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RESEARCH REPORT

Tumor-Induced Tumor Necrosis Factor Production in Macrophages

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ABSTRACT

Tumor-associated tumor necrosis factor (TNF) production in patients as well as a TNF-inducing membrane constituent of tumor cells have been reported. In a murine fibrosarcoma model we analyzed TNF production during growth of a tumor transplant. *In situ* hybridization showed that a gradually increasing number of cells within the tumor tissue became positive for TNFmRNA. Also, in spleen cells of tumor-bearing mice TNFmRNA became more abundant in later stages of tumor growth compared to early stages. In plasma of these animals, however, TNF activity was not detected at any time even after stimulation with bacterial endotoxin. Neutralization with monoclonal antibodies of endogenous TNF during tumor growth did not affect the growth rate of the tumor, indicating that either the antibodies did not reach the relevant TNF production and action sites or that endogenously produced TNF did not play a significant role in this tumor model.

INTRODUCTION

TNF is a product mainly of activated monocytes/macrophages which besides its cytotoxic activity *in vitro* for some tumor cells exerts a plethora of effects on many different kinds of cell types. Due to numerous biologically important functions, TNF is thought to play a key role in regulation of the nonspecific host response in inflammation. Monocytes of cancer patients have been shown to spontaneously release significantly higher amounts of TNF into the supernatant when compared to controls (1). Also, enhanced levels of TNF in serum or plasma of cancer patients have been reported (2,3). In these patients, after resection of the tumors the TNF levels returned to that of controls (3).

In addition, we were able to demonstrate that tumor membrane constituents directly activate human monocytes for TNF production (4). TNFmRNA expression by tumor infiltrating macrophages has also been reported recently (5). In order to investigate whether TNF production correlates with tumor growth, we determined TNF in plasma and TNFmRNA in tumors and spleens of mice inoculated intradermally with fibrosarcoma cells. In addition, to address the question of the role of the observed tumor-induced TNF the animals were treated with anti-murine TNF monoclonal antibodies throughout the experiment.

MATERIALS AND METHODS

Tumor Cells: Methylcholanthrene-induced fibrosarcoma cells CFS1 were generated on a C3H mouse and kept at the German Cancer Research Center. The cells were grown as single cells in tissue culture in RPMI 1640 (Gibco) with 10% FCS. The cells were washed in phosphate buffered saline (PBS) and $1x10^6$ CFS1 cells in 50µl PBS were injected intradermally into the flanks of C3H mice (Staatl. Versuchstieranstalt, Hannover, F.R.G.).

Northern Blot Analysis: Cytoplasmic RNA was prepared from the spleens of individual mice at different times during tumor growth, electrophoresed in 1% agarose-formaldehyde gels. RNA was transferred to nylon filters and hybridized as previously described (4). A TNFcDNA probe (a 750 bp Eco RI-fragment of the coding region of human TNFcDNA) was labeled with ^{32}P by the random primer method. After autoradiography the filters were stripped and hybridized with a human β-actin cDNA probe (a 560 bp Sal 1-Eco RI fragment of β-actin cDNA). The hybridization signals were quantified by scanning the optical density of the autoradiograms. The TNFmRNA signals were evaluated by normalization using the respective actin mRNA signals.

In Situ Hybridization: Tumor tissue was frozen in liquid nitrogen immediately after excision and kept at -70^0C until the preparation of 5mm cryosections. Fixation and hybridization was performed as described recently (4).

TNF Bioassay and ELISA: Blood was collected from the retroorbital plexus at indicated times. Plasma was prepared and stored at -20^0C until tested for TNF activity in the L929 bioassay (7) (in the presence of actinomycin D) and in a specific ELISA for murine TNF. A monoclonal rat anti murine TNF antibody was generated by immunization of rats with purified natural murine TNF and fusion of the spleens of these rats with P3.X63.Ag8.653. One clone (V1q) was selected which neutralized murine TNF. 1µg V1q Ig neutralized 80 pg of recombinant murine TNF (rmTNF, Knoll AG, Ludwigshafen, FRG). In vivo, 20 µg V1q given ip were able to protect mice from LPS-induced lethal shock (Echtenacher, B. et al., manuscript submitted). For the TNF-specific ELISA, plates were coated with purified V1q (10µg/ml). After incubation of the coated wells with serial dilutions of the test samples biotinylated protein A-purified polyclonal rabbit anti-rmTNF (Knoll AG, Ludwigshafen, FRG) Ig (15µg/ml) was applied. The enzymatic activity after reaction with streptavidin-peroxidase and tetramethylbenzidine (Sigma) as peroxidase substrate was determined at 5 to 20 min. Sensitivity of the ELISA was 1 ng/ml for rmTNF.

Interleukin 6 (IL6) Assay: The IL6 determination was performed using a 4 day proliferation assay with the IL6-dependent hybridoma B9 (8).

Immunohistochemistry: Cell-associated TNF in tumor sections was determined with a protein A-purified rabbit anti-rmTNF Ig (Knoll AG, Ludwigshafen, FRG) and peroxidase-coupled goat anti-rabbit antibody (Dianova, Hamburg, FRG). As substrate 3-amino-9 ethylcarbazole (AEC, Sigma) was used. The presence of macrophages/monocytes was determined by staining of tumor sections with anti-Mac-1 antibodies according to the supplier's protocol (Boehringer, Mannheim, FRG).

RESULTS

Mice received 10^6 CFS1 cells intradermally and blood was drawn regularly during tumor growth beginning on day 6. No TNF was detectable in the plasma of these tumor bearing animals at any time neither in the TNF bioassay nor in a TNF-specific ELISA system. Even after an injection of bacterial endotoxin (*S.minnesota* LPS, Sigma, 10µg/animal, ip) 2 hours before bleeding the mice, no TNF was found in the plasma. Low levels of interleukin 6 (IL6) were detectable in the tumor-bearing animals from day 17 on after tumor implantation. In plasma of animals which had received bacterial endotoxin IL6 was always present, but the concentration did not change significantly during tumor growth (data not shown).

mRNA from the spleens of tumor-bearing mice was prepared and analyzed in Northern blots. The signal specific for TNFmRNA increased during tumor growth and was highest at the latest time point (day 24) of the experiment (Fig.1). A peak of TNFmRNA on day 8 indicated a transient rise in TNF expression in the spleen during an early stage of tumor growth.

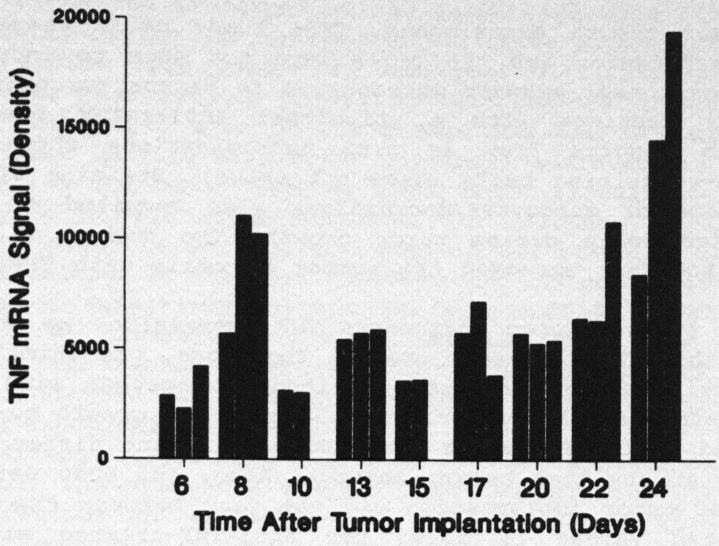

FIGURE 1: TNFmRNA Expression in Spleens of Tumor bearing Mice at Different Time Points after Tumor Implantation. The spleens of the mice were removed at the indicated times and TNFmRNA determined in Northern blot analysis. The TNFmRNA signal from spleens of non tumor bearing mice had a density of 6400.

In situ hybridization of TNFmRNA in tumor tissue revealed very few TNF positive cells at early stages of tumor growth. The number of cells positive for TNFmRNA increased gradually from day 6 on (tested on days 6, 8, 10, 13, 15, 17, 20, 22 and 24) and was maximal at the latest time point tested. Fig. 2 depicts typical TNFmRNA signals in tumor tissue from day 8 and day 20 of tumor growth. Though subjective and difficult to quantify, this gradual increase of cells showing TNFmRNA expression was highly reproducible in different sets of

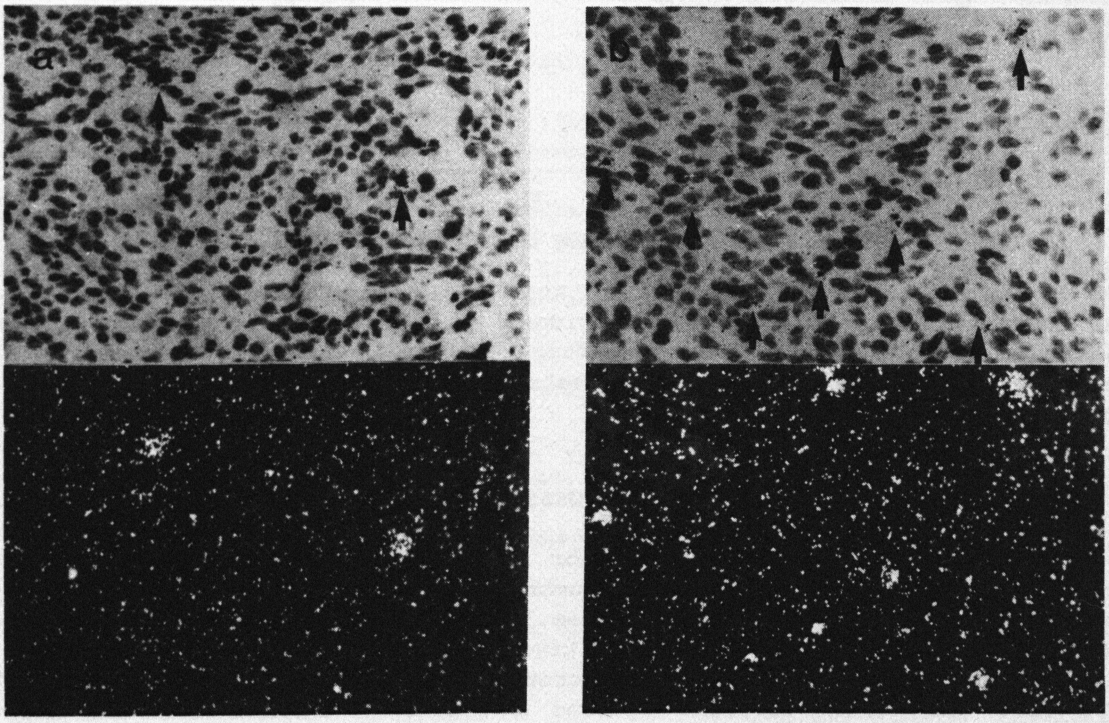

FIGURE 2: *In Situ* TNFmRNA Expression in Tumor Tissue. Tumor tissues from day 8 (a) and day 20 (b) after tumor implantation were hybridized with a TNF-RNA probe. The photographs display the same tissue area x 250 as light and dark field photograph, respectively.

hybridizations and tumor growth experiments. CFS1 tumor cells themselves were negative for TNFmRNA expression and the cells were not able to induce TNFmRNA expression in murine peritoneal exudate macrophages *in vitro*. Immunhistochemical staining of the tumor sections with a polyclonal anti-rmTNF immunoglobulin fraction supported the results from *in situ* hybridizations showing similar numbers of TNF protein-containing cells (data not shown). Staining of the tumor tissues for the presence of monocytes/macrophages also revealed an increasing number of Mac-1-positive cells during tumor growth. The number of phagocytes detected by staining, however, exceeded the number of cells with TNFmRNA signal considerably.

After detection of tumor-induced endogenous TNF expression, we attempted to determine the role of this TNF for tumor growth. Therefore, the animals received an ip injection of murine TNF-neutralizing antibodies together with the tumor transplant and every third day thereafter. Fig. 3 shows the growth curves of the tumors in untreated and anti-TNF-treated mice. No significant difference in the diameter of the tumors at any time became obvious. Also, the body weight of the animals on day 17 after tumor implantation did not vary between the two groups (15.6±1.3g for untreated versus 15.7±0.9g for anti-TNF-treated mice). Thus, application of V1q antibodies had no obvious effect on tumor growth in this mouse model.

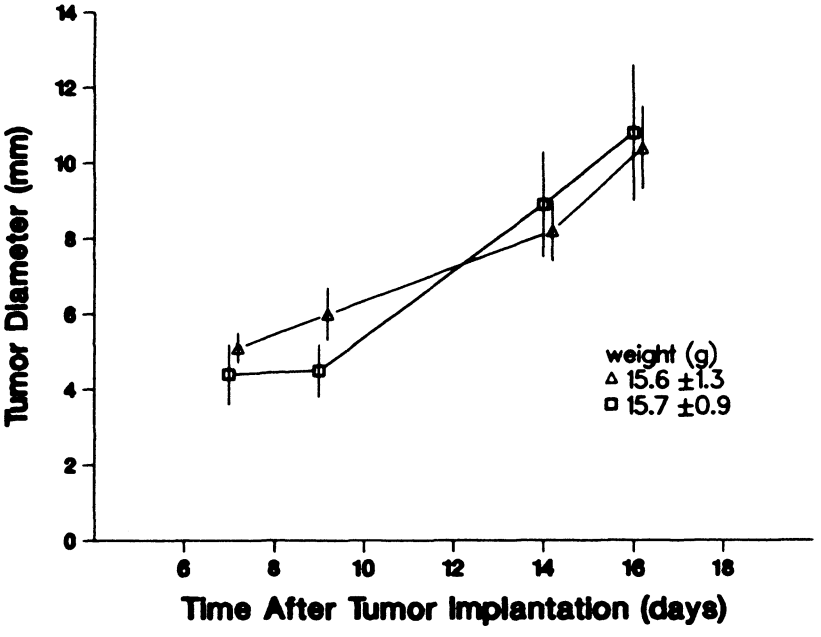

FIGURE 3: Tumor Growth in Untreated and Anti-TNF-Treated Mice. In one group each animal received 100μg in 0.3ml V1q hybridoma supernatant ip on the day of tumor implantation and every third day thereafter. Tumor diameters were measured in the untreated group (□) and the anti-TNF-treated group (△) on the indicated days and are given with standard deviations.

DISCUSSION

The production of TNF during tumor growth *in vivo* as predicted by the literature (1-5) was verified in the tumor model described above. Not only did tumor infiltrating macrophages synthesize TNF but also cells in the spleens of these tumor bearing animals became positive for TNFmRNA. This indicated that 1. physical contact of tumor cells with macrophages induced local production of TNF and 2. that the tumortransplant induced a status of inflammation leading to systemic activation of macrophages/monocytes. The high TNFmRNA expression in spleens after day 20 of tumor growth could also be based on the central necrosis in the tumors which was regularly observed at later stages of tumor growth. The fact that no plasma TNF was detected in the tumor-bearing animals is in agreement with a recent publication (7) but in contrast to the observed enhanced TNF levels in cancer patients (2,3).

This could either be explained by the tumor type used for transplantation or by the sensitivity of the TNF assays used in these experiments. Detection of TNF in biological fluids like plasma is sometimes difficult due to the high protein content of the test samples or due to the presence of inhibitors and/or soluble receptors. This could also be the reason for the failure to detect TNF in the plasma of tumor bearing mice after endotoxin administration. This observation is in contrast to non-tumor bearing mice where circulating TNF can regularly be found after endotoxin administration. The presence of IL6 at later stages of tumor growth might be an indirect sign of the presence of activated macrophages because TNF belongs to the best inducers for IL6 production (7). Tumor burden was shown to be a sensitizing factor for detrimental effects of TNF or endotoxin application (9). Therefore, the failure to detect any TNF release in tumor-bearing mice was rather unexpected. Although soluble TNF was not detected, TNF protein and TNFmRNA was clearly expressed in macrophages in tumor tissue and TNFmRNA was expressed in spleen.

In an attempt to clarify the role of this endogenously produced TNF during tumor growth, the mice were treated with neutralizing antibodies to murine TNF. The concentration of antibodies in the serum was high enough to completely neutralize endotoxin-induced TNF released in lethal shock situations (Echtenacher, B. et al., manuscript submitted) at any time of the experiment. This treatment, however, did not have any obvious effect in regard to the tumor growth or to the behavior of the animals. It is conceivable that transplantation of such a large number of chemically induced tumor cells simply overwhelms the primary defense system in which TNF is meant to play a role as physiological mediator. A more simple explanation would be that the antibodies did not get to the relevant site of TNF production and -action and, therefore, were ineffective. The elucidation of the role of endogenous TNF in tumor growth obviously needs to be investigated in more refined test systems.

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