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Tumor necrosis factor in benign and malign tissue of the kidney

K.-H. Bichler, S. Kleinknecht, H. J. Nelde, and W. L. Strohmaier

Department of Urology, University of Tübingen, Tübingen, FRG

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Summary. The use of tumor necrosis factor (TNF) in immunotherapy of tumor diseases has attracted increasing interest. Since the direct antitumor effect of the TNF is mediated by receptor-bound TNF, we immunohistologically stained both benign and malignant tissue from 35 tumor-bearing human kidneys for TNF. Using a polyclonal anti-TNF-antiserum, paraffin sections were tested in the presence and absence of in vitro preincubation with TNF. Futhermore, all specimens were stained immunohistologically for Tamm-Horsfall protein (THP) because this renospecific glycoprotein can bind TNF in a lectin-like manner. In the absence of TNF preincubation, malignant tissue was TNF-positive in 34 specimens, as was benign tissue from the same tumor-bearing kidneys in 35 cases. In several specimens the staining was so intense that preincubation with TNF did not enhance the reaction. Whereas TNF staining in tumor tissue was relatively homogenous, that in benign tissue was intensive in distal tubuli, moderate in proximal tubuli, and negative in glomeruli. THP staining was negative in malignant kidney tissue but positive in the distal tubuli of benign tissue, i. e., in the regions in which TNF staining was most intense. These results indicate that TNF binds not only to membrane, most likely in a receptor-mediated manner, but also to THP both in vivo and in vitro. In vivo binding of TNF to THP was confirmed in animal experiments in which pigs were given injections of TNF. Immunohistological staining of the animals' kidneys revealed positive reactions for both TNF and THP at the distal tubuli, indicating TNF binding to THP after in vivo TNF administration. The presence of TNF in human kidney tumors implies that renal-cell carcinoma cells in situ are resistant to the direct cytotoxic effect of TNF. This resistance should be taken into account when TNF is considered for use as a possible immunotherapeutic agent in renal-cell carcinoma.

Key words: Tumor necrosis factor – Renal-cell carcinoma – Uromodulin – Tamm-Horsfall protein

Tumor necrosis factor (TNF) is a protein that is primarily secreted by activated macrophages and plays a number of biological roles, exhibiting metabolic, growth-regulating, and immunomodulatory action [18, 25, 27, 29, 42, 44, 46]. In particular, the latter two functions have suggested that TNF might be used in the treatment of tumors (Fig. 1). On the one hand, TNF exerts cytotoxic effects on many tumor cells without impairing the growth of benign cells [52, 55]. On the other hand, it can having the functions as an immunomodulator, activate macrophages and natural killer (NK) cells, i. e., mediators of nonspecific tumor defense, thereby producing antitumor effects [35, 42, 50]. Moreover, the antitumor effects of TNF are correlated with its vasocoagulative action [37].

In animal experiments, TNF has turned out to be highly effective against tumors [20, 30, 57]. In kidney tumor cells, it has proved to be effective in vitro, and in animal experiments in vivo. Various renal carcinoma cell lines are sensitive to TNF [7, 28]; the growth of human renal-cell carcinoma xenografts in nude mice is also inhibited by TNF [8, 23]. However, the results of clinical phase I/II studies on TNF in advanced tumors have

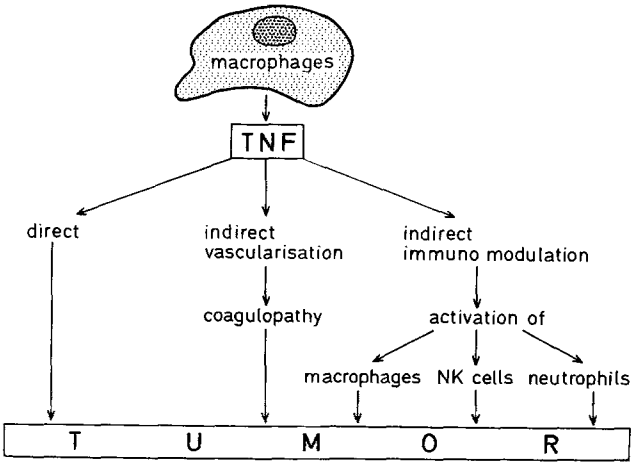


Fig. 1. Antitumoral activities of TNF

Table 1. Animal experiments designed to test the binding of TNF to THP after in vivo administration of TNF to pigs

Animal ($n=4$)	Application	Nephrectomy
S1	Kidney	Before TNF
	TNF i. a. → Kidney	1 h after TNF
S2	TNF i. a. → Kidney	2 h after TNF
	Kidney	2 h after TNF
S3	TNF i. a. → Kidney	0.5 h after TNF
	Kidney	2 h after TNF
S4	TNF i. v. → Kidney	0.5 h after TNF
	Kidney	2 h after TNF

Fig. 2. Porcine kidney tissue used as a negative control in the immunohistological staining series for TNF and THP. All incubation steps were carried out except that using the first antibody. Staining is negative in all parts of the section. ×400

Fig. 3a, b. Immunohistological staining for TNF as carried out in human kidney-tumor tissues without preincubation of the sections. **a** Patient WN, 58-year-old man: staining intensity, ++. **b** Patient RG, 83-year-old woman: staining intensity, +. ×400

Fig. 4. Immunohistological staining for TNF as carried out in benign human kidney-tumor tissue without preincubation of the sections. Patient WN, 58-year-old man: staining intensity, ++. ×400

Fig. 5a, b. Immunohistological staining for THP in human kidney tissue. **a** Benign tissue. *1* Proximal tubule; *2*, distal tubule. **b** Malignant tissue. ×400

Fig. 6a, b. Immunohistological staining for TNF and THP in porcine kidney tissue after in vivo application of TNF. **a** THP staining in the distal tubuli (*2*). **b** TNF staining in the distal tubuli (*2*), no staining of the proximal tubuli (*1*)

mostly failed to meet expectations [5, 16, 31, 43, 56]. In metastazing renal carcinoma, the results obtained using TNF have also been worse than expected based on laboratory findings. Debruyne and colleagues [26] did not observe therapeutic effects following treatment of renal-cell carcinoma with TNF alone or in combination with interferon; however, according to reports presented by Conrad and co-workers [23], TNF is effective only when combined with alpha-interferon.

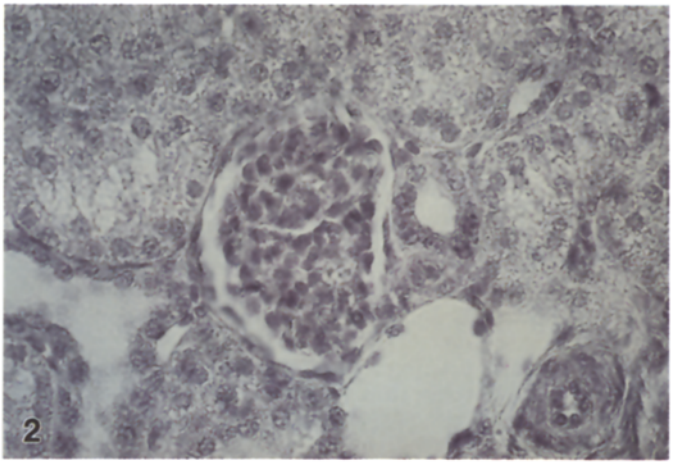
The question arises as to whether the characteristics found for established tumor-cell lines in an in vitro system or in animal experiments are the same as those encountered in vivo. On the basis of the above-mentioned findings, we investigated the question as to whether TNF actually binds to kidney tumor tissue, since a direct antitumor effect of TNF depends on binding to a membrane receptor [2, 42, 55]. An indirect antitumor effect such as the activation of cytotoxic immune cells is probably supported by locally available TNF as well [41]. Sections from 35 tumor-bearing kidneys were examined immunohistologically for the presence of TNF. Malignant as well as benign tissue specimens were taken from the tumors and tested in the presence and absence of in vitro incubation with recombinant TNF. Even in the absence of pre-incubation, we found native TNF is almost all of the kidney tumors examined.

In investigations of kidney tissue, it should be taken into account that lymphokines such as TNF have been shown to undergo binding apart from that mediated by receptors. Muchmore and co-workers [32, 51] have demonstrated that TNF binds to the renospecific glycoprotein uromodulin in a lectin-like manner. Uromodulin is almost identical to Tamm-Horsfall protein (THP) and the uromucoid fraction described elsewhere [10, 17, 32, 49, 51, 53]. THP is expressed in the distal tubuli of the kidney [12, 13, 39, 45]. Following in vitro incubation of renal tissue sections with TNF, the latter lymphokine is identifiable in the THP-positive distal tubuli [32, 51]. For these reasons, the specimens were also examined for both the presence of THP and a possible association of TNF with THP. Moreover, in animal experiments we attempted to determine whether TNF binds to THP in vivo. Pigs were given TNF injections. Prior to and after TNF injection, renal biopsies were taken and investigated immunohistologically in the presence and absence of TNF preincubation for both TNF and its possible association with THP. TNF was found only in the distal tubuli of the kidneys that expressed THP as well. In benign parts of the tumor-bearing kidneys, TNF was also found in THP-negative regions, although the most intensive staining was seen in the THP-positive distal tubuli.

Patients and methods

Benign and malignant tissue samples taken from 35 patients with renal carcinoma who had undergone biopsies between 1986 and 1990 were immunohistologically stained to identify TNF; 17 of the renal carcinomas had metastasized and 18 had not. One section taken from each specimen was preincubated with TNF and another was stained without preincubation [rHu-TNF; Knoll, Ludwigshafen, FRG; diluted 1:1,000 in phosphate-buffered saline (PBS)]. In 1988, an (IRMA) for analysis of serum TNF became commercially available (IRE Medgenix, Brussels, Belgium); since then, we have assayed serum titers prior to surgery.

To determine whether TNF is available in association with THP, we examined all tumor sections immunohistologically for the presence of THP. To settle the question as to whether TNF is found in association with THP after in vivo administration of the former, animal experiments were carried out. Four pigs (Deutsches Hausschwein, 20–25 kg; Haupt- und Landesgestüt Baden-Württemberg, Marbach, FRG) were given 40 mg/kg TNF (rHu-TNF; Knoll, Ludwigshafen, FRG), which was injected either i. v. via the vena cava or i. a. directly into the kidney via the renal artery. Table 1 shows the procedure used for each single pig. In pig 1, one kidney was removed before TNF injection to serve as a control; in the other three animals, kidneys were removed at 30 or 120 min after TNF injection. The serum TNF titer was checked at intervals of 15 min using an IRMA. After the experiment, the pigs were killed, their kidneys were removed, and specimens were taken from the liver, spleen, fatty tissue and spinal marrow for immunohistological analysis.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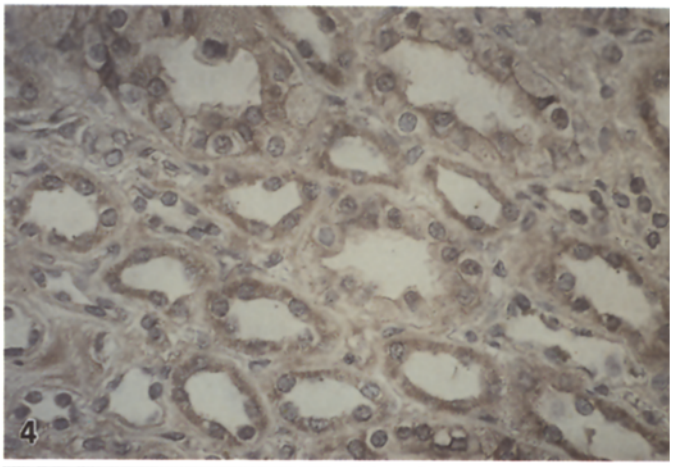
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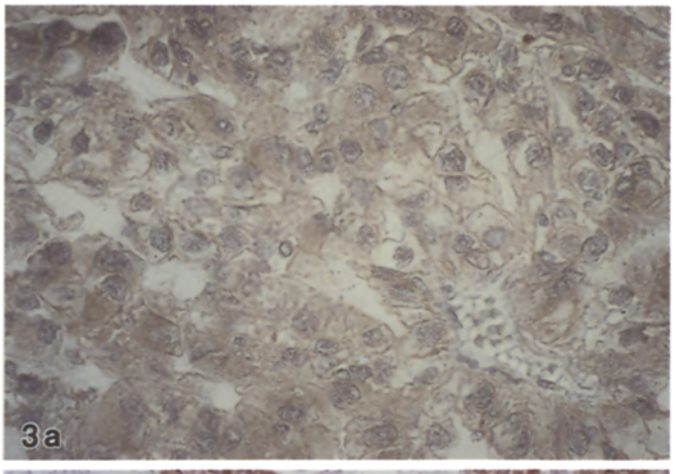
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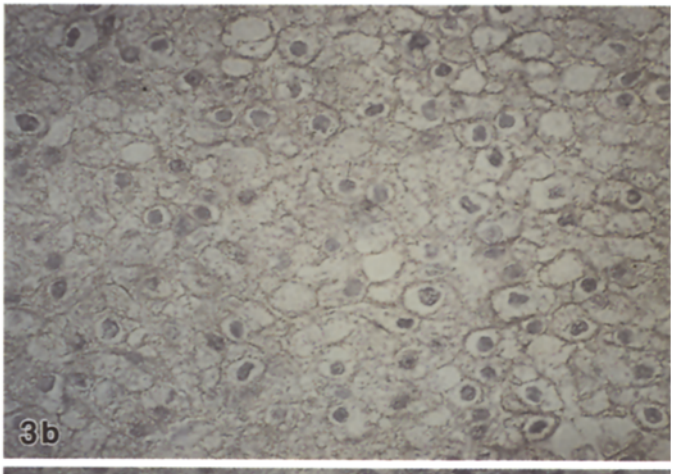
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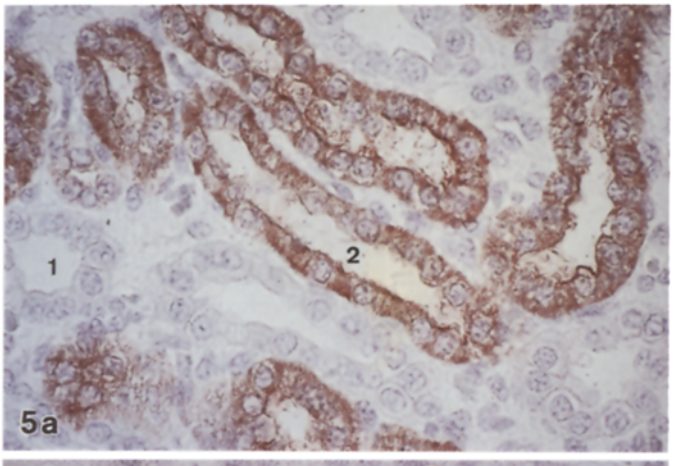
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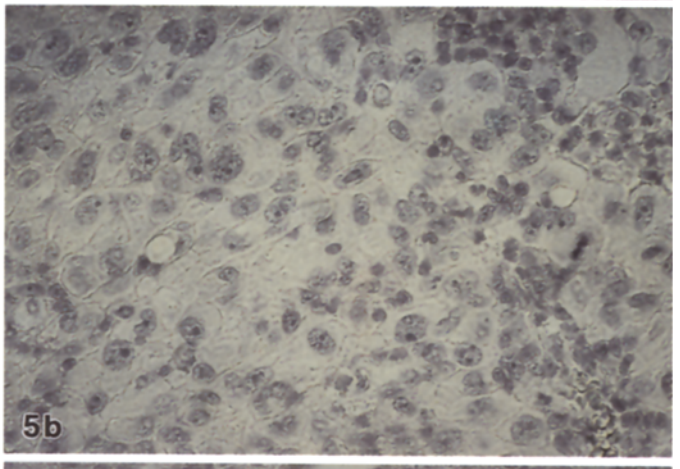
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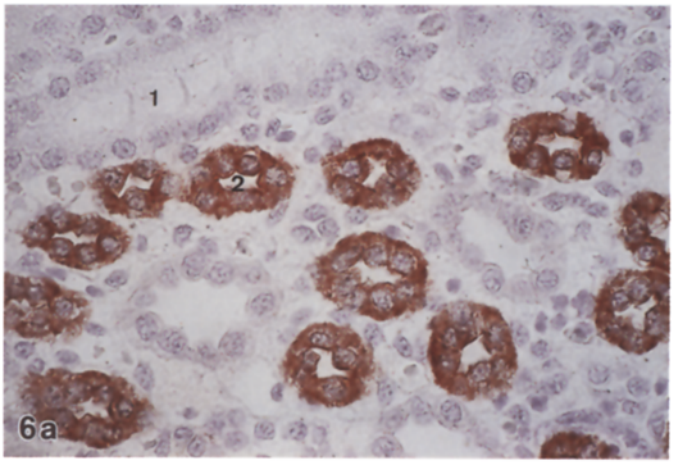
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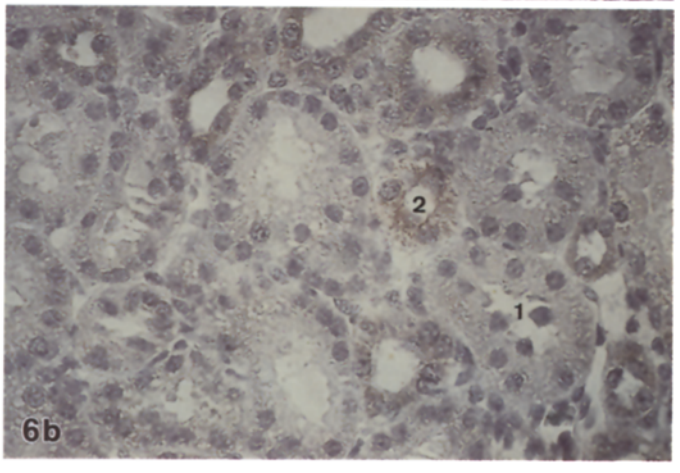
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Table 2. Method of immunohistological staining of TNF and THP by peroxidase reaction in kidney tissue

1. Fixation in formalin	24 h	4% pH 7
2. Wash, ascending alcohol series		
3. Xylene, paraffinization		
4. Cutting of 5-µm sections		
5. Deparaffinization, rehydration		
6. PBS (pH 7.2, 0.1 mol)		
7. H_2O_2	5 min	3%
8. PBS	3×5 min	
9. TNF or PBS	30 min	1:1,000 (v/v) in PBS
10. PBS	3×5 min	
11. Porcine serum	20 min	
12. 1st antibody[a]	30 min	1:20 (v/v) in PBS
13. PBS	3×5 min	
14. 2nd antibody (pig anti-rabbit IgG)	30 min	
15. PBS	3×5 min	
16. PAP complex, peroxidase-linked[b]	20 min	
17. AEC[b]	20 min	
18. H_2O	5 min	
19. Counterstaining with hematoxylin	2 min	
20. Embedding in glycerogelatin		

[a] Polyclonal anti-THP antibody from rabbits (Behring, Marburg, FRG) or polyclonal anti-TNF antibody from rabbits (Knoll, Ludwigshafen, FRG)
[b] PAP kit (Dako, Hamburg, FRG)

Table 3. Results of immunohistological staining for TNF in benign and malignant human kidney tissue as carried out without preincubation of sections with TNF

	Staining intensity				
	Malignant tissue			Benign tissue	
				Dist. tub.	Prox. tub.
	−	+	++	++	+
$n=35$	1	14	20	35	35
%	3	40	57	100	100

++, Intense reaction; t, moderate reaction; −, no reaction; Dist. tub., distal tubuli; Prox. tub., proximal tubuli

Immunohistology

A peroxidase method was used for immunohistological staining for TNF and THP. Table 2 shows the test procedure. The specimens were fixed in 4% buffered formalin (pH 7) and embedded in paraffin prior to section cutting. Then, 5- µm sections were deparaffinized, rehydrated via a descending alcohol series, and incubated with 3% hydrogenperoxid to inactivate endogenous peroxidase. After being washed in buffer (0.1 mol PBS, pH 7.2), one section of each specimen was preincubated for 20 min with TNF (diluted 1:1,000 in PBS) and another was pretreated with buffer. The sections were then incubated with normal pig serum to saturate nonspecific binding. Subsequently, we incubated then with the first antibody. To identify THP, we used an anti-THP antibody from rabbits (see Table 2). For the detection of TNF, an anti-TNF antibody from rabbits (Knoll, Ludwigshafen, BRD) was employed.

Staining with 3-amino-9-ethyl-carbazol (AEC) produced a red-brown color; fo this staining, a commercial test kit was used (Dako, Hamburg, FRG). Following differential staining of the nuclei with hematoxylin, the specimens were embedded in glycerogelatin. In each series, negative controls were tested for nonspecific binding; for this purpose, we carried out the entire immunoperoxidase reaction procedure, except that the sections had not been incubated with the first antibody (anti-TNF, anti-THP). Semiquantitative determination of color intensity was done according to the following classifications: negative (−), positive (+), and doubly positive (++). Each section was examined by two investigators working independently of each other.

Results

The methods used turned out to be specific for the immunohistological staining of TNF and THP, since the control experiments carried out to identify nonspecific binding revealed only faint staining reactions (Fig. 2). Even without preincubation with TNF, tumor tissue was TNF-positive in 34 cases, as was benign kidney tissue in 35 cases. In malignant tissues, the staining intensity was doubly positive (++) in 20 cases and positive (+) in another 14 specimens (Table 3), being independent of the tumor stage. This reaction was found to be intracytoplasmic as well as membrane-based, exhibiting homogeneous distribution (Fig. 3). In the benign tissue, TNF staining was most intense at the distal tubuli, in which the staining intensity was rated as being doubly positive in all 35 cases (Fig. 4, Table 3). The proximal tubuli were stained less intensely and glomeruli were negative. Neither in benign or in malignant tissue did TNF preincubation produce an obvious intensification when the staining reaction had been distinct prior to preincubation.

The TNF serum titer could be assayed preoperatively in eight patients whose kidney tumors had been examined immunohistologically for the presence of TNF. As can be seen from Table 4, TNF could be identified in the tumor even when the preoperative serum titer had tested as negative. Doubly positive (++) reactions were seen in patients whose serum titer had been TNF-positive. THP was detectable at the distal tubuli in all benign biopsy specimens, namely, in the areas in which the staining reaction following TNF incubation was rather distinct. Malignant tissue from the respective kidneys was THP-negative (Fig. 5).

In the animal experiments, the pig kidney taken as a control before the injection of TNF showed a negative response to staining for TNF. Following the application of TNF serum, TNF reached peak levels within 45–69 min after the injection, and urine samples taken 2 h post-injection were TNF-positive. After the (in vivo) injections, TNF was detectable in the pig's kidneys at the distal tubuli. Neither the route of administration i. v. or directly into the kidney via the renal artery) nor the time at which the organ was removed influenced the intensity of the

Table 4. Results of immunohistological staining for TNF in benign and malignant human kidney tissue as carried out in the presence and absence of preincubation of sections with TNF

Patient	Tumor type	Preoperative serum TNF (pg/ml)	Without TNF preincubation		With TNF preincubation	
			Benign	Malignant	Benign	Malignant
GM 420	Metastatic	0	0	+	0	+
BW 540	Metastatic	1.2	+	+	+	+
WN 580	Metastatic	13.8	++	++	++	++
FE 610	Nonmetastatic	1.5	+	+	+	+
GH 410	Nonmetastatic	0	+	++	+	++
RG 830	Nonmetastatic	0	+	+	+	+
LW 540	Nonmetastatic	3.9	++	++	++	++
LK 540	Nonmetastatic	6.7	+	+	+	+

++, Intense reaction; moderate reaction; −, no reaction

staining reaction. However, preincubation with TNF produced intensified staining reactions in the area of the distal tubuli (Fig. 6). The livers, spleens, and fatty and muscular tissues of the pigs were TNF-negative prior to as well as after preincubation. Here and there, stained areas were observed in nerve tissue, although the specificity of this reaction is doubtful.

Discussion

The present immunohistological study, performed to detect TNF in benign and malignant tissue from kidneys of patients suffering from renal-cell carcinoma, demonstrated that TNF was detectable as the native lymphokine in sections that had not been preincubated. The intensity of the staining reaction was often so distinct that preincubation of the respective specimen did not bring about a further enhancement. This finding raises a number of questions, particularly that regarding possible use of TNF in tumor treatment. The evidence of the occurrence of TNF in intact renal carcinoma cells found in the present study indicates that these cells are resistant to a direct cytotoxic effect of TNF. Results such as these may explain the lack of therapeutic success for TNF monotherapy at least.

However, several authors associate the clinical response to other immunotherapeutic approaches with systemic increases in endogenous TNF [15, 30]. On the other hand, our investigations on the immune status of renal cell carcinoma have shown that patients with metastatic disease can exhibit increased levels of serum TNF and that a decrease in TNF may be associated with a better prognosis [14, 40]. Furthermore, monocytes of patients with metastasizing tumors produce more TNF than do those of either patients with locoregional tumor diseases or normal subjects [1, 36]. Moreover, it must be borne in mind that by inducing the production of interleukin 6 (IL-6), TNF is an important mediator of chronic inflammation and cachexia, which occur in patients with advanced malignant disease [6, 9, 29]. Kriegler et al. [41] presume that a chronic systemic increase in soluble TNF causes cachexia or septic shock, whereas TNF produced locally by activated macrophages at the site of inflammation might mediate the cytotoxicity of macrophages against target cells [35, 41, 48].

TNF immunohistology in the kidney tumors was positive, yet the source of the TNF detected in the present study remains unknown. Primarily, activated macrophages are TNF producers (see [18, 27, 42] for review); however, there is reason to assume that the tumor cells themselves can produce the lymphokine [18, 24]. Moreover, the question is whether the TNF in the kidney tumor is systemic and receptor-bound or membrane-associated. Several authors [21, 33, 41] have demonstrated that apart from the soluble 17-kDa form, there is also a transmembrane 26-kDa form of TNF that is excreted by activated macrophages. Nevertheless, renal carcinoma cells seem to be resistant at least to the direct cytotoxic effect of native TNF. The question as to whether the use of TNF would be beneficial in tumor treatment due to its possible immunomodulatory effect also remains open.

The studies performed to provide evidence of the presence of TNF and its association with the renospecific glycoprotein THP in porcine and human kidney tumors demonstrated that after in vivo administration, TNF is available in association with THP in the distal tubuli of pig kidneys and that it also occurs in the same area as THP in benign tissue from human kidney tumors. Therefore, the results reported by Hession et al. [32] and Sherblom and co-workers [51], namely, that lymphokines such as TNF or IL-1 bind to THP following in vitro preincubation, are also valid for in vivo systems. The question as to whether binding to an organ-specific glycoprotein might affect the immunology of kidney tumors remains open. Nonetheless, in an in vitro bioassay, the activity of TNF could be inhibited by uromodulin [19, 47]. Since there are striking glycoprotein patterns in many tumors [3, 11], it would be highly interesting to determne whether lectin binding is also possible between lymphokines and other glycoproteins and whether the local bioavailability of lymphokines might be affected by such lectin binding.

The expression of renal tubular antigens such as THP is of great importance to the histogenesis of renal-cell

carcinoma. Our analysis of the content of THP in renal-cell carcinomas ($n = 35$) corroborates the opinion advocated by Wallace and Nairn [54] and Holthöfer et al. [34], namely, that the lack of THP in these tumors indicates that the lesions derive from fully differentiated proximal tubular cells. In contrast, Apitz [4] has suggested a dysontogenetic origin for renal-cell carcinoma. Recent studies carried out by Cohen et al. [22], who tested the presence of a great number of antigens, have shown that antigens of the distal tubuli are found in renal tumor tissue as well; therefore, these authors suggested that divergent histogenesis from a precursor stem cell is likely. Hence, the histogenesis of renal-cell carcinoma has yet to be clarified.

In conclusion, we find that further investigations are urgently needed to determine the possible significance of TNF in human renal-cell carcinoma before concepts for immunotherapeutic treatment using TNF are developed. One should not use organ or primary cultures, since the sensitivity of renal tumor cells to TNF evidently changes on transition to permanent culture. Another important question is whether TNF can be identified in metastases of the renal tumor and, if so, whether there is a correlation between metastasis and the amount of TNF in the primary tumor. Furthermore, our attention should be directed to the occurrence of non-receptor-mediated binding of lymphokines to glycoproteins and to the meaning of such binding for the local activity or bioavailability of immunomodulatory substances.

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Prof. Dr. K. H. Bichler
Abteilung für Urologie
Universität Tübingen
Hoppe-Seyler-Strasse 3
W-7400 Tübingen
Federal Republic of Germany