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Heterogeneity of Tumour Necrosis Factor Production in Renal Cell Carcinoma

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Endogenous tumour necrosis factor (TNF) production was investigated by *in situ* hybridisation and immunohistochemistry in 8 renal cell carcinoma (RCC) patients at different stages of disease. Analysis of frozen sections of tumour biopsy specimens revealed variable degrees of macrophage infiltration and great heterogeneity in TNF gene expression. Two metastatic tumours investigated showed abundant TNF protein production and marked macrophage infiltration. Based on morphological critera, these TNF-positive cells most likely belong to the macrophage lineage. Two years after nephrectomy the individual survival time was recorded; however, the small numbers did not yet allow any correlation of TNF production to the clinical course of disease. Further studies will be required to eventually reveal the role of TNF in renal cell carcinoma development.
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INTRODUCTION

TUMOUR NECROSIS factor (TNF) has been originally described as a serum activity in lipopolysaccharide-treated mice capable of causing haemorrhagic necrosis of animal tumours [1]. However, it is now recognised that TNF belongs to a group of cytokines that form a complex network of interactive signals regulating their own production and activation of cells involved in inflammation and tissue remodelling [2–4]. The initial euphoria that TNF might be exploitable as a natural host's defence molecule against tumours has been dampened by disappointing results of clinical trials with recombinant TNF. Moreover, the fact that many tumours themselves are able to produce TNF *in vitro* and *in vivo* [5–7] has led to the suspicion that in certain instances TNF might play a possibly adverse, pathogenic role in tumorigenesis. Thus the biological meaning of TNF production in cancer is all but entirely obvious. In order to obtain insights into the pathophysiological role of TNF in tumorigenesis we have begun to investigate the endogenous TNF production in renal carcinoma (RCC). Based on frequent spontaneous regressions or disappearance of metastases following nephrectomy, it has been postulated that immunologic mechanisms may play an important role in controlling this tumour. It was therefore anticipated that the variable clinical courses may be accompanied by differences in endogenous TNF production. In this study we show by immunohistochemistry and *in situ* hybridisation great heterogeneity of TNF gene expression in the tumours of 8 individual RCC patients at different stages of disease.

MATERIALS AND METHODS

Tumour specimens

The tumour material was obtained from 8 renal cell carcinoma patients during nephrectomy. The mean age of tumour patients (4 women and 4 men) was 62 years (range 47–78 years).

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RNA blotting analysis

Tumour samples were lysed in guanidine isothiocyanate and total cellular RNA was pelleted over a CsCl cushion as described [8]. RNA was size-separated on formaldehyde gels, transferred to nitrocellulose filters, and hybridised to a TNF-specific cDNA probe (an 800-base pair *Eco*RI fragment of λ42–4) [9] or to an HLA-B7-specific cDNA [10] labelled with ^{32}P-dCTP by random priming.

In situ *hybridisation*

In situ hybridisation was performed essentially as recently described [11]. Briefly, freshly isolated tumour samples were frozen immediately without any fixation. Sections of 8 μm were cut from specimen blocks at −20°C. Serial frozen sections were dried for 10 min at 55°C and fixed in 4% paraformaldehyde. Slides were incubated in 2 × sodium saline citrate (SSC) (1 × SSC = 0.15 mol/l NaCl/0.0015 mol/l sodium citrate, pH 7.0) for 30 min at 70°C, rinsed in phosphate-buffered saline (PBS, 20 mmol/l sodium phosphate/0.7% NaCl, pH 7.4) and digested with Pronase (0.2 mg/ml) for 10 min at room temperature. Slides were then refixed, acetylated, dehydrated and finally air-dried.

RNA probes were synthesised using either SP6 RNA or T7 RNA polymerase and [^{35}S]-UTP as described [11]. Hybridisation was performed at 50°C in a humid chamber. Slides were washed in 50% (vol/vol) formamide/2 × SSC followed by digestion with RNase A (20 μm/ml) and three further washes in 50% formamide/2 × SSC. For autoradiography, air-dried slides were coated with NTB2 emulsion (Eastman Kodak) and exposed at 4°C for 5 days. Slides were developed and counterstained with Giemsa.

Immunohistochemistry

For immunostaining frozen sections were incubated as recently described [11] with either monoclonal antihuman TNF antibody 154/6 (a kind gift of Dr Trinchieri, Philadelphia) or monoclonal antibody (Mab) 63D3 (American Type Culture Collection, USA), recognising a monocyte-specific antigen [12]. Alkaline phosphatase-anti-alkaline phosphatase complexes were prepared as described by Cordell *et al.* [13]. Sections were counterstained with haematoxylin/eosin.

Table 1. Patients' characteristics

Case no. (age, sex)	TMN classification	Date of nephrectomy	Survival (months)
1 (62, m)	T2N0M0	18/12/87	>28
2 (47, f)	T2N0M1	23/ 2/88	>26
3 (47, m)	T3N2M0	26/ 2/88	9
4 (78, f)	T2N0M0	29/ 2/88	>26
5 (60, m)	T3N0M0	17/ 3/88	>25
6 (72, f)	T3N0M0	14/ 4/88	>24
7 (62, f)	T2N2M0	5/ 5/88	16
8 (67, m)	T3N2M1	29/ 6/88	15

RESULTS

Tumour specimens were obtained during tumour nephrectomy from 8 patients with renal cell carcinoma (RCC,Table 1). In order to assess TNF gene expression in the individual tumour tissue, the examination procedures included (i) RNA blotting analysis of total cellular RNA extracted from whole tumour; (ii) *in situ* hybridisation of frozen sections to demonstrate TNF mRNA expression at the single cell level, and (iii) immunohistochemistry to detect TNF protein synthesising cells. In addition, the degree of monocyte/macrophage infiltration was investigated by immunohistochemistry. Several microscopic fields of distinct tumour areas were examined to realise possible intratumoral heterogeneity. A representative analysis of case No. 3 is illustrated in Figs 1 and 2. To obtain a first impression of the overall TNF gene expression in the tumour tissue, total RNA was extracted from the tumour and analysed for its TNF mRNA content. As shown in Fig. 1(a), the tumour RNA did not show any signs of degradation. Hybridisation to a ^{32}P-labelled, TNF-specific cDNA revealed a readily detectable signal of the appropriate size (Fig. 1b). Control hybridisation to an HLA-A,B,C-specific cDNA probe confirmed the integrity of the RNA samples loaded. This kind of RNA blotting analysis indicated

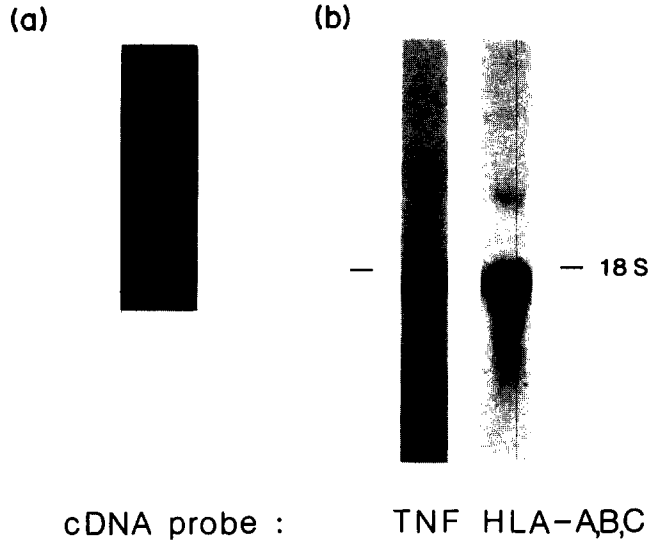

Fig. 1. Northern blot analysis of TNF mRNA in the renal cell carcinoma from patient number 3. (a) Ethidium bromide stain of total RNA (20 μg) that has been extracted from the tumour specimen and size-separated by formaldehyde-agarose gel electrophoresis. (b) RNA was transferred to nitrocellulose filters and hybridized to ^{32}P-labeled TNF and HLA-B7-specific cDNA probes.

that the TNF gene was transcribed in the tumour tissue yet did not reveal the cellular source of TNF mRNA production.

In situ hybridisation of frozen tumour sections using a ^{35}S-labelled antisense TNF RNA probe demonstrated that approximately 20% of mononuclear cells expressed TNF mRNA (Fig. 2a). Control hybridisations to insert less plasmid RNA probes defined a background of < 15 grains/cell (Fig. 2b). Upon higher magnification and based on morphological criteria the TNF mRNA-expressing cells could be identified to belong to the monocyte/macrophage lineage. An example is shown in Fig. 2(c), where two TNF mRNA-positive cells are caught during diapedesis through the endothelial layer of a blood vessel. Of note, in no instance TNF mRNA was detected in renal carcinoma cells. Control hybridisation to a ^{35}S-labelled HLA-A,B,C specific RNA probe stained greater than 85% of nucleated cells (Fig. 2f) indicating that most of the cells contained intact mRNA accessible for effective hybridisation.

To detect TNF protein production immunohistochemistry was performed utilising the anti-TNF monoclonal antibody 154/6 (provided by Dr G. Trinchieri, Wistar Institute, Philadelphia). This anti-TNF antibody stained approximately 11% of mononucleated cells (Table 2) with a dense cytoplasmic staining pattern (Fig. 2d). To determine the extent of monocytic infiltration, tumour sections were incubated with the monoclonal monocyte-specific antibody 63D3. Immunohistochemical analysis showed heavy mononuclear infiltration (Fig. 2e). The abundance of monocytic cells present in the tumour correlated well to the frequency of TNF mRNA and TNF protein containing cells. Together, these results indicate that the tumour was heavily infiltrated by monocytes/macrophages. Importantly, a high proportion of these infiltrating monocytic cells exhibited an activated state, that is, actively transcribed the TNF gene and synthesised TNF protein.

When tumour specimens from 7 other patients were investigated by the same type of analysis, a heterogeneity of TNF production in RCC became apparent (Table 2). As in patient number 3, the tumour of patient number 7 was infiltrated by macrophages and active TNF protein production corresponded to TNA mRNA expression. Strikingly, while both mononuclear infiltration and significant TNF mRNA expression was also observed in patients 2, 5 and 6, these tumours were largely devoid of detectable TNF protein. Since TNF gene transcription and mRNA translation require different stimuli [14], we conclude that the mononuclear infiltrates of these tumours were only suboptimally activated. In the remaining cases, neither TNF mRNA nor TNF protein was detectable independent of the degree of mononuclear infiltration. In no instance, TNF mRNA expression by tumour cells was observed. As expected, the frequency of TNF mRNA positive mononuclear cells correlated well with the level of TNF mRNA expression detected by Northern analysis of total cellular RNA (data not shown). Case number 8 represents an exception, in that this tumour exhibited great intratumoral heterogeneity. When several sections from distinct parts of the tumour were investigated the pattern of monocytic infiltration differed significantly. Tumour areas with minor monocytic infiltration were devoid of both TNF mRNA and TNF protein. Unfortunately, frozen sections of the second, infiltrated part of the tumour were not initially prepared for *in situ* hybridisation purposes. Later attempts to investigate TNF gene expression in this tumour area by *in situ* hybridisation and immunostaining were not successful. Since cells could not be stained by the HLA-B7 RNA probe either, which is routinely used as a positive control, this negative result was probably due

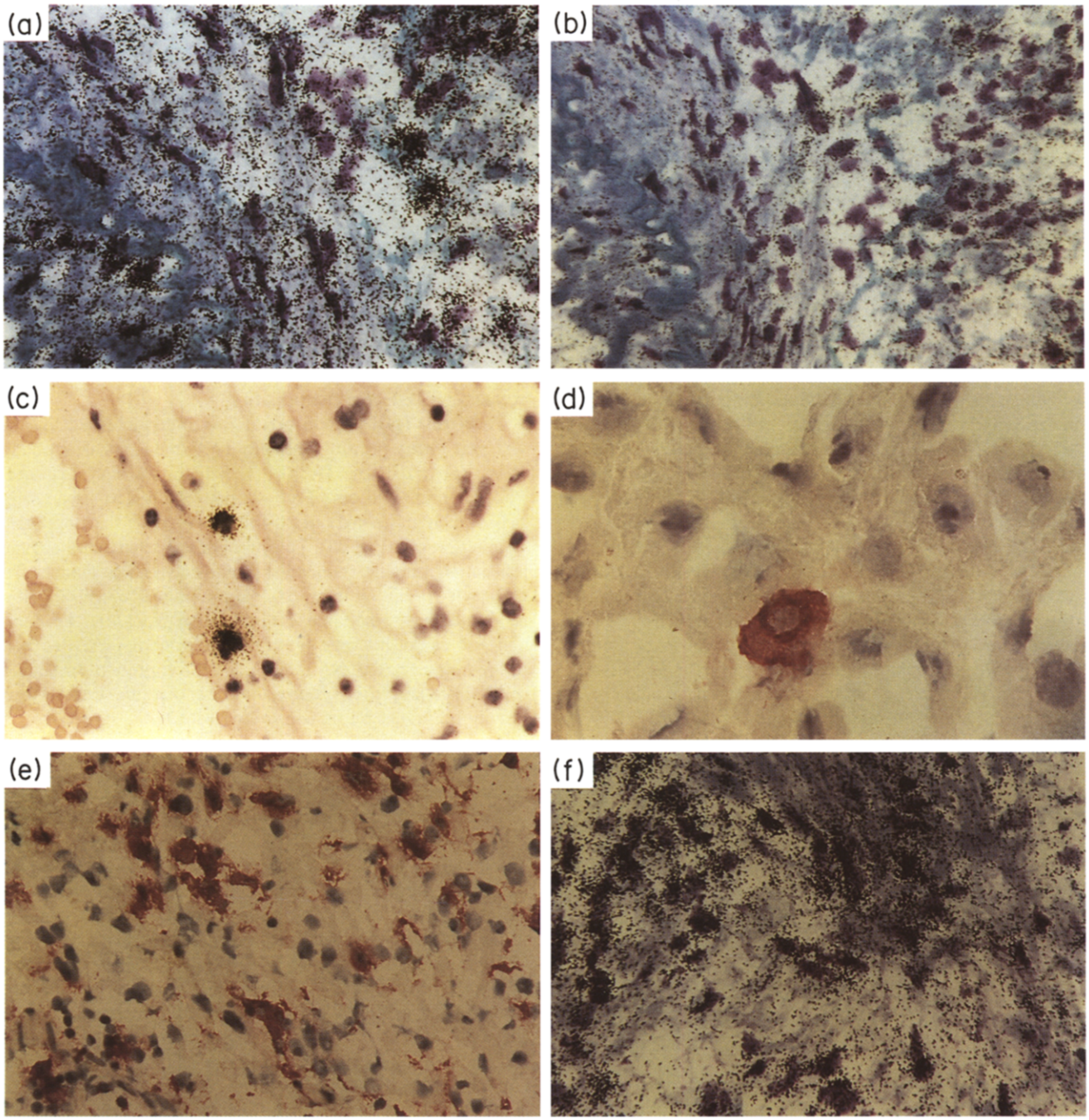

Fig. 2. ***In situ*** **analysis of TNF gene expression.** ***In situ*** **hybridisation was performed using antisense TNF (a, × 250, and c, × 400), insertless pSP64 (b, × 250) or antisense HLA-B7 RNA probes (f, × 250). Frozen sections from patient number 3 were immunostained with either monoclonal anti-TNF antibody 154/6 (d, × 600) or monocyte-specific monoclonal antibody 63D3 (e, × 250).**

to autolytic degradation of the tumour tissue. In any event, patient number 8 illustrates, that the mononuclear infiltrate can vary in one given tumour sample and stresses the necessity, that several areas of a tumour sample should be investigated in every instance.

DISCUSSION

In the present study we have investigated by immunohistochemistry and *in situ* hybridisation the extent of both mononuclear infiltration and TNF gene expression in 8 cases of RCC. Three major conclusions can be drawn from the results obtained (i) in RCC the extent of monocyte/macrophage infiltration is highly variable and can intratumorally differ depending on the area investigated, (ii) RCC displays great heterogeneity with respect to the level of TNF gene expression, and (iii) in RCC, TNF-mRNA and -protein appears to be produced by infiltrating monocytes/macrophages rather than by the tumour cells themselves.

With respect to TNF gene expression the mononuclear infiltrate can be functinally divided into three categories. First, the monocytes/macrophages can be totally devoid of both TNF mRNA or TNF protein (patients 1, 4 and 8a); second they may express TNF mRNA but do not synthesise measurable amounts of TNF protein (patients 2, 5 and 6), and finally, infiltrating

Table 2. Activation status of the mononuclear infiltrate in renal cell carcinoma

Case no.	Frequency of infiltrating macrophages (%)*	TNF mRNA positive cells (%)†	TNF-producing cells (%)‡
1	20–30§	<1	<1
2	15–25	5	1–2
3	35–40	20	11
4	3–7	<1	<1
5	30–40	6	1–2
6	35–60	17	1–2
7	35–45	9	6
8a	4–10	<1	<
8b	35–55	ND‖	ND

*Cryostat sections of tumour biopsy specimens were immunostained with the monocytic specific antibody 63D3. Frequencies of infiltrating macrophages were revealed by microscopic evaluation of several visual fields corresponding to more than 1000 nucleated cells.
†TNF mRNA-positive cells were visualised by *in situ* hybridisation with an antisense TNF cRNA probe. Frequencies of TNF mRNA-positive cells were estimated by evaluating 2000 cells.
‡ TNF protein-producing cells were detected by immunostaining with anti-TNF antibody. Frequencies of positive cells were estimated by evaluating 2000 cells.
§Given are the ranges of monocyte infiltration, which varied within a given tumour specimen depending on the particular section investigated.
‖ND = not done.

macrophages/monocytes can contain both TNF mRNA and TNF protein (patients 3 and 7). This distinction is of particular interest, because the level of TNF gene expression reflects different states of monocyte/macrophage activation. It is well documented that a sequential stimulation by distinct signals is required to induce TNF gene transcription, TNF mRNA translation, and TNF cleavage from its membrane associated 26 kD form, respectively [6, 14, 15]. Thus the mononuclear infiltrates of case numbers 3 and 7 probably represent a fully activated state, while macrophages of the remaining cases appear only suboptimally stimulated. How can one explain these differences? As has been recently reported, one site of macrophage activation and induction of TNF production may be the tumour itself [16]. On the other hand, in the case of colorectal adenocarcinoma, the tumour microenvironment appeared to provide insufficient macrophage activation signals [11]. Strikingly, in the present study, TNF protein production was observed in those patients, where tumour metastasis involved the draining lymph nodes. One could suggest that optimal macrophage activation may occur only after neoplastic antigens have been presented properly in a primary or secondary immunologic site. It will be interesting to perform further studies with RCC patients on a more extended basis to prove or disprove, whether macrophage activation and TNF production depend on tumour spread to the draining lymph nodes.

The finding that RCC cells did not synthesise TNF is not entirely trivial. First, it has been shown, that a number of tumour cell lines either constitutively express or can be induced to transcribe TNF mRNA *in vitro* [5–7]. Moreover, Naylor *et al.* have recently shown by *in situ* hybridisation, that ovarian cancer cells *in vivo* transcribe the TNF gene and produce TNF protein [7]. While TNF production by the mononuclear infiltrate may be viewed as a proctective host reaction against tumour development, TNF production of tumour cells has been implicated in the neoplastic progression and enhanced tumour metastasis [17]. It should be noted, however, that the biological significance of intratumoral TNF production is expected to be neither dependent of, nor determined by the cellular source of TNF. If tumour cell-derived TNF can act as a tumour progression factor, the same must hold true for monocyte-derived TNF. Indeed, TNF production by infiltrating monocytes was observed in RCC patients with advanced stages of disease and short survival time (patients 3 and 7, compare Tables 1 and 2). In contrast, patients with tumours devoid of TNF protein achieved clinical remissions > 2 years. Case number 8 should not be considered, because no information is available about TNF production within the infiltrated part (Table 2, number 8b). While the number of patients studied is far too small to draw any conclusions, the notion that TNF production in RCC may be associated with advanced stages of disease has been already put forward by Bichler *et al.* [18]. These investigators reported, that significant increased TNF serum levels were found in 13 RCC patients with metastatic disease but not in any of 16 patients with non-metastatic disease. However, further studies will be required to reveal a possible functional relationship between TNF production and progression of RCC, which may have important clinical implications.

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Antitumour Effect and Symptomatic Control with Interferon α_{2b} in Patients with Endocrine Active Tumours

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26 patients with progressive neuroendocrine tumours received 3 × 10^6U/m² interferon alfa (IFN-α_{2b}) subcutaneously thrice weekly, until progression, as outpatients with moderate toxicity. 4/16 carcinoids and none out of 10 endocrine pancreatic tumours showed objective regression. Another 17 patients (68%) had no change. For a median of 34 weeks symptom control was excellent: 9 of 17 patients had major relief from pain, 11 of 13 from diarrhoea, and 7 of 7 from flushing. Thus, low-dose INF-α_{2b} given thrice weekly might be as effective as daily treatment with higher dosages. Treatment was only administered to patients with progression or major symptoms and this did not seem to adversely affect remission quality and survival.
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INTRODUCTION

ENDOCRINE GUT tumours are relatively rare but they are of great interest to oncologists because their survival even in metastatic disease can be long even with little or no therapy. Subjective symptoms arising from ectopic hormone activity could become deleterious [1, 2]. Therefore, subjective response and 50% decrease of hormone levels were judged as the aims of treatment [3].

Systemic treatment with cytotoxic agents has been disappointing. In vipomas it offers the prospect of long-lasting remissions [4]. Antiproliferative treatment with the hormone-like peptide somatostatin [3, 5] or interferons are promising alternatives.

Updated results from OBERG with intermediate dosage of interferon in 32 patients with endocrine pancreatic tumours gave an overall response rate of 22% [3]. In a review of the literature, patients with carcinoid tumours achieved only 10/111 (9%) remissions defined by WHO criteria [3, 6–16]. Subjective

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