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Mechanisms Involved in Metastasis Enhanced by Inflammatory Mediators

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The enhancement of tumor metastasis by concurrent inflammatory processes is mainly due to the cytokines TNF and IL-1. In the case of TNF this effect is not restricted to metastasis models as measured by in vivo colony formation but also found in experimental model systems of spontaneous metastasis. Direct effects on the tumor cells or interference with the host NK cell system did not seem to account for the observed TNF effect. Experimental evidence from different test systems rather points to TNF- or IL-1-induced enhanced adhesion of tumor cells to the endothelial cell layer as the underlying mechanism. Blocking of integrin-matrix interactions with monoclonal antibodies or competing peptides inhibited tumor cell adhesion to endothelioma cells in vitro and lung colony formation of tumor cells in vivo.

Key words: tumor necrosis factor, interleukin-1, lipopolysaccharide, LPS, endothelial cells, NK activity, adhesion

INTRODUCTION

The process of metastasis is a multistep event through which tumor cells have to pass in order to colonize a target tissue. Deriving from a primary tumor, they are bound to get into the blood or lymph circulation and later to adhere to the endothelial cell layer within the target organ of metastasis. In order to get some insight at the molecular level in the complexity of the many events of cell-cell interaction taking place, experimental systems have to be established which dissect the entire event of metastasis into individual steps. The endeavour to dissect such a complex series of interactions by using simplified assay systems bears the danger of concentrating on epiphenomena or artifacts which might be of no relevance to the in vivo situation. Therefore, the findings from in vitro assays or from simplified in vivo experimental systems have to be validated in the more complex in vivo experimental systems of spontaneous metastasis which are closer to the clinical situation or best in biopsies from clinical studies.

Since inflammatory cytokines like TNF, IL-1, IFNγ, etc. became available for clinical use, attempts have been made to utilize their antitumoral or immunomodulatory capacity for cancer therapy. These cytokines, however, due to their pleiotropic actions, also had the potential to exert effects adverse to the attempted goal of anticancer efficacy. Accordingly, several investigators have reported that the usage of inflammatory cytokines in vivo might actually enhance metastasis [1–5]. In order to clarify the basis for these observations, we established in vitro and in vivo adhesion assays for different tumor cells on endothelial cell layers to evaluate the contribution of some of the TNF-modulated molecules to the tumor cell-endothelial cell interactions. Furthermore, we intended to find out whether the observations made under these relatively artificial conditions have impact on experimental or spontaneous metastasis in mouse models.

MATERIALS AND METHODS

Mice

Female mice, 5–7 weeks of age were obtained from the Institut für Versuchstierforschung (Hannover, Germany) or from Charles River Wiga GmbH (Sulzfeld, Germany). The NK-deficient beige mice on a C57Bl/6 background were a generous gift from Dr. H. Mossmann, Max-Planck-Institut für Immunbiologie (Freiburg, Germany). The animals were kept in the central animal facility of the German Cancer Research Centre under specific-pathogen-free conditions or at the animal facility of the University Clinic of Regensburg throughout the experiments.

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Tumor Cells

CSF1 is a methylcholanthrene-induced fibrosarcoma cell line of C3H/HeN mouse origin. ESb is a highly metastatic subline of the methylcholanthrene-induced DBA/2 lymphoma L5178YE (Eb) [6]. L929 is a TNF-sensitive mouse fibrosarcoma cell line. Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 5 mM Hepes, 100 IU/ml penicillin, 0.1 mg/ml streptomycin (all from Gibco-BRL, Eggenstein, Germany). The mouse bEnd3 endothelioma cells [7] were kindly provided by Dr. W. Risau and were maintained in DMEM with high glucose (Gibco-BRL) containing 10% FBS (Gibco-BRL, low endotoxin). For adhesion assays cells were transfered to Labtek glass chamber slides (Nunc) and grown to confluency. All cells were kept at 37°C, 5% CO_2, and 90% humidity in air.

Reagents

Recombinant human tumor necrosis factor (rhTNF) with a specific activity of 9×10^6 U/mg and recombinant mouse tumor necrosis factor (rmTNF) with a specific activity of 8×10^7 U/mg were generous gifts from BASF/Knoll AG (Ludwigshafen, Germany). TNF was injected i.p. at different time points as indicated in the legends. The rat anti-mouse TNF mAb V1Q was purified and used as recently described [8]. Polyclonal rat IgG and LPS (from *Salmonella minnesota*) were purchased from Sigma Chemical Co. (St. Louis, MO).

Quantification of Metastases

The nonadherent tumor cells were washed twice and resuspended in HBSS. Either 2×10^5 cells/50 µl were inoculated subcutaneously into the neck or 1×10^5 cells/200 µl were injected into the lateral tail vein of C3H/He, DBA/2, or other mice, respectively. For quantification of lung metastases the animals were killed by cervical dislocation on day 11 or 12. After staining by endotracheal infiltration with 15% china ink solution (Rotring Werke KG, Hamburg, Germany), lungs were removed, fixed, and bleached in Fekete's solution as described [3]. For histological detection of lung or liver metastases mice were killed on day 4 after tumor inoculation, and the livers were removed and fixed in 4% buffered formalin. Paraffin sections were cut and stained with haematoxylin-eosin. Number and diameter of metastases were determined by analyzing the metastatic foci using a microscope.

Tumor Cell Adhesion Assay

CFS-1 cells (5×10^5/ml) in HBSS containing 2 mM Ca^2Cl_2 and 2 mM Mg^2Cl_2 and 10 mM Hepes were allowed to bind to bEnd3 endothelioma cells grown in Labtek chamber slides. bEnd3 cells were stimulated with rmTNF for 4 or 16 hr, respectively. The binding assay was performed for 60 min at room temperature without shaking. The slides were fixed in 2% glutaraldehyde/PBS after a brief wash in PBS. Cell binding was measured by counting 4 independent 40× fields by video microscopy using IMAGE 1.42 software. The counted area is approximately 0.25 mm^2.

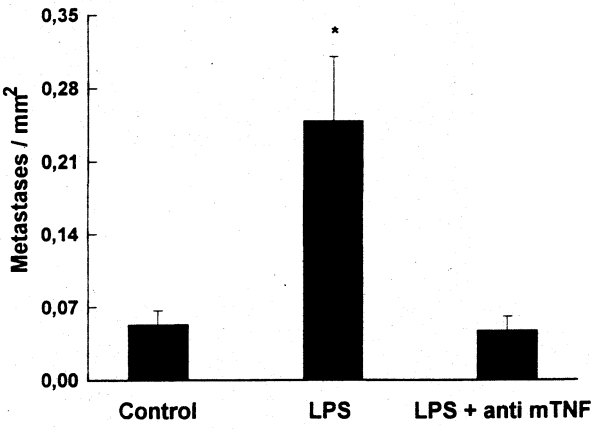

Fig. 1. Enhancement of fibrosarcoma CFS-1 colony formation in the lungs of animals after treatment with LPS and reversal of the metastasis-enhancement by antibodies to TNF. The animals received 20 µg LPS, 40 µg anti-mTNFmAk V1q, or 100 µl PBS, respectively, i.p., before i.v. application of the tumor cells. The number of colonies was determined in histological sections of the lungs 4 days after tumor cell application.

RESULTS AND DISCUSSION

Metastasis and Inflammation

Reports can be found in the literature focusing on the fact that inflamed tissue is a favored target of tumor cell colonization [9,10]. Enhanced tumor cell colonization to the lung was measured when we injected bacterial lipopolysaccharide (LPS) into mice 3 hr prior to the iv inoculation of tumor cells (Fig. 1). This LPS effect was reversed by blocking endogenous TNF production in the LPS-injected mice with mouse TNF-neutralizing antibodies, indicating that TNF was a mediator in this metastasis-enhancing effect of LPS. Other investigators found inhibition of LPS-enhanced metastasis by using IL-1ra [11]. Very clearly, dose-dependently enhanced tumor cell colonization to the lungs was observed after application of rhTNF, as well as rmTNF with i.v.-injected fibrosarcoma (CFS-1) [3] or with melanoma (B16) cells. Also, metastasis to the liver and spleen after i.v. injection of the highly metastatic thymoma cells (ESb) was enhanced by exogenous TNF (Fig. 2). TNF had to be given within 24 hr of tumor cell inoculation to exert its metastasis-enhancing effect.

TNF enhanced metastasis not only in the simplified tumor cell colonization model using i.v. tumor cell injection but also in a model of spontaneous metastasis. Spon-

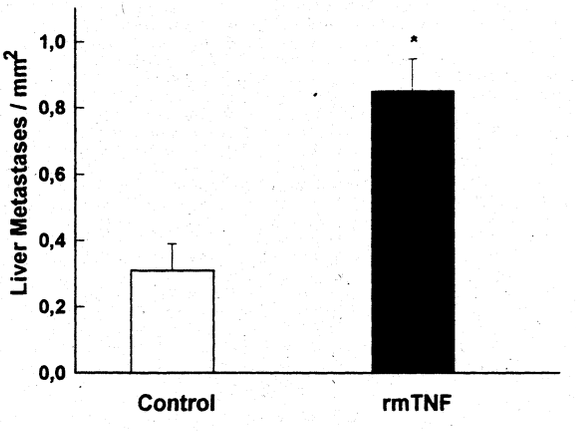

Fig. 2. Enhancement of thymoma ESb colony formation in the livers of animals after treatment with either 100 µl PBS (Control) or 10 µg rmTNF i.p. before i.v. application of the tumor cells. The number of colonies was determined in histological sections of the livers 4 days after tumor cell application.

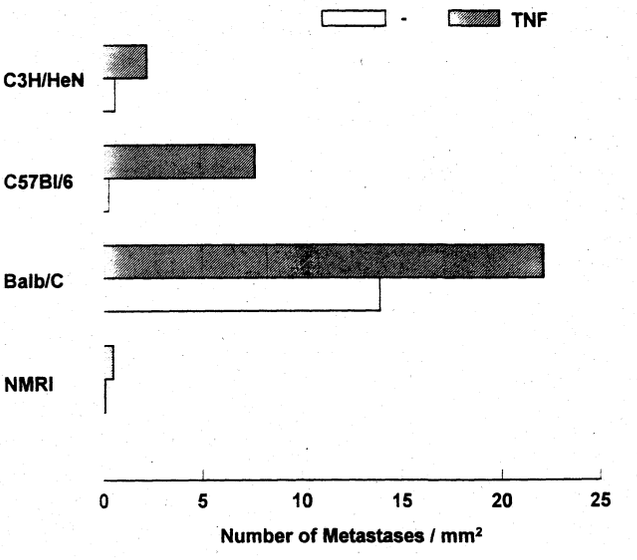

Fig. 3. Enhancement of fibrosarcoma CFS-1 colony formation in the lungs of mice of different strains after treatment with either 100 µl PBS (open bars) or 5 µg rmTNF (hatched bars) i.p. before i.v. application of the tumor cells. The number of colonies was determined in histological sections of the lungs 4 days after tumor cell application.

taneous liver colonization of ESb thymoma cells originating from an established tumor was similarly enhanced by TNF. In this case, TNF had to be injected at the time when individual ESb tumor cells were dislodged from the solid primary tumor and found in circulation (day 7) [12] (Orosz et al., manuscript submitted).

Taken together, enhanced metastasis has been observed in several different systems of experimental metastasis with IL-1, TNF, and IFN-γ [1–4.13]. Tumor cells were either coinjected with cytokines [1.3] or tumor cells stably transfected with cytokine genes were used [4,5]. In some of these models even in vitro pretreatment of tumor cells with IL-1 or TNF, respectively, enhanced colony formation in vivo. When this was tried with CFS-1 or ESb with rmTNF as activator in our systems we could not detect any significant enhancement of metastasis. This could possibly be explained by the fact that other tumor cells were used in the described experiments. In the case of Lollini et al. [13] the B16 melanoma cells displayed enhanced MHC class I antigen expression after exposure to TNF which could possibly have lead to reduced NK sensitivity. Such enhanced class I antigen expression was neither detectable in the CFS-1 nor the ESb cells used by us.

Mechanisms Involved in TNF-Enhanced Metastasis

T-cell responses based on MHC incompatibilities did not seem to play a role in the enhancement of colony formation by i.v. injected tumor cells. The metastatic capacity of C3H-derived fibrosarcomas (CFS-1) differed in the tested mouse strains, inducing hardly any lung colonies in NMRI animals (Fig. 3). In the other strains used, clear differences in the extent of metastasis were found with the following hierarchy of metastasis formation: C3H < C57Bl/6 < BalbC. However, in all cases, significant stimuation of colony formation was induced by the application of TNF independently of the basic levels of tumor cell colonization in the respective mouse strain (Fig. 3).

In vitro as well as in vivo some enhanced proliferative activity of the tumor cells was found after TNF treatment [3]. However, the average size of the lung colonies was not different in TNF- versus un-treated mice which indicates that direct growth promoting activity of TNF probably did not account for the observed enhanced metastasis.

Participation of the NK Cell System

NK activity is an important protective mechanism that accounts to a large degree for tumor cell destruction in the circulation. Since we found enhanced NK activity in spleen cells from animals which had been injected i.v. with fibrosarcoma cells, impairment of such NK activity could explain the enhanced metastasis observed after TNF treatment. Indeed, spleen NK activity was reduced 24 hr after TNF injection supporting the idea of an impaired NK system by TNF being the reason for enhanced metastasis. However, when NK-deficient mice were used for the experiments, the number of metastases in general was higher than in normal mice, as had been expected and published [14,15]; and, as shown in Figure 4, TNF still exerted a metastasis-enhancing effect in these animals. This made it clear that the enhancing effect of TNF

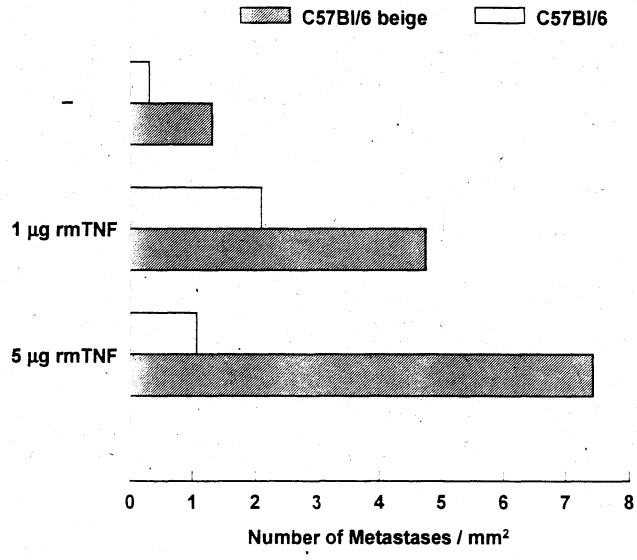

Fig. 4. Enhancement of fibrosarcoma CFS-1 colony formation in the lungs of C57Bl/6 mice (open bars) or NK-deficient C57Bl/6 beige mice after treatment with either 100 µl PBS or 1 µg or 5 µg rmTNF i.p. before i.v. application of the tumor cells. The number of colonies was determined in histological sections of the lungs 4 days after tumor cell application.

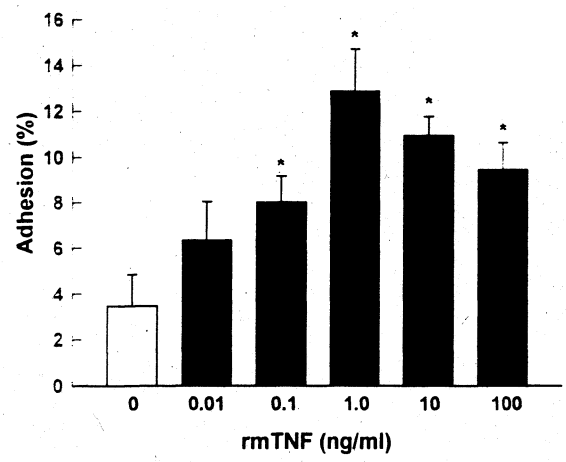

Fig. 5. Adhesion of fibrosarcoma CFS-1 tumor cells to TNF-activated endothelioma bEnd3 cells. A confluent layer of bEnd3 cells was exposed to different concentrations of rmTNF for 12 hr before the tumor cells were added. Adherent tumor cells were counted after 60 min.

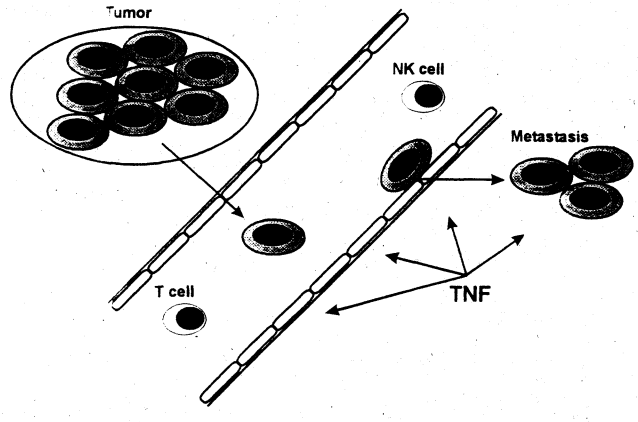

Fig. 6. Schematic presentation of cells and cell-cell interactions possibly affected by TNF during extravasation of tumor cells.

on metastasis formation was not only due to impairment of NK activity.

Tumor Cell Endothelial Cell Adhesion

Another facet of the spectrum of TNF activities which has to be kept in mind as possibly contributing to the enhancement of tumor cell metastasis is the strengthened adhesion of tumor cells to endothelium after TNF exposure. In several in vitro adhesion assays it has been shown that pretreatment of endothelial cells with TNF leads to enhanced adhesion of tumor cells [16–18]. We also found that preexposure of mouse endothelioma cells (bEnd3) resulted in a TNF-dose-dependent enhancement of adhesion of ESb and CFS-1 tumor cells (Fig. 5). In the case of ESb cells this adhesion was blocked by the addition of monoclonal antibodies which interfered with the interaction of VLA-4 on the tumor cells and VCAM-1 on the endothelioma cells (Orosz et al., manuscript submitted). It remains to be tested whether the VLA-4-VCAM-1 interaction is of importance for formation of spontaneous liver metastases by ESb also in vivo.

Preliminary experiments showed that lung colony formation using the CFS-1 fibrosarcoma cells was abolished by the injection of peptides containing the RGD motif, an amino acid sequence which is common in extracellular matrix proteins and responsible for integrin binding. Similarly, it has been published that a TNF fusion protein which contains the laminin-specific YIGSR motif on the N-terminus did not exhibit enhancement of metastasis any longer [19]. This indicates that integrin interactions via the fibronectin or laminin receptors are important for the observed TNF effect. Experiments utilizing neutralizing monoclonal antibodies to ICAM-1 or LFA-1 molecules have to be performed in NK-deficient mice in order to avoid interference with the TNF-responsive NK system. These experiments would discriminate between the enhancing effect due to impaired NK activity and the metastasis inhibitory effect based on reduced tumor cell adhesion. The contribution of other adhesion molecules like CD44 [20] or E-selectins [21] for metastasis are well documented. Whether these surface structures are also involved in the phenomenon of TNF-enhanced metastasis has not been tested so far.

CONCLUSION

The complex process of metastasis can become affected at many steps by mediators of inflammation.

Figure 6 only depicts some of the cells and cell-cell interactions which are relevant to the tumor cell extravasation. We have attempted to analyze which steps are involved in TNF-enhanced metastasis using different experimental models. Our findings are in good accordance with the literature, indicating that the most important mechanism for enhanced metastasis under inflammatory conditions seems to be the TNF- or IL-1-increased adhesion of tumor cells to the capillary endothelial cells.

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