-CSF or rhIL-3, respectively.
† Concentrations given in ng/ml, S.D. was < 20% for the values indicated.
‡ At all times.

RESULTS

We have incubated increasing concentrations of rhIL-3 and rhGM-CSF with HTB 38 and HTB 119 in the $HTCA_{cap}$. Both factors stimulated the clonal growth of HTB 38 and rhGM-CSF stimulated clonal growth of HTB 119 in a concentration-dependent fashion as previously described (details not shown). IL-3 concentrations of 40 ng/ml and GM-CSF concentrations of 150 ng/ml stimulated clonal growth of these cell lines 2-fold with 15 ng/ml of rhGM-CSF and 5 ng/ml of rhIL-3 producing the first significant growth stimulation. Whereas we have not looked for CSF receptors in the HTB 38 line, GM-CSF receptors have been found in the HTB 119 line [5].

Because of the *in vitro* responsiveness of these cell lines, the cytokines were then assayed for *in vivo* growth modulation on both of these cell lines growing as tumour xenografts in nu/nu mice. In six experiments cell numbers of 5×10^5, 1×10^6, 5×10^6, and 1×10^7 of the HTB 38 cell line and 4×10^7 cells of the HTB 119 cell line were injected at transplantation. Subcutaneous treatment with the cytokines was given daily for 20 days, beginning 1 day after tumour transplantation. Doses of the cytokines used were 0.5 mg/m² and 1 mg/m² daily. With reference to the doses used for the clinical trials, higher doses were not applied. In none of the experiments was there a significant and reproducible growth stimulation or growth retardation of the tumours by rhIL-3 and rhGM-CSF. Stimulation by rhIL-3 of HTB 38 tumour growth reached significance levels only in one experiment at an initial tumour burden of 5×10^6 cells and only on day 20 of observation (see Fig. 1). This however was not reproducible. Examples of the experiments are shown in Figs 1 and 2. In addition, there was no difference in the low metastatic potential between controls and treated animals (details not shown). In some of the experiments with low tumour burden (5×10^5 cells injected) few mice had no tumour-take. Interestingly, this occurred preferentially in the CSF-treated groups (data not shown).

Plasma levels of the cytokines obtained 0.5 h, 5 h and 24 h after the last injection showed peak concentrations well within the range of being sufficient for *in vitro* stimulation of clonal growth (Table 1 and ref. 3).

Toleration of the cytokines by the animals was good. No deaths or visible toxicity occurred during the treatment interval. Monitoring of body weight in one experiment however, showed a mean value of 23.06(2.0 g) in the control group and 20.0 (1.8 g) in the group treated with rhGM-CSF at the last day of therapy.

DISCUSSION

Previous reports [3, 8–12] indicate that various haematopoietic growth factors can stimulate the clonal growth of some non-haematopoietic tumour cell lines *in vitro*. This effect was also observed in fresh tumour specimens by one laboratory [13], whereas at lower concentrations others could not find similar activity [14, 15]. In order to further study the implications of this observation for the clinical trials with CSF in tumour patients, we have xenotransplanted responsive tumour cell lines into BALB/c athymic mice and have studied the tumour growth with and without subcutaneous treatment of the mice with rhIL-3 and rhGM-CSF at daily doses up to 1 mg/m². There was no significant and reproducible growth-modulating effect of both cytokines in these experiments (see Figs 1 and 2).

At the moment there is no convincing explanation for the difference between the *in vitro* and *in vivo* results. Since we could show, that growth of HTB 38 cells is not modulated by autocrine CSF production [3], autocrine receptor blockade should not be interfering with the experiments of this study. On the other hand, the effects of GM-CSF and IL-3 preferentially occurred under suboptimal growth conditions and could be partially masked by higher serum concentrations *in vitro* [3]. This together with the comparatively short half-life of the cytokines in this experimental setting (see Table 1, ref. 16) could represent pharmacokinetic reasons for their lacking *in vivo* activity. In humans 10 μg/kg subcutaneous GM-CSF yields plasma peak levels of approx. 16 ng/ml, which is much lower than that observed in our experiments on mice [16]. However, plasma half life of GM-CSF at this dose in humans was > 6 h [16] and thus comparatively longer than was found in our experiments. In addition, tumour cell populations are heterogenous with respect to their response to peptide growth factors [17] and the clonogenic subpopulation of cells being sensitive for the haematopoietic CSF *in vitro* may not necessarily be the relevant cell population dominating the intracutaneous growth of the cell line as xenograft. Since on the other hand, this subpopulation may be relevant for certain metastatic patterns of a tumour such as bone marrow metastasis, the dependency of bone marrow metastasis from receptors for haematopoietic CSF is under further study in our laboratory.

It should be noted in addition that in artificial *in vitro* systems the *in vivo* environment, such as the tumour stroma with host response cells, is lacking. Thus, *in vivo* effects which these cytokines may have, such as enhancement of antibody-dependent cellular cytotoxicity by GM-CSF [18], alteration of human neutrophil migration by GM-CSF [19] or enhancement of monocyte/macrophage tumoricidal activity by M-CSF [20, 21] and GM-CSF [22] may be acting partially against direct effects of this material on the tumour cells. However, these effects may be operative in the clinical trials, but should not be operative in human xenografts growing in nu/nu mice when human IL-3 and GM-CSF is used because of the species specificity of these molecules.

In conclusion, the fact that haematopoietic growth factors can be involved in the growth regulation of non-haematopoietic malignant cells *in vitro* as well as observations with CSF's in other non-haematopoietic tissues [23] may give more insight into the biology of growth regulation inside the network of peptide growth factors and into tumour biology and metastasis. However, the *in vivo* experiments of this study do not show tumour growth stimulation by rhGM-CSF and rhIL-3 under the specific pharmacokinetic parameters studied. With due caution this supports the notion that potential hazards for the

clinical application of CSFs in cancer patients in conjunction with cytotoxic chemotherapy are unlikely.

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