Brief Definitive Report

Expression of Tumor Necrosis Factor by Different Tumor Cell Lines Results either in Tumor Suppression or Augmented Metastasis

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Summary

Tumor necrosis factor (TNF) produced by tumor cells after gene transfer can effectively suppress the growth of locally growing tumors. We wanted to test the effects of "local" TNF on the growth of a highly metastatic cell line. Therefore, a recombinant retrovirus allowing expression of the TNF gene by the β-actin promotor has been constructed and used to infect the two tumor cell lines EB and ESB, which grow as solid tumor or metastasize, respectively. Expression of TNF by EB cells resulted in their rapid and dose-dependent rejection. In sharp contrast, mice injected with ESB cells producing similar amounts of TNF showed no signs of tumor suppression, but rather had reduced survival rates that correlated with enhanced hepatic metastases. The accelerated formation of liver metastases by ESB TNF cells could be reversed by an anti-TNF mAb. These results demonstrate the opposite effects TNF may have on tumor growth: suppression of a locally growing tumor and promotion of metastasis formation.

TNF has been identified by its direct cytotoxic effects on some tumor cells. In the meantime, however, it has become clear that TNF is a multifunctional, immunoregulatory cytokine with a broad spectrum of activities on hematopoetic and nonhematopoetic cell types (for review, see references 1 and 2). Its tumoricidal activity in vivo seems to be mediated largely by immunological mechanisms (3, 4). Several TNF gene–modified apparently nonmetastasizing tumor lines have been shown to be suppressed in vivo involving both T cell–independent and –dependent mechanisms (5–10). It is currently not known which molecular mechanisms are involved in the TNF-induced antitumor response. However, it is reasonable to assume that the rapid and strong inflammatory reaction in TNF-producing tumors (6, 10) is influenced by the action of TNF on endothelial cells, e.g., the induction of chemotactic factors for neutrophils or macrophages (11, 12), which could facilitate invasion of tumor-reactive cells. Moreover, TNF has been shown to induce several adhesion molecules on endothelial cells (13) and modulates endothelial cell coagulant properties, thereby increasing vascular permeability (14). Similar mechanisms that cause inflammation in TNF-producing tumors could also contribute to metastasis, which involves invading the host tissue, penetrating the blood vessels' endothelium, and establishing growth at a secondary site. Due to these potentially opposite effects of TNF on tumor growth, we have addressed the question of which activity of TNF would dominate, its tumor suppressive effect or its possible involvement in tumor cell movement when expressed by a cell line that has the potential to metastasize.

Materials and Methods

Cell Lines. EB is a methylcholanthrene-induced DBA/2 T lymphoma formerly called L5178Y, and ESB (previously L5178Y-M) is a spontaneous metastasizing variant of EB (15, 16). Both cell lines were cultured in RPMI 1640, 10% FCS, and antibiotics. The culture medium of ESB cells contained additionally 50 μm 2-ME. Helper virus–containing cell lines psi2 (17) and PA317 (18) were cultured in DME, 10% FCS.

Generation of Recombinant Retrovirus and Viral Infection. The mouse TNF cDNA was isolated by reverse PCR using primers specific for regions that contain translation start or stop signals, respectively, and additionally BamHI sites to facilitate cloning (5' CTGGATCCAGAAAAGACACCATGAGCACA 3' and 5' ATGGATCCACACCCATTCCCTTCACAGA 3'). Substrate for PCR was cDNA derived from LPS-stimulated mouse spleen cells. Activation of spleen cells by LPS, RNA preparation, cDNA synthesis, and PCR was done as described (19). The 0.74-kb TNF cDNA BamHI fragment was used to replace the neomycin gene of vector pBA-neo (20). This plasmid (pBA-TNF; 6) was digested with XhoI and partially with BamHI, a ~1-kb fragment containing the β-actin promotor, and the mouse TNF cDNA was provided with XhoI linker (1030; New England Biolabs, Schwalbach, FRG) and cloned into vector N2 (21). The resulting vector pNBT was supposed to express TNF by the β-actin promoter and the neomycin gene by

the retroviral LTR. Retroviruses were generated as described (6). Briefly, the amphotropic packaging line PA317 was transfected using an eucaryotic transfection kit (200285; Stratagene, La Jolla, CA) with plasmid pNBT, and cells resistant to G418 (1 mg/ml) were selected (PA317-NBT). Then, the ecotropic packaging line psi2 was infected by overnight exposure to the culture supernatant of PA317-NBT cells in the presence of 5 μg/ml polybrene, and G418-resistant cells were selected (psi-NBT). Cell-free conditioned medium (subconfluent monolayers cultured for 24 h) contained 5×10^4/ml (PA317-NBT cells) or 3×10^6/ml (psi-NBT cells) $G418^R$ CFU, which was determined by infection of NIH3T3 cells with serial dilutions of supernatant of either cell culture. Similarly, EB and ESB cells were infected (10^6 cells in 4 ml culture medium supplemented with 4 ml virus-containing culture supernatant of psi-NBT cells and 5 μg/ml polybrene for 16 h) and cloned after G418 selection. All infected EB or ESB cells used in this study were negative for replication-competent helper virus as judged by the inability of culture supernatants of the cells grown for at least 2 wk to generate $G418^R$ colonies upon exposure to NIH3T3 cells.

TNF Bioassay. TNF activity was measured by the L929 cytotoxicity assay as described (6) with recombinant mouse TNF (B1243; Genzyme, Cambridge, MA) as standard. A neutralizing anti-TNF mAb (V1q; 22) was used at a concentration of 1 μg/ml for specificity control. Detection limit of the L929 assay was 4 pg/ml.

Analysis of Tumorigenicity. An indicated number of cells was injected subcutaneously into the belly region of 6–8-wk-old female DBA/2 mice (Bomholtgard, Ry, Denmark) in a volume of 0.2 ml in Dulbecco's PBS. Tumor size was measured as described (6). Hepatic metastases were macroscopically evaluated at different time points after tumor cell injection. In some experiments, primary tumors or metastases were excised and recultured in order to determine TNF expression after in vivo growth. To analyze ESB metastasis in the presence of anti-TNF mAb, 200 μg V1q mAb or isotype-matched control mAb were injected together with tumor cells subcutaneously, and the same dose of mAbs was injected intraperitoneally on days 0, 2, 4, 6, and 8.

Results

TNF Expression by Tumor Cell Lines EB and ESB after Infection with a TNF-specific Retrovirus. For expression of TNF in tumor cell lines EB and ESB, a Moloney murine leukemia virus–based retroviral vector named pNBT was constructed that contains the neomycin gene as selectable marker and the mouse TNF gene under the control of viral LTR and β-actin promotor, respectively (Fig. 1). Virus-producing cells (psi-NBT) were established and used to infect EB and ESB cells. After infection, $G418^R$ cells were cloned and TNF activity in the culture supernatant was determined. Neither EB nor ESB cells produced detectable amounts of TNF (Table 1). The bulk culture of infected EB cells (EB-TNF) or clones derived thereof (EB-T9 and EB-T11) produced between 15 and 80 pg/ml TNF. The bulk culture of infected ESB cells (ESB-TNF) and derived clones (ESB-T2 and ESB-T7) produced up to 200 pg/ml TNF. The cytotoxic activity on L929 cells of all retrovirally infected cells could be neutralized by an anti-TNF mAb (not shown). It should be noted that both EB and ESB cells were resistant to TNF, and TNF expression did not alter the growth rates of the cells in vitro. As control, ESB cells were also infected with control virus N2,

NBT :

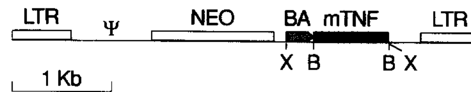

Figure 1. Structure of recombinant retrovirus used for TNF expression. *LTR*, long terminal repeat; *neo*, neomycin gene; *BA*, β-actin promotor; *mTNF*, mouse TNF cDNA; *X*, XhoI; *B*, BamHI.

Table 1. *TNF Expression after Gene Transfer into Cell Lines EB and ESB*

Cell line	*TNF activity*
	pg/ml per 10^6 cells/24 h
EB	–
EB-TNF (bulk)	15
EB-T9	80
EB-T11	15
ESB	–
ESB-TNF (bulk)	20
ESB-T2	200
ESB-T7	130
ESB-N (clones 1–5)	–

Table 2. *Tumor Suppression Resulting from TNF Expression by EB Cells*

Exp.	Cell line	Injected cells	No. of mice with tumor/ no. of mice injected
		$\times 10^6$	
1	EB	2.5	5/5
	EB-TNF (bulk)	2.5	2/4 (2)
2	EB	2.5	10/10
		1.0	9/10
		0.5	5/5
		0.1	4/5
	EB-T9	2.5	3/8 (3)
		1.0	0/5
		0.5	0/5
		0.1	0/5
	EB-T11	1.0	3/5 (2)

Cells at the indicated number were injected subcutaneously into DBA/2 mice and tumor growth was observed for 4 mo. The numbers of mice that transiently developed a tumor are given in parentheses.

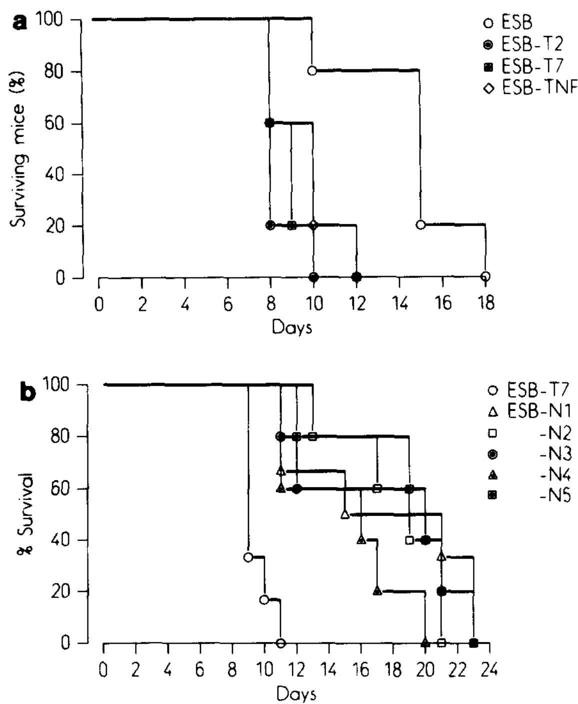

Figure 2. Mortality curves of mice injected subcutaneously with the indicated cells. (*a*) Parental ESB cells have been compared with TNF-transduced clones ESB-T2 and ESB-T7 and bulk culture ESB-TNF. (*b*) TNF-transduced ESB-T7 cells have been compared with five clones of mock-infected ESB cells. Each group consisted of five or six mice.

and five G418^R clones were established (ESB-N1 to -N5) that did not produce TNF.

Tumor Growth of TNF-producing EB Cells Is Suppressed. EB cells and their TNF-producing derivatives were injected into syngeneic DBA/2 mice and tumor growth was analyzed (Table 2). Injection of 2.5 × 10^6 EB and EB-TNF (bulk) cells showed that EB cells grew as a tumor in all cases, whereas EB-TNF cells were either rejected or developed to a tumor with delay. In a second experiment tumor growth in mice injected with clones EB-T9 and EB-T11 was compared with that of EB cells. The low TNF–producing EB-T11 cells grew as a tumor with significant delay or were rejected. EB-T9 cells (producing higher amounts of TNF) were completely rejected when up to 10^6 cells were injected. At 2.5 × 10^6 cells rejection occurred in five of eight mice. In contrast, nearly all mice injected with parental EB cells developed a tumor when as few as 10^5 cells were injected. These results confirm

Table 3. *Survival Rates of DBA/2 Mice Injected with Decreasing Numbers of Cells*

Cell line	Cell number	50% survival in days (± SD)
ESB	10^5	28.2 ± 9.1 (5/5)
	10^4	31.8 ± 11.5 (5/5)
	10^3	37.3 ± 14.8 (4/5)
ESB-T7	10^5	13.2 ± 3.8 (5/5)
	10^4	21.0 ± 6.5 (5/5)
	10^3	19.6 ± 5.4 (5/5)

The numbers of mice having died per number of mice in experiments are given in parentheses.

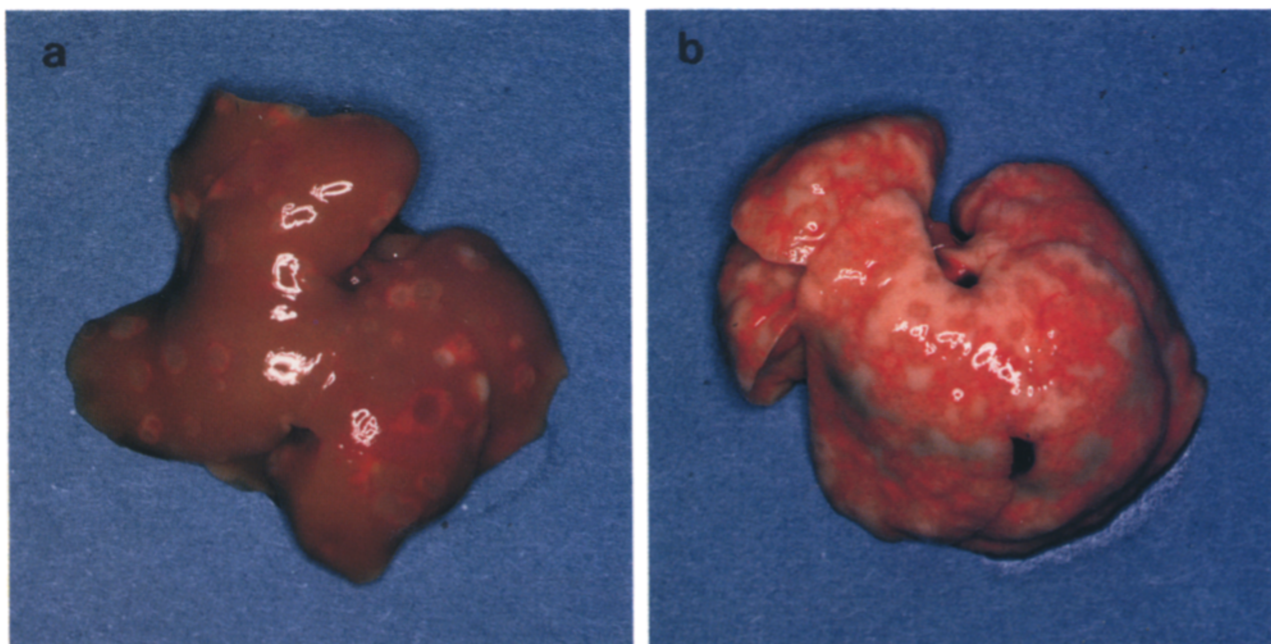

Figure 3. Liver metastases in mice injected subcutaneously with 2.5 × 10^6 ESB (*a*) or TNF-producing ESB-T2 cells (*b*) 8 d before. This result was confirmed in at least five other mice per group injected with ESB compared with ESB-T2 or ESB-T7 cells.

Table 4. *Treatment with an Anti-TNF mAb of Mice Injected with Indicated Cells Results in Reduction of Liver Metastases*

Cell line	mAb injected	Mean survival	Metastases in liver of moribunds	No. of livers analyzed
		d		
ESB-T2	–	9.6 ± 0.5	+ + +	3
ESB-T2	Anti-TNF	8.2 ± 0.4	+	5
ESB-T2	Control	8.6 ± 0.9	+ + +	4
ESB-T7	–	8.2 ± 0.4	+ + +	3
ESB-T7	Anti-TNF	8.4 ± 0.5	+	4
ESB-T7	Control	7.8 ± 0.4	+ + +	2

Mice were injected subcutaneously with 2.5 × 10^6 cells and mAbs as indicated. The experiment was done with five mice per group. Those mice that were accessible when moribund were analyzed for liver metastases. +, liver was clearly enlarged with <100 macroscopically visible metastases; + + +, metastases spread over the whole liver (>1,000).

observations with several other TNF gene–transfected locally growing tumors that rejection occurred in a TNF dose– and cell dose–dependent manner.

TNF Production by ESB Cells Accelerates Metastasis and Animal Death. ESB cells and their derivatives were injected into DBA/2 mice. At 2.5 × 10^6 cells, all ESB-injected mice died, with a mean survival of 14.6 ± 2.9 d (Fig. 2 *a*). All mice injected with TNF-producing cells ESB-TNF, -T2, or -T7 also died, however, within 8–10 d on average. Similar results were obtained in three other experiments. The difference in survival rates of mice was even more obvious when lower numbers of ESB-T7 in comparison with ESB cells were injected (Table 3). To exclude the possibility that the reduced survival of mice injected with TNF-producing cells resulted from cloning artifacts, mice were injected with mock-infected clones ESB-N1 to -N5 in comparison with ESB-T7 cells (Fig. 2 *b*). Mice from all groups injected with non-TNF-producing cells survived significantly longer ($p < 0.005$) than those injected with TNF-producing ESB-T7 cells.

Since metastasis of ESB cells into a number of organs, most obviously into the liver, is well established (16), livers of mice injected 8 d before with either ESB-T2 or ESB cells were macroscopically analyzed. Whereas livers of ESB cell–injected animals contained <100 metastases, ESB-T2 cell injection led to livers spread with >1,000 metastases. An example that was similar in all analyzed cases (each with five livers of ESB-, ESB-T2-, and ESB-T7-injected mice) is shown in Fig. 3. Recultivation of tumors and liver metastases from mice injected with ESB-T2, ESB-T7, and ESB cells and detection of TNF activity in the culture supernatant confirmed that ESB-T2 and ESB-T7 tumors, but not ESB tumors or metastatic cells, still produced TNF (not shown).

To directly attribute the increasing number of liver metastases to TNF, ESB-T2 and -T7 cells were injected into DBA/2 mice in parallel with a neutralizing anti-TNF mAb, a control mAb, or without mAb. Mice that were moribund were analyzed for liver metastases (Table 4). While mice injected with ESB-T2 or -T7 cells alone or control mAb had widespread liver metastases, the liver of those mice injected with the anti-TNF mAb were enlarged but barely showed metastases. However, the reduction in liver metastases by the anti-TNF mAb did not correlate with a longer survival time in any of the groups.

Discussion

By TNF gene transfer into ovary (5), skin (7), plasmacytoma (6), or sarcoma tumor cell lines (8), it has been shown that TNF locally expressed at the tumor site effectively suppresses tumor growth. This observation is extended here to EB cells previously classified as T cell lymphoma (16). The results with EB cells also confirm that low nontoxic amounts of TNF produced by the tumor cells are sufficient to exert effective antitumor activity. In previous studies the antitumor effect of transfected TNF has consistently been shown to be immunologically mediated and $CD8^+$ T cells were required for complete tumor eradication (8, 10). A similar mechanism seems likely for EB cells, which are immunogenic and elicit an effective cytolytic $CD8^+$ T cell response in the absence of $CD4^+$ T cells (23).

The ESB tumor arose in a mouse after injection of EB cells (15) and, therefore, both cell lines seem to be clonally related. ESB cells are characterized by a particularly fast metastatic spread into various organs, most pronounced in the liver (16). In contrast to EB, TNF expression by ESB cells did not cause tumor suppression but rather enhanced metastatic spread and reduced the survival time of mice injected with these cells. The acceleration of hepatic metastasis of TNF-producing ESB cells was revertible by an anti-TNF mAb, which also slightly reduced liver metastasis in mice injected with parental ESB cells (data not shown), suggesting some contribution of host's TNF for tumor cell spread. The reason why anti-TNF mAb application did not prolong survival of mice injected with TNF-producing cells is not clear, but could be explained by a failure of the mAb to efficiently prevent metastasis into other organs. However, it argues against the possibility that reduced survival resulted from liver toxicity by TNF.

The mechanism by which TNF increased hepatic metastases may involve any of its multiple effects on endothelial cells. TNF upregulates vascular cell adhesion molecule, intercellular adhesion molecule, and endothelial leukocyte adhesion molecule 1 on endothelial cells (13) and directly causes transendothelial cell migration of PMN (14). Notably, ESB cells are LFA-1$^+$, VLA-4$^+$, and Mel14$^+$, whereas EB cells are LFA-1$^+$, VLA-4$^-$, and Mel14$^-$ (data not shown). Thus, ESB cells express ligands for adhesion molecules on endothelial cells, which could facilitate early arrest in the circulatory system and enable ESB-derived TNF to allow stabilization of this interaction by upregulation of adhesion molecules on endothelial cells. Furthermore, local TNF may stimulate transendothelial migration of ESB cells, similar as has been observed for neutrophils in in vitro systems (for review, see reference 14). In this regard it is of interest that TNF-activated vascular endothelium increases adhesion of human melanoma cells (24) and that some melanoma lines express TNF (25). The precise mechanism, however, by which TNF promotes metastasis of ESB cells has to be determined.

The failure of TNF to suppress ESB metastasis could relate to the rapid dissemination of ESB cells and/or to the inability of ESB-derived TNF to induce an efficient inflammatory response to the tumor, unlike what had been observed in other models (6, 8, 10). Evidence for the latter possibility was obtained from immunohistologic analysis. Like ESB cells, ESB-T7 tumors were not infiltrated by T cells (CD4$^+$ and CD8$^+$) within the first 8 d, nor was the CR3$^+$ cell infiltrate increased in comparison to ESB tumors (data not shown). Furthermore, we did not observe any growth retardation of TNF-producing ESB cells at the injection site until death of the animals.

TNF has previously been implicated in metastasis. Giavazzi et al. (26) have described augmented experimental metastases in nude mice injected intravenously with TNF. Malik et al. (27) have observed that intraperitoneally injected TNF-producing CHO cells killed the mice earlier than control CHO cells and showed a more pronounced tumor infiltration of the peritoneal wall. The peritoneum in this system seems to be a privileged site since the same cells injected subcutaneously were suppressed in growth (5). The site of TNF production has been shown to determine its metabolic effects (28). Our results differ from previous ones by: (*a*) analyzing metastasis in subcutaneously rather than intravenously injected animals; (*b*) providing TNF locally and at an injection site that (with the exception of ESB cells) consistently revealed an effective antitumor response; and (*c*) analyzing metastasis in immunocompetent mice. Therefore, our results allow direct comparison of the tumor-suppressive with the tumor-promoting effects of TNF. However, we cannot exclude that with other metastatic tumor cells that spread slower and via different mechanisms the tumor-suppressive activity of TNF dominates over its involvement in metastases.

Finally, our results may have implications for immunotherapeutic approaches in cancer treatment. The use of TNF gene–transfected tumor cells as vaccines (29) raises the question as to whether such cells are reliably able to elicit an antitumor response and, if tumor cells have already metastasized, whether TNF expression might favor the metastatic process. On the other hand, the genetic engineering of tumor-infiltrating T lymphocytes to produce TNF could indeed alter their in vivo characteristics (30), in particular with respect to their motility. At least for ESB cells, <100 pg/ml secreted TNF was sufficient to promote cell movement. Whether TNF-producing tumor-specific T cells localize more efficiently to the tumor is under investigation.

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