Urol Res (1990) 18:245–250

Urological Research

Enhanced in vivo cytotoxicity of recombinant human tumor necrosis factor with etoposide in human renal cell carcinoma*

Evaluation in a pre-clinical model

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Accepted: November 1, 1989

Summary. The combination of tumor necrosis factor (TNF) and etoposide (ETP) was evaluated for potential cytotoxic efficacy against a human renal cell carcinoma xenograft using an in vivo assay employing an athymic mouse host with tumor implanted a the subrenal capsule site. Both antitumor efficacy (relative survival or RTS) and toxicity (weight loss) of TNF and ETP alone and in combination were evaluated. While TNF and ETP alone were mildly inhibitory (RTS 90% and 71%, respectively), the combination caused marked tumor inhibition (45% of controls). Host toxicity encountered with the combination did not exceed the toxicity associated with ETP alone, suggesting that the therapeutic index may have been augmented. It is concluded that enhanced antitumor activity without substantial augmentation of toxicity is observed with this combination, providing a rationale for further evaluation of tumor necrosis factor-based regimens for the treatment of advanced renal carcinoma.

Key words: Tumor necrosis factor – Etoposide – Renal adenocarcinoma – Athymic nude mice

The identification of an effective therapeutic regimen in the management of advanced renal cell carcinoma remains an important but elusive objective for the urologic oncologist. Few chemotherapeutic agents either singly or together have demonstrated any consistent effect (only 15–20% response rate) [12, 18]. Because renal carcinoma is one of a small group of tumors recognized as biologic response modifier (BRM)-sensitive (i.e., amenable to immunomodulatory therapeutic strategies), a variety of immunotherapeutic approaches have been evaluated over the last two decades [12, 20, 23]. While only modest clinical activity has been shown with nonspecific stimulators, a low but objective clinical activity has been observed with the interferons (particularly alpha-interferon). More recently, renal carcinoma was noted to be the most responsive tumor histiotype to high dose therapy with interleukin-2, a cytokine in the family of BRM's [24]. Nevertheless, the response rate at best remains less than 35%; further development of effective therapeutic approaches is clearly necessary in renal carcinoma.

Tumor necrosis factor (TNF) is a naturally occurring cytokine produced by marophages which has been shown to induce hemorrhagic necrosis in tumors borne by mice [5, 8, 21, 22]. The human form (rhTNF) is now available in large quantities as a result of advances in recombinant DNA technology. Our laboratory has studied the efficacy of rhTNF alone and in combination with several chemotherapeutic drugs using an in vivo model of human bladder cancer using human tumor xenografts in an athymic nude mouse subrenal capsule assay (SRCA) [11]. Such an approach provides the ability to evaluate the impact of a new therapeutic strategy on the therapeutic index by providing an assessment of both the relative tumor cytotoxic efficacy and the host toxicity. Of the binary combinations tested, rhTNF/etoposide (ETP) proved the most promising in this assay system [11]. This approach has also allowed the delineation of an optimal in vivo dose schedule maximizing this effect [14].

Because of its known BRM-sensitivity, renal carcinoma is an important focus for potential application of new cytotoxic BRM regimens. While TNF has been demonstrated to be active in vitro using cytotoxicity assays with renal carcinoma cell lines, whether in vivo activity exists (within acceptable toxicity limits) is dependent on many considerations. Both components of the binary combination (TNF and ETP) must be able to impact on their respective intracellular sites of activity in a pharmacokinetically achievable manner; intra-tumoral tissue concentration of all agents resulting from extravascular compartment distribution is a critical parameter of efficacy. The well-vascularized subrenal capsule site is an excellent model simulating clincial tumor micro-environment. To assess the potential utility of this promising combination in the treatment of renal cancer, we carried out a nude

* Supported by a Merit Review grant, VA Medical Research Service, Durham, NC 27710, USA

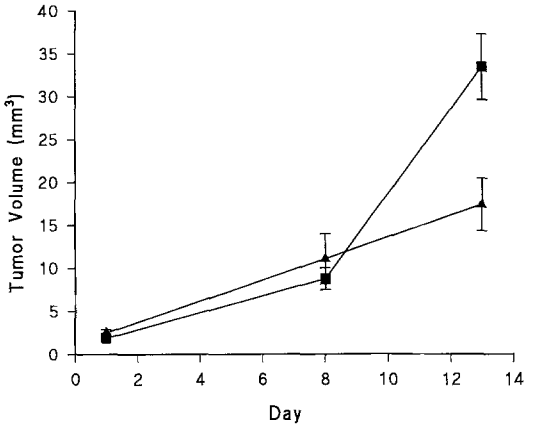

Fig. 1. Growth of untreated and treated explants of human renal cell cancer xenograft (DU11983M) implanted in the subrenal capsule site of athymic nude mouse hosts. Data points represent mean volumes in mm^3 of tumors in treatment cohorts of 11–12 mice and error bars represent one standard error of measurement (SEM). Curve with solid squares – untreated controls (2% BSA); Curve with solid triangles – cohort treated with both ETP (45 mg/kg) and rhTNF (50 μg/kg)

mouse subrenal capsule assay utilizing a human renal cancer xenograft. The findings would support further study of this regimen in renal carcinoma.

Materials and methods

Cytotoxic agents

Recombinant human TNF (bioactivity 2.4×10^6 U/mg, purity > 99% by SDS-PAGE, endotoxin contamination 0.02 ng/ml by LAL assay) was kindly provided by Cetus Corporation (Emeryville, CA). Solutions were prepared using 2% BSA as carrier protein and stored at −20°C before and −70°C freshly prepared in 0.9% saline before each use.

Human tumor xenografts

Tumor tissue was obtained from heterotransplants of human renal cell carcinoma (RCC) line DU11983M maintained by serial subcutaneous passage in nu/nu athymic mice. The tumor line was established from a lung metastasis in a patient with high grade RCC and was used in its 15th passage. The subcutaneous doubling time is 8 days. Athymic nude mice (BALB/c AnBom Urd) from our inbred colony maintained in the Animal Isolation Facility (true barrier facility) of Duke University were utilized for all tumors. Tumor was removed from mice and bathed in RPMI 1640 until implantation within 2–3 h.

Modified subrenal capsule assay

A modification of the subrenal capsule assay (SRCA), previously described by Das et al. [11] was performed. Direct in situ tumor measurement was made through the transparent subrenal capsule utilizing an Olympus stereoscopic zoom microscopic (Olympus Corporation, Lake Success, NY, Model SZH) with an ocular micrometer. Briefly the following protocol was observed:

Day 1. Six to eight week old mice from our breeding colony were weighed after induction of anesthesia with sodium pentobarbital. Using sterile technique the left kidney was externalized through a flank incision and a small incision then made in the renal capsule. Utilizing fine forceps, a tumor fragment (1 mm^3) was placed subcapsularly and exact measurements recorded. The kidney was then replaced and the incision closed with clips.

Day 8. Mice were anesthetized, clips removed, and kidneys exteriorized for precise measurement before re-closure. Volume determinations were made using a formula approximating the volume of a spheroid ($v = (D1 \times D2^2)/2$, where D1 is the larger diameter and D2 is the smaller diameter), and any mice whose tumors regressed were rejected. Remaining mice were then randomized into groups of 11–12.

Days 9 and 10. Animals received therapy in the following cohorts: 1) Control-2% BSA IP, 2) rhTNF-50 μg/kg IV, 3) ETP-45 mg/kg IP and 4) ETP 60 min prior to rhTNF.

Day 13. Mice were sacrificed, weighed, and tumor bearing kidneys excised. Final length and width determinations were made before specimens were sectioned and fixed in 10% formalin for microscopic histologic review.

A 13 day protocol was used in this study in order to allow adequate tumor growth. We have previously reported an 11 day SRCA for a human bladder cancer line (DU4284) with a more rapid doubling time of 4 days [11]. The Day 9 and 10 treatment protocols used in this study were derived from prior studies in our laboratory evaluating the efficacy of rhTNF with various chemotherapeutic agents in the treatment of human bladder cancer. Das et al. [11] identified the ETP/rhTNF combination as most promising among combinations tested in vivo. Larger scale studies of the time course of administration of this binary combination showed enhancement of antitumor effect at several time intervals and both orders of administration, but peak efficacy was achieved when ETP (45 mg/kg IP) was given one hour before rhTNF (50 μg/kg IV) [14].

Analysis of data

Growth ratio and relative tumor survival (RTS). After tumor volume determinations at days 1, 8 and 13 had been made, Day 8/Day 1 and Day 13/Day 8 growth ratios were calculated. Relative tumor survival (RTS) was calculated using the formula $RTS = (Rt/Rc) \times 100$, where Rt is the Day 13/Day 8 ratio of the treatment cohort and Rc is the Day 13/Day 8 ratio of the control (BSA) cohort. RTS of the control group is therefore 100%.

Statistical analysis. Tumor volume ratios were analyzed using the nonparametric Kruskal-Wallis one way analysis of variance [25] to confirm that all cohorts were not statistically different prior to therapy. The nonparametric Mann-Whitney U test was used to determine statistical significance in differences among RTS values of certain cohorts since data contains ratios and is not normally distributed. Data was analyzed using the STATGRAPHICS statistical graphics system (Statistical Graphics Corporation and STSC, Inc.)

Results

Growth of tumor implants

The growth of untreated tumor implants over the test period is illustrated in Fig. 1 (solid squares). For purposes of comparison, the growth curve of tumors treated with both ETP and rhTNF is included (solid triangles). Variability in tumor volume is expressed as one standard error

Table 1. Impact of treatment on tumor volume

	Mean volume day 8	Mean volume day 13
Control (BSA)	8.73 ± 1.26[a]	33.3 ± 3.82[a]
ETP	7.15 ± 0.83	23.6 ± 2.56
rhTNF	7.35 ± 1.01	22.23 ± 3.37
ETP + rhTNF	11.04 ± 2.9	17.2 ± 3.04

[a] ± 1 standard error of measurement

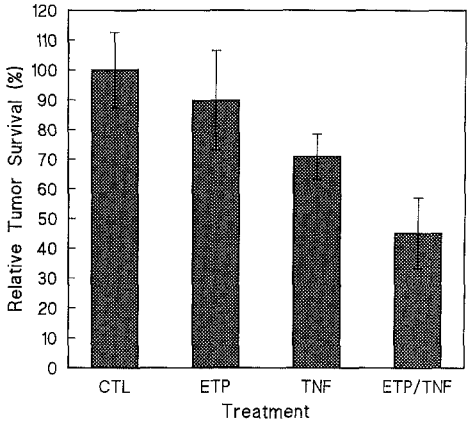

Fig. 2. Evaluation of relative tumor survival (RTS) in cohorts treated with 2% BSA (CTL), 45 mg/kg ETP, 50 μg/kg rhTNF, or both ETP and rhTNF (ETP/TNF). RTS is normalized to control cohort (100%) Error bars represent one standard error of measurement (SEM)

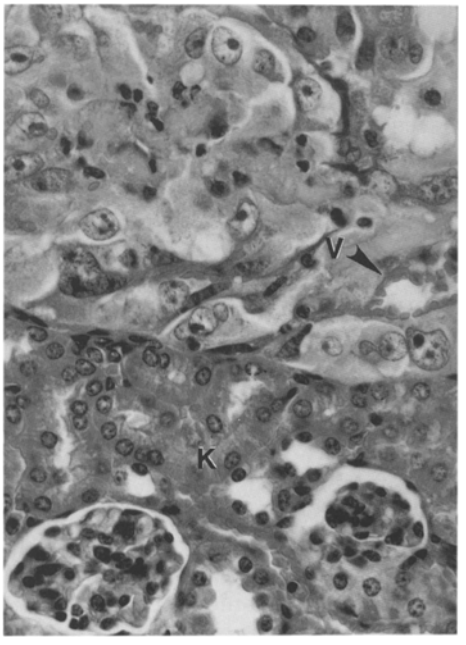

Fig. 3. High power view of a histologic section (H+E stain) of untreated tumor explant at Day 13. The tumor-kidney interface demonstrating normal kidney (K) and poorly differentiated renal adenocarcinoma, with features of primitive glandularity and regions of vascularization (V) (Reduced from 400×)

of measurement (error bars). Tumor explants treated with carrier protein only grew from a mean volume of 1.9 mm^3 (+/− 0.2 mm^3) on Day 1 to 8.7 mm^3 (+/− 1.3 mm^3) on Day 8 and 33.3 mm^3 (+/− 3.8 mm^3) by Day 13. The inhibitory effect of ETP with rhTNF on tumor growth between Days 8 and 13 is evident by comparison of untreated tumor volumes with dual agent treated tumor volumes at Day 13.

Single and dual agent efficacy

To compare single and dual agent impact on tumor progression, pre- and post-treatment tumor volumes of all treatment groups are listed in Table 1. While there is no difference in volumes prior to therapy, and minimal difference between single agent treated tumors and untreated tumors after therapy, a statistically significant difference is noted between untreated tumors and those given both ETP and rhTNF ($p < 0.02$). Figure 2 illustrates the impact of various treatments expressed as Relative Tumor Survival (RTS), an index of growth inhibition normalizing treatment outcome as a ratio of tumor growth of controls (defined as 100% RTS). While ETP and rhTNF appear to have mild to moderate cytotoxic effects, with RTS values of 90% and 71% respectively, there is no statistically significant difference from relative tumor survival in BSA treated mice ($p = 0.25$ and 0.06 by Mann-Whitney U). In contrast, a pronounced and statistically significant tumor inhibitory effect is demonstrated when the agents are combined, with an RTS value of 45% ($p = 0.0005$).

Histologic assessment of therapeutic impact

A representative high power view of a section of BSA treated xenograft fixed at Day 13 is shown in Fig. 3. The appearance is consistent with that of the original tumor: poorly differentiated renal adenocarcinoma, highly vascularized with primitive glandular elements. As seen in Fig. 3, the kidney-tumor interface is distinct with normal kidney parenchyma and glomeruli clearly evident. The cytologic features of high grade malignancy (anisonucleosis, hyperchromasia, irregular nucleoli) are noted in the tumor cells. Figure 4a–d show representative views of each study cohort, illustrating the regression in tumor volume in mice treated with ETP/TNF relative to tumors borne by mice treated with BSA, ETP or rhTNF alone.

Toxicity

An assessment of toxicity of the treatment regimens based on weight loss is outlined in Table 2. Tumor necrosis factor alone produced minimal and insignificant weight loss. Conversely, etopside caused a 16% weight loss alone and 17.5% when combined with rhTNF. Statistically, the difference in toxicity of ETP and ETP/rhTNF is insignificant. All mice survived except one in the ETP/rhTNF group (1/12) which died on Day 10.

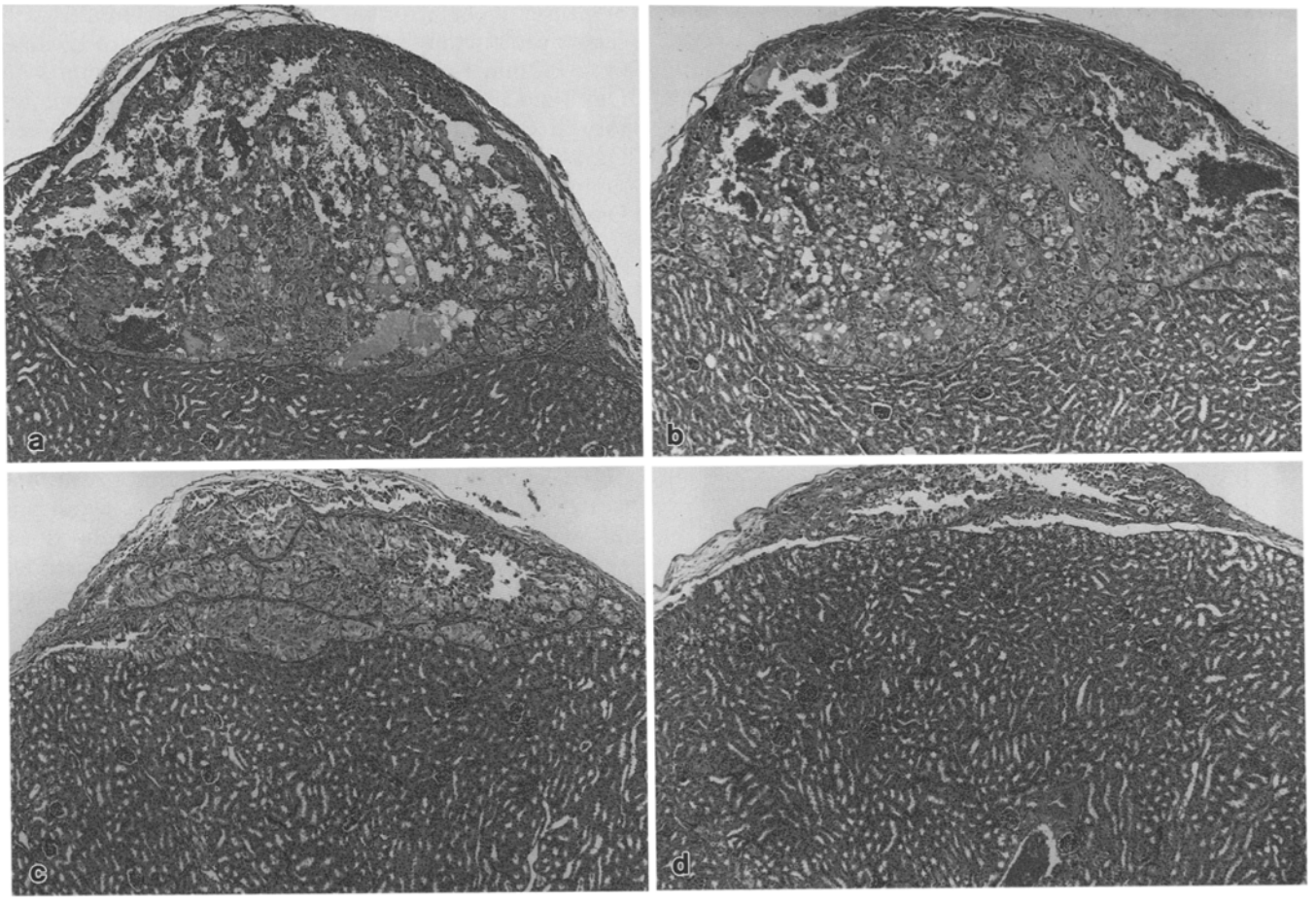

Fig. 4a–d. Representative histologic sections (H+E stain) of treatment cohorts taken at Day 13. **a** Untreated control tumor, **b** ETP alone, **c** rhTNF alone, **d** ETP/rhTNF combination therapy (All reduced from 52×)

Table 2. Host toxicity

Treatment	Weight loss
Control (BSA)	0.0%[a]
ETP	16.0%
rhTNF	0.2%
ETP + rhTNF	17.4%

[a] % pretreatment weight

Discussion

Three primary conclusions can be derived from this experiment which suggest that chemoimmunotherapeutic regimens incorporating rhTNF may prove clinically valuable in the treatment of advanced adenocarcinoma. First, marked cytotoxic enhancement occurs with the coadministration of ETP and TNF in an in vivo assay system utilizing a human renal adenocarcinoma xenograft. Secondly, this tumor line showed minimal sensitivity to either ETP or rhTNF alone, findings which are concordant with clinical testing of these agents against renal carcinoma. The finding in our studies of marginal single agent efficacy is concordant with clinical trials in renal cancer treatment. Etoposide has minimal activity clinically [19]. Likewise, little striking clinical efficacy of rhTNF has been seen in renal cell carcinoma; of the limited number of patients with renal cancer included in phase I and II trials of rhTNF, none have shown any clinical response [6, 9]. Finally, careful consideration of toxicity, the second parameter of therapeutic index, reveals a limited (<20% weight loss) toxicity which appears predominantly attributable to ETP; the lack of substantial augmentation of toxicity with the combination would suggest that the therapeutic index of TNF has been improved and may be suitable for clinical application at acceptable maximal tolerated doses (MTD's).

The augmented antitumor activity seen in this in vivo study correlates well with our previous experience with human bladder cancer xenografts [11, 14] in which enhanced interaction between rhTNF and ETP was also seen; such work in both in vivo tumor models demonstrates that the enhanced interaction initially defined in vitro with murine sarcoma and bladder lines [1, 2] is attainable in vivo. The extent of this enhancement is still not known. Such an enhancement when initially observed in vitro had been termed "synergistic" by previous investigators [1, 2], but whether the interaction is truly "synergis-

tic" would require formal isobolographic [10] or median effect [7] analysis. The actual biochemical mechanism of positive antitumor interaction between rhTNF and ETP also remains conjectural. Renal cell carcinoma cell-lines have demonstrated heterogeneity in responsiveness to rhTNF alone, and TNF resistance could be induced by prolonged cell culture [3, 17]. It has been suggested that certain tumor phenotypes are inherently sensitive to TNF, while some require sensitization by a second agent such as an inhibitor of protein synthesis [13]; whether ETP functions in this way will require extensive further work. Nevertheless, a combined inhibitory effect of 55% (100%-RTS) in the presence of only 29% and 10% single agent inhibition in our study with renal carcinoma xenografts is substantial, and it would suggest the occurrence of a pronounced positive therapeutic drug interaction in vivo by a mechanism yet to be determined.

The importance of evaluating the impact of a novel therapeutic strategy on the in vivo therapeutic index (i.e., the "ratio" of efficacy versus toxicity) is highlighted by this work as well as other recent work from our laboratory. For instance, despite observations by other investigators of enhanced cytotoxic efficacy achieved when actinomycin D and TNF were combined in vitro [1, 10], the addition of actinomycin D to rhTNF in vivo in our SRCA model resulted in an unacceptable level of host toxicity (i.e., uniform lethality at LD_{10} doses) [4, 11]. In the present study with ETP + rhTNF, weight loss data suggest that toxicity is *not* significantly increased above single agent toxicity and remains below the toxicity threshold generally utilized in preclinical animal studies (>20% weight loss) considered consistent with clinical MTD's. Since toxicity observed in this experiment is primarily attributable to etoposide, clinical evaluation of this combination in phase I trials would appear feasible, with anticipated TNF-related host toxicity not significantly greater than single agent toxicity.

While laboratory and preclinical work with TNF as a single agent has not shown any striking activity thus far in advanced renal cancer, our results suggest that a new strategy regarding clinical applicability of TNF may be necessary (i.e. use in conjunction with agents such as ETP). Moreover, several other biologic response modifiers have also demonstrated an enhanced cytotoxicity with TNF, which may be particularly significant in light of the BRM-sensitivity of renal carcinoma. Augmented cytotoxicity of rhTNF by interferon-γ (IFN-γ) was noted in a variety of cell-lines (renal cancer not tested) despite variation of intrinsic sensitivity to rhTNF alone [15]. Substantially enhanced inhibition of colony formation in cells from two human renal cancer xenografts was achieved with the combination of interferon-α (IFN-α) and rhTNF in an in vitro study of renal carcinoma xenografts [3]. These findings may provide the basis for future pursuits towards improved TNF-based BRM combinations. For instance, it would appear important to evaluate the impact of ETP in a ternary combination with TNF and IFN-α, allowing full expression of TNF cytotoxicity. Such studies are ongoing in our laboratory.

In conclusion, we have demonstrated substantial cooperativity between etoposide and tumor necrosis factor in an in vivo model of advanced human renal carcinoma. Toxicity of the combination appears limited and is not significantly greater than the agent toxicity of etoposide. The findings are of particular single significance in light of the accumulating evidence supporting a role for biologic response modifiers in the management of advanced renal cancer and the urgent need for an effective systemic management. While additional studies carefully evaluating therapeutic index and dose and schedule dependency of this promising combination are needed, our findings would support a reassessment of the role of rhTNF as a component of therapy in the treatment of renal carcinoma.

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