Enhancement of Experimental Metastasis by Tumor Necrosis Factor

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Summary

The influence of endogenous and exogenous tumor necrosis factor (TNF) on metastasis was investigated in an experimental fibrosarcoma metastasis model. A single intraperitoneal injection of recombinant human (rh) TNF or recombinant mouse (rm) TNF into mice 5 h before intravenous inoculation of methylcholanthrene-induced fibrosarcoma cells (CFS1) induced a significant enhancement of the number of metastases in the lung. Dose responses of rmTNF and rhTNF demonstrated a stronger metastasis-augmenting effect by rmTNF compared with rhTNF. This effect was time dependent, as administration of rmTNF 5 h before or 1 h but not 24 h after tumor cell inoculation caused an increase of tumor cell colony formation on the lung surface, suggesting an influence of TNF on the vascular adhesion and diapedesis of tumor cells. Since tumor-bearing mice showed an enhanced ability to produce TNF after endotoxin injection compared to control mice, tumor-bearing mice were treated with anti-mTNF antibodies. Neutralization of endogenous tumor-induced TNF led to a significant decrease of the number of pulmonary metastases. Histological analysis of micrometastases in the lung on day 5 by silver staining of proteins associated with nucleolar organizer regions revealed more metastatic foci and augmented proliferative activity of the tumor cells after rmTNF pretreatment of mice. However, no direct effect of rmTNF on the proliferation rate of tumor cells was seen in vitro. These findings suggest that low doses of endogenous TNF or administered TNF during cytokine therapy might enhance the metastatic potential of circulating tumor cells.

TNF, a cytokine predominantly produced by activated macrophages, exerts a cytotoxic or cytostatic effect on some tumor cell lines in vitro and has the capacity to induce necrosis of solid tumors in some animal models (1–4). TNF has, in addition to the antitumor effects, a broad spectrum of biological activities, implicating a major role in immune and inflammatory responses. For instance, TNF induces the production of IL-1 (5), IFN-β (6), as well as CSF (7) and enhances the expression of HLA genes in tumor cells (8). Furthermore, TNF stimulates cells to express cell-surface adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) (9, 10), vascular cell adhesion molecule 1 (11, 12), and endothelial leukocyte adhesion molecule 1 (13). Because of some of these actions, TNF has beneficial antitumor activity (1, 2). However, TNF might also contribute to tumor progression. TNF is able to induce enzymes that may enhance tumor spread, e.g., collagenases (14), to stimulate angiogenesis in vivo (15), to stimulate bone resorption (16), and to increase the adherence of tumor cells to endothelium in vitro (17–19). It has also been shown that TNF promotes the invasive growth of tumor cells in the peritoneum and their establishment as tumor nodules below the mesothelial surface (20, 21). The role of endogenous TNF in the pathophysiology of cancer and metastasis is still controversially discussed and only limited information is available demonstrating enhanced TNF levels in the serum of cancer patients (22, 23) and tumor-bearing animals (24, 25). We therefore investigated the effects of exogenous and endogenous TNF on metastasis in an experimental tumor model in mice.

Materials and Methods

Mice. Female C3H/He and female DBA/2 mice 5–7 wk of age were obtained from the Institut für Versuchstierforschung (Hannover, FRG). The animals were kept in the central animal facility of the German Cancer Research Center under specific-pathogen-free conditions throughout the experiments.

Cell Cultures. CFS1 is a methylcholanthrene-induced fibrosarcoma cell line of C3H/HeN mouse origin, generated and kept at the German Cancer Research Center. ESb, a highly metastatic subline of the methylcholanthrene-induced DBA/2 lymphoma L5178YE (Eb), was kindly provided by V. Schirrmacher (German Cancer Research Center). L929 is a TNF-sensitive mouse fibrosar-

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coma cell line. All cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 5 mM Hepes, and 100 IU/ml penicillin–0.1 mg/ml streptomycin (all from Gibco, Eggenstein, FRG), in a humidified atmosphere containing 5% CO_2 at 37°C. Cultures were routinely verified as being mycoplasma free. The tests were performed with a mycoplasma-DNA hybridization kit according to the manufacturer's instructions (Gen Probe, San Diego, CA).

Reagents. Recombinant human (rh)[1]TNF-α (9×10^6 U/mg sp act) and recombinant mouse (rm)TNF-α (8×10^7 U/mg sp act) were a generous gift from BASF/Knoll AG (Ludwigshafen, FRG). The rat anti-mouse TNF mAb V1Q was purified as previously described (26). Briefly, serum-free hybridoma supernatant was precipitated at 45% ammonium sulfate saturation. The precipitated material was centrifuged at 15,000 *g*, dissolved in 10 mM NaCl/50 mM Tris/HCl, pH 8, and dialyzed against this buffer. This buffer was also used as the starting buffer for anion exchange HPLC (Spherogel TSK DEAE-5PW; Beckman, München, FRG). The material was applied to the HPLC column in a volume of 10 ml at a flow rate of 1 ml/min. The column was washed with loading buffer until baseline absorption at 280 nm was reached. The adsorbed material was eluted using a linear gradient from 10 to 300 mM NaCl in 50 mM Tris/HCL pH 8. 1-ml fractions were collected and the TNF neutralizing capacity was measured using the L929 TNF bioassay. The most active fractions were pooled and dialyzed against PBS. R4-6A2, a rat anti-IFN-γ mAb (27), was precipitated from serum-free hybridoma supernatant at 45% ammonium sulfate saturation, centrifuged, dissolved in distilled water, and dialyzed against PBS. Polyclonal rat IgG and LPS (from *Salmonella minnesota*) were purchased from Sigma Chemical Co. (St. Louis, MO).

Quantitation of Experimental Metastases. CFS1 tumor cells were harvested by exposure to 0.05% trypsin–0.02% EDTA solution (Gibco), washed twice, and resuspended in HBSS. The suspension was filtered through a cell strainer (Becton Dickinson & Co., Lincoln Park, NJ) and adjusted to a concentration of 1.5×10^6 cells/ml 0.2 ml (3×10^5 CFS1 cells) of the single cell suspension (>90% viability by trypan blue exclusion) was injected into the lateral tail vein of C3H mice. Animals were killed by cervical dislocation on day 11 or 12. After staining by endotracheal infiltration with 15% china ink solution (Rotring Werke Riepe KG, Hamburg, FRG), lungs were removed, fixed, and bleached in Fekete's solution (28). The number of macroscopic pulmonary tumor nodules was counted in each of the five lobes.

The nonadherent ESb cells were washed twice and resuspended in HBSS. 10^5 cells/0.2 ml were injected into the lateral tail vein of DBA/2 mice. Mice were killed at day 4, and the livers were removed and fixed in 4% buffered formalin. Paraffin sections were cut and stained with haematoxylin–eosin. Liver metastases were determined by counting the metastatic foci under the microscope.

Induction of TNF Production. At the indicated days, mice were injected intraperitoneally with 10 μg LPS in 0.2 ml PBS 1.5 h before blood collection.

Preparation of Serum. Blood samples collected from the tail vein were allowed to clot at room temperature for 15 min and were then kept at 4°C for at least 1 h. Sera were clarified by centrifugation (5,000 rpm for 2 min) and stored at −20°C until needed.

L929 TNF Bioassay. The assay was performed as previously described (29). Briefly, 2.2×10^4 L929 cells/100 μl per well of a 96-well microtiter plate were incubated overnight. After replacing the culture medium with medium containing 2 μg/ml of actinomycin D (Sigma Chemical Co.), 100 μl of serum (diluted 1:4 with PBS) was added and sequential twofold dilutions were made. After 18 h of incubation at 37°C, remaining cells were stained with crystal violet (Sigma Chemical Co.). After washing, the stain was dissolved in 33% acetic acid (100 μl) and the OD at 550 nm was determined using an ELISA reader (Easy Reader EAR 400; SLT-Labinstruments, Gröding, Austria). An internal rhTNF-α standard was included in each assay.

Measurement of Tumor Cell Proliferation In Vivo. Silver staining of argyrophilic proteins associated with nucleolar organizer regions (AgNOR), whose number and size correlate with cell proliferation and differentiation, was performed as described previously (30). Briefly, lungs were removed at the indicated times and fixed in 4% buffered formalin. Paraffin sections were cut at 3–4 μm onto glass slides. These were dewaxed in xylene and rehydrated through descending concentrations of ethanols to distilled, deionized water. The staining time was about 26 min and the silver staining was carried out in the dark. The AgNOR were evaluated by means of a digital image analysis system (Cue2; Olympus Optical Corp., Hamburg, FRG). At least 50 cells of each slide were examined, choosing fields at random and avoiding nontumorous areas. The mean number of AgNOR per cell (N), and the mean AgNOR area per silver stained NOR particle (A) were calculated as follows: N = No. AgNOR/No. cells; A = area of AgNOR/No. AgNOR. Since the NOR number and area are inversely correlated, the ratio of these two parameters was regarded as an equivalent of the AgNOR content of a given cell ($C = N/A$). Since the area of AgNOR is in the range of 0.1–2 μm^2, a relatively high final magnification of 4,000 was used on the monitor of the image analyzer; the microscope magnification was 1,000. To minimize the problem of nucleolus-biased sampling, the measuring field of the image digital video board was reduced to a central portion, so that only one or two cells could be measured at once.

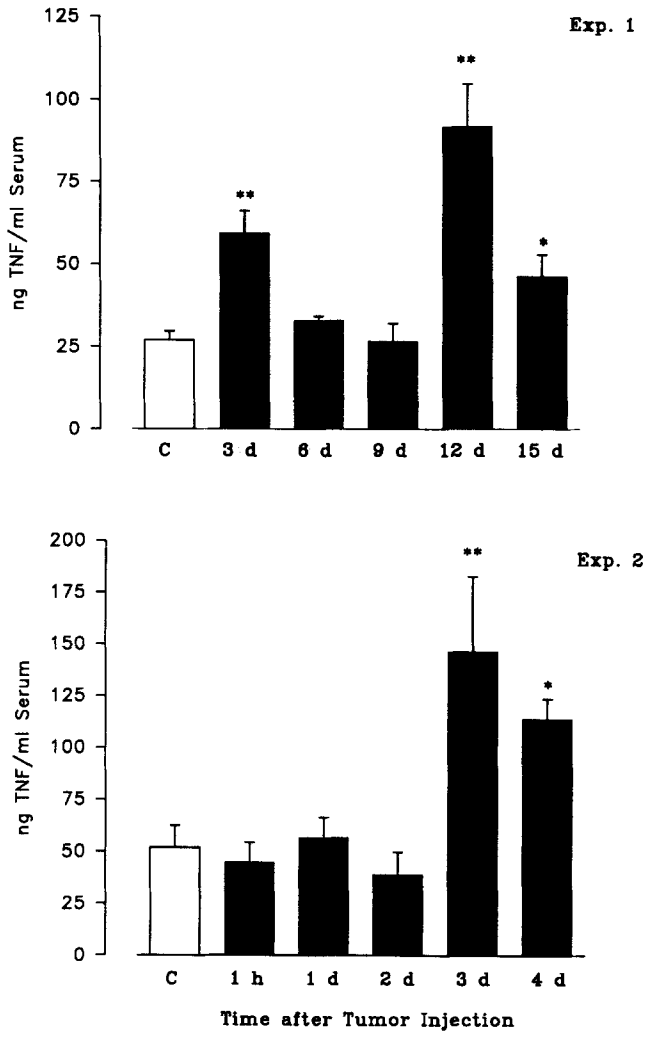
Statistical Analysis. The probability of significant differences between groups was determined by Student's t test. Differences in the numbers of lung colonies and AgNOR content were analyzed using the Mann-Whitney U test. Results are representative of at least two different experiments.

Results

Endotoxin-induced TNF Production after Intravenous Inoculation of Tumor Cells. Mice were injected with endotoxin in order to determine whether tumor-bearing mice show an increased capacity to produce TNF. Fig. 1 shows that 3 d after injection of CFS1 tumor cells, mice had enhanced serum TNF levels in response to LPS compared with control mice. A second peak developed on day 12. Endotoxin-induced serum TNF levels before day 3 and on days 6 and 9 after tumor implantation did not differ from endotoxin-induced serum TNF levels of normal mice.

Effects of rmTNF and rhTNF on the Number of Metastases. Since tumor-bearing mice had a higher capacity to produce TNF, we investigated whether exogenous TNF would influence the number of lung metastases. Mice were inoculated intravenously with CFS1 tumor cells. 5 h before inoculation, they were injected intraperitoneally with 10 μg per mouse of either rmTNF or rhTNF. After 12 d, mice were killed and the pulmonary metastases were counted. Fig. 2 shows a dramatic increase in the number of metastases in TNF-treated

[1] *Abbreviations used in this paper:* AgNOR, nucleolar organizer regions demonstrated by silver staining; rhTNF, recombinant human tumor necrosis factor; rmTNF, recombinant mouse tumor necrosis factor.



mice. Histological examinations revealed no metastases in other organs. To exclude that this effect was due to contaminating LPS in the TNF preparations, the preparations were heated to 100°C for 15 min. This treatment completely destroyed their metastasis-enhancing capacity (data not shown).

The augmentation of the number of tumor colonies on the surface of the lung was greater with increasing concentrations of rTNF (Fig. 3). The figure also shows that rmTNF had a two- to fivefold higher capacity to enhance the metastatic potential of tumor cells than had rhTNF. Whereas 0.4 μg rhTNF (mean number of metastases ± SEM [range]: 33.2 ± 7.9 [5–56]) had no influence on the number of lung metastases on day 11, 0.4 μg rmTNF (82.0 ± 13.4 [28–123]) already caused a significant increase. Pretreatment of the mice with 25 μg rhTNF (197.7 ± 15.4 [135–250]) resulted in an equally significant enhancement of metastases as pretreatment with 10 μg rmTNF (221 ± 18 [145–250]).

To investigate the time course of rmTNF-induced augmentation of lung metastasis, mice were injected intraperitoneally with 7.5 μg rmTNF at various times before and after intravenous tumor cell implantation. Fig. 4 shows that a significant increase of tumor colonies on the lung compared with control mice was only observed when rmTNF was given 5 h before (185.7 ± 31.7 [86–250]) or 1 h after (169.3 ± 4.3 [151–182]) CSF1 tumor cells. No significant influence on lung colony formation was seen when TNF was given 24 h before

Figure 1. Time course of endotoxin-induced TNF production in tumor-bearing mice. At the indicated times after intravenous CFS1 tumor cell injection, 10 μg endotoxin/200 μl PBS was injected intraperitoneally. Serum was obtained 1.5 h later and TNF levels were determined by L929 TNF bioassay. Results are expressed as mean ± SEM of five mice per tumor-bearing group. Control groups (C) n = 13. (**) $p < 0.005$; (*) $p < 0.025$.

Figure 2. Enhancement of experimental lung metastasis by rmTNF and rhTNF. Mice received single intraperitoneal injections of 10 μg rmTNF/mouse. 10 μg rhTNF/mouse, or 100 μl PBS 5 h before tumor cell injection (3 × 10^5 CFS1 fibrosarcoma cells intravenously). Lungs were stained on day 12 and metastases were counted. Representative lungs taken from one experiment are shown. (*Top*) PBS pretreatment (n = 10); (*center*) rmTNF (n = 6); (*bottom*) rhTNF (n = 6).

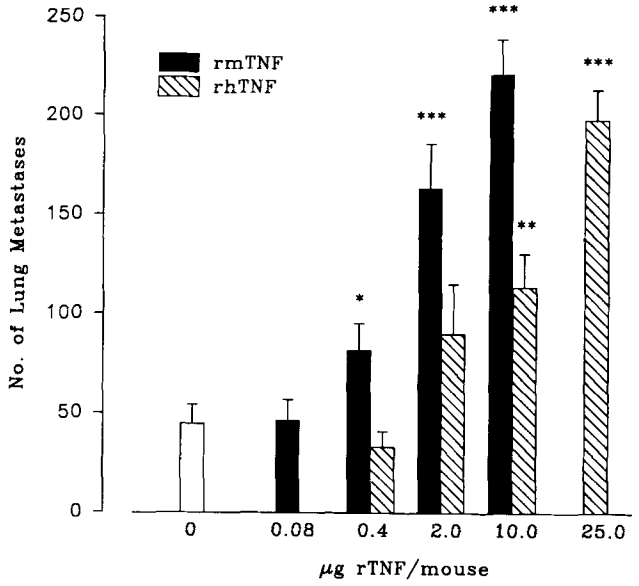


Figure 3. Increase of experimental lung metastasis by different concentrations of rmTNF and rhTNF. Mice were given intraperitoneal injections of different concentrations of rmTNF or rhTNF 5 h before intravenous CFS1 tumor cell injection (3×10^5). Control mice received the same volume of PBS. Results are expressed as mean ± SEM. $n = 12$ for control group; $n = 6$ for TNF-treated groups. (***) $p < 0.001$; (**) $p < 0.005$; (*) $p < 0.05$.

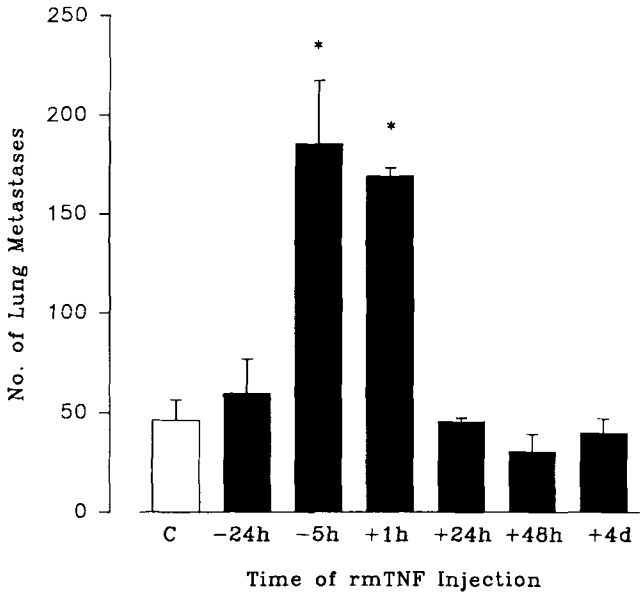


Figure 4. Kinetics of rmTNF-induced enhancement of lung metastasis. rmTNF (7.5 μg/mouse i.p.) was administered at different times before or after intravenous injection of 3×10^5 CFS1 tumor cells. Lungs were removed and stained on day 11. Results are expressed as mean ± SEM. $n = 6$ for TNF-treated groups; $n = 12$ for control group (*C*). (*) $p = 0.001$.

or 24 h up to 4 d after tumor cell injection. In control mice 46.2 ± 10.2 (1–99) metastases were counted per lung.

To obtain information whether these effects were host mediated or due to a direct influence of TNF on the tumor cells, CFS1 cells were cultured for 24 h with rmTNF (0.2 μg/ml) or rat anti-mTNF mAb (V1Q)(0.8 μg/ml) at 37°C. After trypsinizing and washing, 3×10^5 cells were injected intravenously in untreated mice. On day 12 after tumor cell implantation, the number of lung metastases from mice given rmTNF- or V1Q-pretreated tumor cells was not significantly different compared with the control group (data not shown).

In a second metastasis model, application of 10 μg rmTNF intraperitoneally 5 h before intravenous inoculation of 10^5 ESb cells caused an approximately threefold enhancement of tumor infiltrates into the liver (mean number of metastases/mm² ± SEM: 0.85 ± 0.098) compared with the control group (0.31 ± 0.077) (data not shown).

Decrease of Metastasis by Neutralization of Endogenous TNF. As tumor-bearing mice showed an enhanced capacity for TNF

Figure 5. Decrease of lung metastasis by neutralization of endogenous TNF. Rat anti-mTNF mAb (40 μg) or PBS were injected intraperitoneally 5 h before intravenous CFS1 tumor cell inoculation. Mice were autopsied on day 12. Representative lungs taken from one experiment are shown. (*Top*) PBS pretreatment ($n = 10$); (*bottom*) rat anti-mTNF mAb treatment ($n = 6$).

Table 1. *Reduction of Experimental Metastasis by Neutralization of Endogenous TNF*

Pretreatment	No. of lung metastases			
	n	Mean ± SEM	Range	*p*
PBS-control	8	58.5 ± 7.9	22–82	
Rat anti-mTNF mAb (V1Q)	6	9.6 ± 5.6	1–31	<0.005
Rat anti-mIFN-γ mAb (R4-6A2)	6	48.7 ± 16.3	0–116	
Rat IgG	6	66.9 ± 18.0	23–114	

Mice were treated intraperitonally with the indicated substances 5 h before intravenous CFS 1 tumor cell injection (3×10^5). Animals were killed on day 11, the lungs were removed, and the number of surface colonies was counted.

Table 2. *Enhancement of the Tumor Cell Proliferation Rate In Vivo by rmTNF*

AgNOR parameter	Pretreatment		
	PBS control	rmTNF	*p*
No. of AgNOR/cell	1.2 ± 1.2*	8.4 ± 0.7	<0.001
AgNOR area (μm^2)/AgNOR	0.02 ± 0.02	0.13 ± 0.01	<0.001
AgNOR content/cell	9.4 ± 9.4	65.8 ± 5.6	<0.001

Mice received 10 μg rmTNF or 100 μl PBS 5 h before intravenous tumor cell inoculation. Lungs were removed on day 5, fixed in formalin, and the AgNOR parameters were determined.
* $n = 6$ per group; data represent mean ± SEM.

production and since exogenous TNF increased metastasis, we investigated the influence of endogenous TNF on the formation of lung metastases. Mice received a single intraperitoneal injection of 40 μg of rat anti-mTNF mAb (V1Q) in 0.2 ml PBS 5 h before CFS1 tumor cell inoculation. Control mice received either polyclonal rat IgG (100 μg) or 0.2 ml PBS. After 11 d, tumor cell colonies on the lung surface were counted. Fig. 5 and Table 1 show a significant decrease of metastasis in the anti-mTNF pretreated group in comparison with the control IgG- and PBS-treated mice.

As IFN-γ is able to enhance LPS-induced TNF production of macrophages (31), we examined whether endogenous IFN-γ might also be involved in the enhancement of metastasis induced by endogenous TNF. For that purpose, rat anti-mIFN-γ mAb (R4-6A2) was administered 5 h before tumor cell inoculation. As can be seen in Table 1, this mAb was not effective in preventing the formation of tumor cell colonies on the lung, in contrast with the mTNF-neutralizing mAb V1Q.

Augmentation by rmTNF of Tumor Cell Proliferation In Vivo. One possible reason for finding more and larger tumor cell colonies on the lung surface in rTNF-treated mice could be

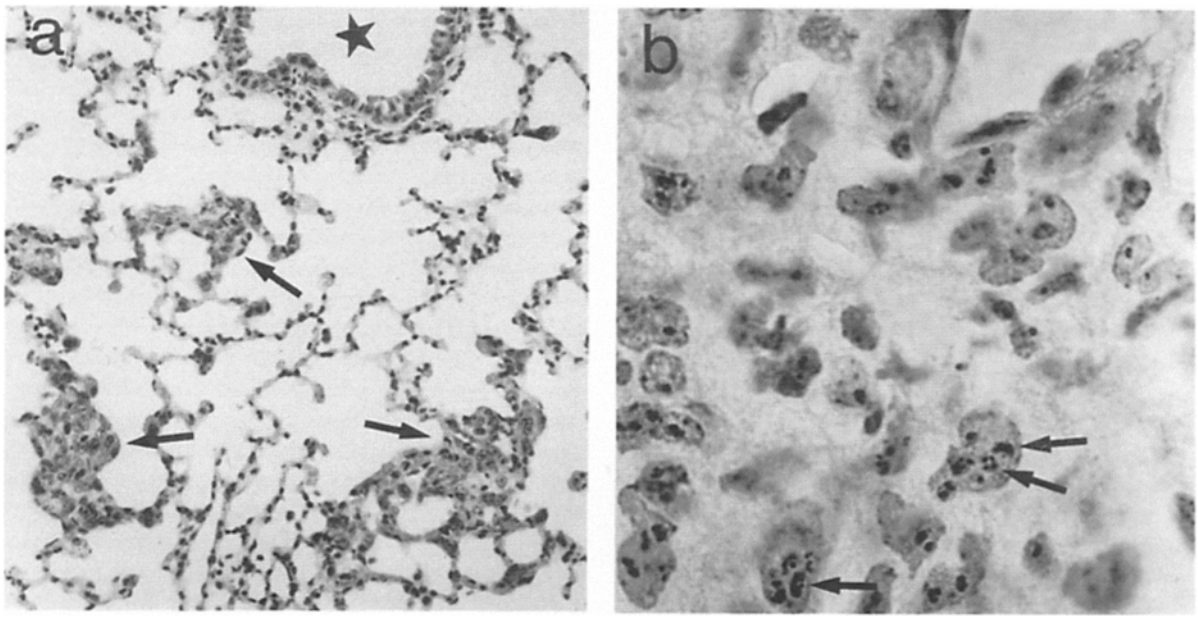


Figure 6. (*a*) Histological section of lung tissue exhibiting small metastatic tumor nodules within alveolar septae (*arrows*). Terminal broncheolus without pathological changes (*). (Haematoxylin/eosin stain, original ×200). (*b*) Nodule of metastatic tumor cells showing multiple silver-stained NOR dots (AgNOR) within nuclei (*arrows*). (AgNOR stain, original ×1,000).

an enhanced proliferative activity of the tumor cells. A reliable parameter for in vivo proliferation is the number and size of silver-stained proteins associated with AgNOR per cell (30). Therefore, we investigated the effect of 10 μg rmTNF given 5 h before intravenous CFS1 tumor cell inoculation on the AgNOR parameters per tumor cell. The lungs were removed on day 5 and the AgNOR parameters were determined on paraffin sections by digital image analysis. Sections from the lungs of rmTNF-pretreated mice showed a significantly enhanced number of AgNOR dots, AgNOR area, and AgNOR content per cell, indicating an augmented proliferation rate compared with the PBS-treated control group (Fig. 6, Table 2). AgNOR parameters measured on day 7 after tumor cell inoculation showed a comparable result (data not shown). This effect of rmTNF on CFS1 tumor cells seems to be specific for in vivo conditions for we could not demonstrate any influence of rmTNF on CFS1 proliferation in vitro measured by [^{3}H]thymidine incorporation (data not shown).

Discussion

The influence of exogenous TNF administered during cytokine therapy and of endogenous TNF produced, for example, by tumor-infiltrating macrophages and peripheral blood monocytes on the metastatic behavior of circulating tumor cells is of considerable importance. In this study we have shown that rmTNF and rhTNF injected 5 h before intravenous inoculation of CFS1 fibrosarcoma cells caused a significant augmentation of pulmonary metastases. Similarly, application of rmTNF before intravenous injection of ESb cells resulted in an increase of liver metastases. In addition, we do have preliminary data indicating that enhanced metastasis occurs under the influence of TNF when the metastasizing tumor cells started from a solid primary tumor nodule, e.g., Lewis lung carcinoma or ESb (our own unpublished results).

The effect of TNF on CFS1 cells was dose dependent and rmTNF revealed a higher metastasis-enhancing activity than did rhTNF. Increased formation of tumor cell colonies on the lung was only seen when rmTNF was given 5 h before or 1 h after, but not 24 h or later after tumor cell implantation. Tumor cells in TNF-pretreated mice showed an enhanced proliferation rate as determined by the number of AgNOR dots. Accordingly, neutralization of endogenous tumor-induced TNF led to a decrease of the number of lung metastases.

Augmented serum TNF levels in tumor-bearing mice after LPS injection have also been shown in other studies (24, 25). However, the time course of TNF production was different, suggesting an influence of tumor cell immunogenicity, the way of application, and/or the mouse strain used on TNF secretion. In this study, endogenous TNF production peaked at day 3 after tumor cell injection (Fig. 1). With respect to the metastasis-enhancing effect of TNF, this finding does not correlate with the observation that TNF led to an augmentation of tumor cell colonies on the lung surface only when given 5 h before or 1 h after, but not 3 d after tumor cell inoculation (Fig. 4). A possible explanation for this finding could be that 1 h after tumor implantation, when TNF exerts its metastasis-enhancing effect, tumor-induced TNF either remained membrane bound or was only present in very low amounts and thus was not detected in the serum with our TNF assay system.

Nevertheless, a critical role of endogenous TNF on the formation of pulmonary metastases was demonstrated by using anti-mTNF mAb. Neutralization of endogenous TNF caused a significant decrease of tumor cell colonies on the lung surface (Table 1, Fig. 5). Few other studies indicate a contribution of endogenous TNF to the metastatic behavior of tumor cells. Malik et al. (21) transfected Chinese hamster ovary cells with the gene for hTNF and showed that these TNF-secreting cells exhibited a greatly enhanced ability to invade peritoneal surfaces. Even though this experimental design differs from our study with host-produced endogenous TNF, it demonstrates TNF-caused increased capacity of tumor cells for invasive growth.

Experiments concerning the influence of exogenous TNF on metastasis had rather controversial results. Tomazic et al. (32) noted that TNF inhibited the development of pulmonary metastases in a murine fibrosarcoma model if TNF was injected within 3 d of tumor cell inoculation. Similar results were described in a murine lung cancer model (33) and in a murine melanoma model (34). On the other hand, administration of hTNF led to an increase in the number of pulmonary metastases when given together with tumor cells (35) or 1 h before intravenous inoculation of a human melanoma cell line (36). Furthermore, hTNF promoted the implantation of human ovarian cancer xenografts in the peritoneal cavity, leading to a pathological appearance similar to that of metastatic human cancer (20). The latter findings confirm our results revealing an increased metastatic capacity of tumor cells induced by TNF.

As shown in Fig. 3, rmTNF exhibited a higher metastasis-enhancing capacity than rhTNF. This fact is not surprising considering the partial species specificity of TNF (37, 38). In addition, it is conceivable that the effect of enhanced tumor cell colony formation on lungs of mice is in part mediated via the type II TNF receptor (75 kD) which binds mTNF species specifically (TNF receptors reviewed in reference 39).

A variety of mechanisms can be envisaged to play a role in the metastasis-augmenting activity of TNF. Metastasis is a complex process involving the release of tumor cells from a primary tumor entering of the vascular or lymphatic circulation and extravasation to specific sites distant from the original tumor. These processes require tumor cell attachment to the endothelium of vessels or capillaries before they can perform diapedesis and migrate through the vessel wall. Morphological studies on the interaction of intravascular tumor cells with endothelial cells revealed an initial tumor cell arrest with extensive contact to the endothelial cells during the first 8 h after intravenous tumor cell injection (40). Penetration by arrested tumor cells of vascular endothelial cell junctions commenced already after 4 h and intravascular proliferation was first noted 24 h after the initial arrest of the circulating tumor cells. Our results showing enhancement of metastasis by endogenous TNF and by exogenous TNF given 5 h before or 1 h after but not 24 h after tumor cell injection indicate an influence of TNF on one of the above

mentioned steps. This hypothesis is supported by in vitro experiments demonstrating an increased expression of cell surface adhesion molecules (9–13) and an augmented adherence of tumor cells to endothelial cell lines (17–19) induced by TNF. Miyata et al. (35) recently reported that a TNF mutein with a laminin-derived cell-adhesive peptide reduced experimental pulmonary metastases in contrast to wild type TNF. Furthermore, Giavazzi et al. (41) demonstrated that TNF and other cytokines induced the release of soluble ICAM-1 by tumor cells in vitro and that the serum level of ICAM-1 in nude mice showed a positive correlation with the tumor weight, suggesting an involvement of ICAM-1 release in the recognition of the tumor by the immune system.

A further possible mechanism leading to more and larger pulmonary metastases relies on an enhanced proliferation rate caused by TNF of the extravasated tumor cells. The number and content (ratio of number and area) of silver-stained proteins associated with AgNOR correlate with the cell proliferation (30). Rüschoff et al. (30) demonstrated a significant correlation between the AgNOR number and tumor cell proliferation, as determined by staining of tumor cells with Ki67, an immunohistochemical proliferation marker. Similar results were reported for neuroblastoma cell lines (42). In Table 2 we show that TNF treatment led to an increase of the AgNOR parameters in tumor cells 5 d after tumor implantation, indicating an augmented metabolic and proliferative activity. This effect was host dependent, for we could not demonstrate any influence of rmTNF on the tumor cell proliferation in vitro when tritiated thymidine uptake was measured (data not shown). However, recent data by Lollini et al. (43) demonstrated that TNF can enhance the metastasizing capacity by direct action on the tumor cells. In our experimental system, we did not see such an effect after pretreatment of tumor cells with TNF in vitro before inoculation.

Recent reports of a positive correlation of stimulated immune status determined by enhanced levels of neopterin, a macrophage-derived product, and IL-6 in tumor patients with bad prognoses and enhanced metastases are well in line with the data obtained in this study (44, 45). The observations reported here, enhancement of metastasis by endogenous and exogenous TNF and increased tumor cell proliferation in vivo induced by TNF treatment, suggest a promoting activity of TNF on the secondary spread of neoplastic cells and recommend a careful administration of this cytokine in cancer therapy.

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References

1. Carswell, E.A., L.J. Old, R.L. Kassel, S. Green, N. Fiore, and B. Williamson. 1975. An endotoxin-induced serumfactor that causes necrosis of tumor. *Proc. Natl. Acad. Sci. USA.* 72:3666.
2. Haranaka, K., N. Satomi, and A. Sakurai. 1984. Antitumour activity of murine tumour necrosis factor (TNF) against transplanted murine tumours and heterotransplanted human tumours in nude mice. *Int. J. Cancer.* 34:263.
3. Old, L.J. 1985. Tumor necrosis factor (TNF). *Science (Wash. DC).* 230:630.
4. Palladino, M.A., Jr., M.R. Shalaby, S.M. Kramer, B.L. Ferraiolo, R.A. Baughman, A.A. Deleo, D. Crase, G. Marifino, B.B. Aggarwal, I.S. Figari, et al. 1987. Characterization of the antitumor activities of human tumor necrosis factor alpha and the comparison with other cytokines: induction of tumor-specific immunity. *J. Immunol.* 138:4023.
5. Dinarello, C.A., J.G. Cannon, S.M. Wolff, H.A. Bernheim, B. Beutler, A. Cerami, I.S. Figari, M.A. Palladino, and J.V. O'Connor. 1986. TNF (cachectin) is an endogenous pyrogen and induces release of interleukin-1. *J. Exp. Med.* 163:1433.
6. Kohase, M., D. Hendricksen-DeStefano, L.T. May, J. Vilcek, and P.B. Sehgal. 1986. Induction of β2-interferon by tumor necrosis factor: a homeostatic mechanism in the control of cell proliferation. *Cell.* 45:659.
7. Munker, R., J. Gasson, M. Ogawa, and P. Koeffler. 1986. Recombinant TNF induces production of granulocyte-monocyte colony-stimulating factor. *Nature (Lond.).* 323:79.
8. Pfizenmaier, K., P. Scheurich, C. Schluter, and M. Krönke. 1987. Tumor necrosis factor enhances HLA-A,B,C and HLA-DR gene expression in human tumor cells. *J. Immunol.* 138:975.
9. Dustin, M.L., and T.A. Springer. 1988. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured cells. *J. Cell Biol.* 107:321.
10. Rothlein, R., M. Czajkowski, M.M. O'Neill, S.D. Marlin, E. Mainolfi, and V. Merluzzi. 1988. Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by pro-inflammatory cytokines: regulation by pharmacologic agents and neutralizing antibodies. *J. Immunol.* 141:1665.
11. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell.* 59:1203.
12. Marlor, C.W., D.L. Webb, M.P. Bombara, J.M. Greve, and M.L. Blue. 1992. Expression of vascular cell adhesion molecule-1 in fibroblastlike synoviocytes after stimulation with tumor necrosis factor. *Am. J. Pathol.* 140:1055.
13. Bevilacqua, M.P., S. Stengelein, M.A. Gimbrone, Jr., and B. Seed. 1989. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science (Wash. DC).* 243:1160.

14. Dayer, J.M., B. Beutler, and A. Cerami. 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. *J. Exp. Med.* 162:2163.
15. Frater-Schröder, M., W. Risau, R. Hallmann, P. Gautschi, and P. Böhlen. 1987. Tumor necrosis factor type α, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. *Proc. Natl. Acad. Sci. USA.* 84:5277.
16. Bertolini, D.R., G.E. Nedwin, T.S. Bringman, D.D. Smith, and G.R. Mundy. 1986. Stimulation of bone resorption and inhibition of bone formation in vitro by human TNF. *Nature (Lond.).* 319:516.
17. Rice, G.E., M.A. Gimbrone, and M.P. Bevilacqua. 1988. Tumor cell–endothelial interactions: increased adhesion of human melanoma cells to activated vascular endothelium. *Am. J. Pathol.* 133:204.
18. Dejana, E., F. Bertocchi, M.C. Bortolami, A. Regonesi, A. Tonta, F. Breviario, and R. Giavazzi. 1988. Interleukin 1 promotes tumor cell adhesion to cultured human endothelial cells. *J. Clin. Invest.* 82:1466.
19. Bereta, M., J. Bereta, S. Cohen, K. Zaifert, and M.C. Cohen. 1991. Effect of inflammatory cytokines on the adherence of tumor cells to endothelium in a murine model. *Cell. Immunol.* 136:263.
20. Malik, S.T, D.B. Griffin, W. Fiers, and F.R. Balkwill. 1989. Paradoxical effects of tumour necrosis factor in experimental ovarian cancer. *Int. J. Cancer.* 44:918.
21. Malik, S.T.A., S.M. Naylor, N. East, A. Oliff, and F.R. Balkwill. 1990. Cells secreting tumour necrosis factor show enhanced metastasis in nude mice. *Eur. J. Cancer.* 26:1031.
22. Naylor, S.M., S.T.A. Malik, W.H. Stamp, T. Jobling, and F.R. Balkwill. 1990. In situ detection of tumour necrosis factor in human ovarian cancer specimens. *Eur. J. Cancer.* 26:1027.
23. Balkwill, F., R. Osborne, F. Burke, S.M. Naylor, D. Talbot, H. Durbin, J. Tavernier, and W. Fiers. 1987. Evidence for tumour necrosis factor/cachectin production in cancer. *Lancet.* 2:1229.
24. Rakhmilevich, A.L., and R.J. North. 1991. Rapid acquisition of an enhanced capacity to produce tumor necrosis factor, alpha/beta interferon, and interleukin-6 after implantation of tumor cells. *Cytokine.* 3:398.
25. Männel, D.N., R. Jänicke, U. Westenfelder, B. Echtenacher, A. Kist, and W. Falk. 1990. Tumor-induced tumor necrosis factor production in macrophages. *Lymphokine Res.* 9:485.
26. Echtenacher, B., W. Falk, D.N. Männel, and P.H. Krammer. 1990. Requirement of endogenous tumor necrosis factor/cachectin for recovery from experimental peritonitis. *J. Immunol.* 145:3762.
27. Spitalny, G.L., and E.A. Havell. 1984. Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. *J. Exp. Med.* 159:1560.
28. Wexler, H. 1966. Accurate identification of experimental pulmonary metastases. *J. Natl. Cancer Inst.* 36:641.
29. Andus, T., P.C. Heinrich, J. Bauer, T.A. Tran-Thi, K. Decker, D. Männel, and H. Northoff. 1987. Discrimination of hepatocyte-stimulating activity from human recombinant tumor necrosis factor α. *Eur. J. Immunol.* 17:1193.
30. Rüschoff, J., K. Neumann, H. Contractor, K. Plate, and C. Thomas. 1990. Assessment of nucleolar organizer regions by automatic image analysis in breast cancer: correlation with DNA content, proliferation rate, receptor status, and histopathological grading. *J. Cancer Res. Clin. Oncol.* 116:480.
31. Beutler, B., V. Tkacenko, J. Milsark, N. Krochin, and A. Cerami. 1986. Effect of gamma interferon on cachectin expression by mononuclear phagocytes. Reversal of the lps-d (endotoxin resistance) phenotype. *J. Exp. Med.* 164:1791.
32. Tomazic, V.J., M. Farha, A. Loftus, and E.G. Elias. 1988. Antitumor activity of recombinant tumor necrosis factor on mouse fibrosarcoma in vivo and in vitro. *J. Immunol.* 140:4056.
33. Schultz, R.M., and M.G. Altom. 1990. Protective activity of recombinant murine tumor necrosis factor- alpha and interferon-gamma against experimental murine lung carcinoma metastases. *J. Interferon. Res.* 10:229.
34. Sylvester, D.M., S.Y. Liu, and G.G. Meadows. 1990. Augmentation of antimetastatic activity of interferon and tumor necrosis factor by heparin. *Immunopharmacol. Immunotoxicol.* 12:161.
35. Miyata, K., M. Kato, H. Shikama, K. Nishimura, N. Sakae, K. Kawagoe, T. Nishikawa, K. Kuroda, K. Yamaguchi, Y. Aoyama, et al. 1992. A YIGSR-containing novel mutein without the detrimental effect of human TNF-α of enhancing experimental pulmonary metastasis. *Clin. Exp. Metastasis.* 10:267.
36. Giavazzi, R., A. Garofalo, M.R. Bani, M. Abbate, P. Ghezzi, D. Boraschi, A. Mantovani, and E. Dejana. 1990. Interleukin 1-induced augmentation of experimental metastases from a human melanoma. *Cancer Res.* 50:4771.
37. Kramer, S.M., B.B. Aggarwal, T.E. Eessalu, S.M. McCabe, B.L. Ferraiolo, I.S. Figari, and M.A. Palladino. 1988. Characterization of the in vitro and in vivo species preference of human and murine tumor necrosis factor-α. *Cancer Res.* 48:920.
38. Brouckaert, P., C. Libert, B. Everaerdt, and W. Fiers. 1992. Selective species specificity of tumor necrosis factor for toxicity in the mouse. *Lymphokine Cytokine. Res.* 11:193.
39. Tartaglia, L.A., and D.V. Goeddel. 1992. Two TNF receptors. *Immunol. Today.* 13:151.
40. Crissman, J.D., J.S. Hatfield, D.G. Menter, B. Sloane, and K.V. Honn. 1988. Morphological study of the interaction of intravascular tumor cells with endothelial cells and subendothelial matrix. *Cancer Res.* 48:4065.
41. Giavazzi, R., R.G. Chirivi, A. Garofalo, A. Rambaldi, I. Hemingway, R. Pigott, and A.J. Gearing. 1992. Soluble intercellular adhesion molecule 1 is released by human melanoma cells and is associated with tumor growth in nude mice. *Cancer Res.* 52:2628.
42. Derenzini, M., A. Pession, F. Farabegoli, D. Tere, M. Badiali, and P. Dehan. 1989. Relationship between interphasic nucleolar organizer regions and growth rate in two neuroblastoma cell lines. *Am. J. Pathol.* 134:925.
43. Lollini, P.L., C. deGiovanni, G. Nicoletti, A. Bontadini, P.L. Tazzari, L. Landuzzi, K. Scotlandi, and P. Nanni. 1990. Enhancement of experimental metastatic ability by tumor necrosis factor-alpha alone or in combination with interferon-gamma. *Clin. & Exp. Metastasis.* 8:215.
44. Reibnegger, G., H. Hetzel, D. Fuchs, L.C. Fuith, A. Hausen, E.R. Werner, and H. Wachter. 1987. Clinical significance of neopterin for prognosis and follow-up in ovarian cancer. *Cancer Res.* 47:4977.
45. Reibnegger, G., M. Krainer, M. Herold, H. Ludwig, H. Wachter, and H. Huber. 1991. Predictive value of interleukin-6 and neopterin in patients with multiple myeloma. *Cancer Res.* 51:6250.