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Anti-tumor effect of tumor necrosis factor combined with electrotherapy on mouse sarcoma

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Anti-tumor effectiveness of tumor necrosis factor (TNF)-α applied peritumorally was assessed in combination with local electrotherapy on subcutaneous SA-1 tumors in mice. TNF and electrotherapy each induced significant tumor growth delay. In combined treatment using TNF and electrotherapy, a synergistic anti-tumor effect was observed, regardless of whether TNF was injected before or after electrotherapy. An extra anti-tumor effect was achieved when TNF in the same total dose (2×10^5 U) was split into a priming dose (0.5×10^5 U) 1 h before electrotherapy and the other (1.5×10^5 U) 24 h thereafter. As the result of this therapeutic combination survival rate of the animals was 40%. No animal survived more than 50 days in groups subjected to TNF or electrotherapy treatment alone. Combined treatment with TNF and electrotherapy induced massive tumor destruction, confirmed by histological examination 2 days after the treatment. The results indicate that TNF and electrotherapy interact and that they can be effective in control of local tumor growth.

Key words: Electrotherapy, sarcoma experimental, tumor necrosis factor.

Introduction

Tumor necrosis factor (TNF)-α, one of the biological response modifiers, has demonstrated cytotoxicity in a number of experimental tumor systems.[1,2] In addition to direct cell killing, TNF has been shown to perturb tumor vascular supply, cause hypoxia and decrease pH in the tumor microenvironment.[3,4] Immunomodulatory activity of TNF has also been demonstrated, which activates anti-tumor mechanisms of the organism.[2]

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The results of clinical trials with systemic TNF application have demonstrated that the side effects are severe, with minor impact on tumor growth.[2] Therefore effectiveness of local TNF application, either intratumoral or peritumoral, on tumor growth was tested.[5,6] Studies on animal tumor models have demonstrated that local TNF application is at least as effective as systemic application.[7,8] In addition, some clinical studies indicated that local TNF application is more effective and with less side effects.[5,6] The effects of TNF on the tumor seem to depend mostly on the concentration of TNF at the tumor site.

Several studies have demonstrated that the anti-tumor effectiveness of TNF can be improved in combination with other treatments. Most of the studies demonstrated an interaction of TNF with other biological response modifiers,[2] as well as with cytotoxic treatments such as chemotherapeutic drugs,[9] radiotherapy[7] or hyperthermia.[10]

A new treatment modality which has recently drawn much attention is electrotherapy.[11] Electrotherapy with low-level direct current has been demonstrated to control tumor growth effectively.[12,13] Besides experimental results,[12–14] there are also reports on its clinical use.[15,16] Because its effects are not sufficient to eradicate all clonogenic tumor cells, combinations with other treatments have been suggested, either with chemotherapeutic drugs[16,17] or biological response modifiers, such as interleukin-2[18] and interferon-α.[19] In all these combined treatments, the anti-tumor effect was improved.

The aim of our present study was to determine the anti-tumor effectiveness of TNF combined with electrotherapy. Subcutaneous tumors in mice were treated with TNF peritumorally in combination with local direct current electrotherapy. An *in vivo* tumor growth assay was used to evaluate the anti-tumor effect of these two therapies on a transplantable fibrosarcoma.

Materials and methods

Animals

Inbred A/J mice were purchased from Rudjer Bošković Institute, Zagreb, Croatia. Animals were maintained at constant room temperature (24°C) on a natural day/night cycle, in a standard animal colony. Mice in good condition, without signs of fungal or other infections, 8–12 weeks old, were used in the experiments. Each experimental group consisted of eight to 11 mice.

Tumors

The SA-1 tumor used in this study represents a transplantable spindle-cell sarcoma. It is an immunogenic tumor, originally induced with dibenzanthracene in strain A mice.[20] Viable tumor cells were obtained from the ascitic form of the tumor. Solid subcutaneous tumors, transplanted dorsolaterally, were initiated by injection of 5×10^5 viable SA-1 cells into A/J mice. After the tumors reached 30–40 mm^3 in volume, animals were randomly divided into experimental groups on day 0. On each consecutive day the tumor volume was calculated from orthogonal tumor diameters measured by a vernier caliper gauge and presented as arithmetic means and SEM. Tumor doubling time was determined for individual tumors and the mean doubling time of experimental groups was calculated. Animals that were tumor free 100 days after the treatment were considered cured and were not included in tumor growth curves and tumor doubling time data. The non-parametric Mann–Whitney rank sum test was employed for comparison of tumor doubling times, with Bonferroni adjustment for multiple comparison between experimental groups. Survival data were analyzed by Mantel–Cox statistics.

Tumor necrosis factor therapy

Recombinant human TNF-α analog lacking one to three amino acids from the N-terminal end (TNF) was used (Zimet, Jena, Germany). Specific activity was 2.2×10^7 U/mg, tested on L929 cells in the presence of actinomycin-D. Other properties of this TNF have been described before.[21] TNF was diluted in phosphate-buffered saline (PBS, pH 7.4) before use. TNF was administered subcutaneously 5–7 mm from the tumor margin (peritumorally) in 0.1 ml on two opposite sites of the tumor. TNF application dose was 2×10^5 U per animal, which gave 10561 ± 93 U of TNF/g of animal. The treatment was started when the tumors reached 30–40 mm^3 in volume and was administered in bolus either 1 h before or 1 h after electrotherapy. When the dose was split into two, the first dose (0.5×10^5 U) was administered on day 0, 1 h before electrotherapy, and the second (1.5×10^5) 24 h later, *i.e.* on day 1.

Electrotherapy

The DC source was designed and manufactured at the Faculty of Electrical and Computer Engineering Ljubljana, Slovenia. Current and voltage were continuously monitored during electrotherapy with 0.6 mA DC of 1 h duration. Current was delivered through Pt/Ir (90/10%) alloy needle electrodes (1.0 mm diameter, 22.0 mm long) with rounded tips which were inserted subcutaneously 5–10 mm from the margin of the tumor on the two opposite sites.[22] The control group was treated in the same way as the experimental group, except that no current was administered. During electrotherapy, animals were firmly restrained, with no obvious discomfort; therefore no anesthesia was necessary.

Histologic assessment

Two days after the beginning of treatment, tumors were excised and fixed in 10% formalin. Tumors were cut along the greatest tumor diameter and embedded in paraffin. Hematoxylin and eosin stained slides were evaluated for the presence of necrosis. The extent of necrosis was defined as the percentage of necrotic region compared with the whole area of the tumor section.[13,18]

Results

SA-1 tumors, growing subcutaneously in the flank, were used to test the anti-tumor activity of TNF, electrotherapy or a combination of both. TNF was injected when the tumors reached 30–40 mm^3 in volume, subcutaneously in the vicinity of the tumors, in a concentration of 2×10^5 U. The effect of the treatment was noticeable already 1 day after the treatment: tumor volume was reduced, but thereafter tumors continued to grow and remained smaller than in the control group

(Figure 1, Table 1). There were no serious side effects of local TNF treatment on animals and their weight was not changed for more than 10% during the treatment.

The same tumor growth delay as with TNF treatment was achieved with electrotherapy (0.6 mA for 60 min) (Figure 1 and Table 1). Two electrodes of the opposite polarity were placed subcutaneously 5–10 mm from the tumor margins. Tumors were smaller already 1 day after the treatment, but thereafter regrew and remained smaller than in the control group during the entire period of observation. During and after the treatment no side effects were observed and animal weight was not changed.

To test the importance of TNF timing in combination with electrotherapy, TNF was administered in one group of animals 1 h before electrotherapy and in the other group 1 h after electrotherapy (Figure 1). No difference in anti-tumor effectiveness was observed ($p > 0.10$). Regardless of timing, the effect of combined treatment was better than after single treatments with TNF or electrotherapy (Table 1). Tumors regressed for 5 days, but thereafter regrew again. Although the effect of combined treatment was good, no animals were tumor free and all tumors regrew to the size of 200 mm^3 within 20 days after the treatment. After combined treatment no side effects were observed in the animals.

In order to improve the anti-tumor effect, TNF treatment was split into two doses: the first dose administered 1 h before and the second dose 24 h after electrotherapy. The cumulative dose was the same as in the previous experiment (2×10^5 U), 0.5×10^5 U TNF being injected before and

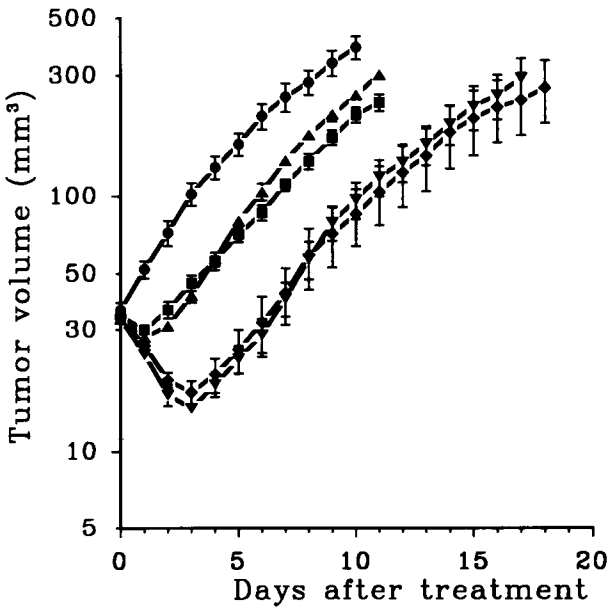


Figure 1. Anti-tumor effect of a single TNF peritumoral application (2×10^5 U) and electrotherapy (ET) (0.6 mA, 60 min) on subcutaneous SA-1 tumors. Combined treatment was performed with TNF applied 1 h before or 1 h after ET. ●, Control; ■, ET; ▲, TNF peritumoral; ◆, TNF 1 h before ET; ▼, TNF 1 h after ET.

1.5×10^5 U after electrotherapy. The anti-tumor effect of TNF in split doses was slightly better than after bolus treatment ($p \geq 0.10$) (Table 1).

In combined treatment with TNF and electrotherapy, the effect was again better with a split TNF dose than in combination treatment with bolus TNF treatment. The tumors regressed for 9 days and tumor doubling time was prolonged from 8.9 to 15.4 days (Figure 2 and Table 1). Combined treatment with TNF before and after electrotherapy also im-

Table 1. Comparison of tumor doubling times in mice treated with TNF peritumorally, electrotherapy (60 min, 0.6 mA) and a combination of both

Treatment	*n*	Doubling times (mean ± SEM)	Control *p*	Electrotherapy *p*	TNF bolus *p*	TNF split dose[a] *p*
Control	18	2.3 ± 0.2				
Electrotherapy	20	5.1 ± 0.2	0.001			
TNF bolus (2×10^5 U) electrotherapy	10	4.4 ± 0.2	0.001	NS		
TNF split dose[a]	7	5.6 ± 0.6	0.001	NS	NS	
TNF bolus 1h before	8	9.3 ± 1.5	0.001	0.004	0.001	NS
TNF bolus 1h after electrotherapy	10	8.9 ± 0.6	0.001	0.001	0.002	0.003
TNF split dose and electrotherapy	8	15.4 ± 3.4	0.001	0.001	0.001	0.006

[a] TNF treatment: 5×10^4 U on day 0, and 1.5×10^5 on day 1.
NS, not significant ($p > 0.10$)

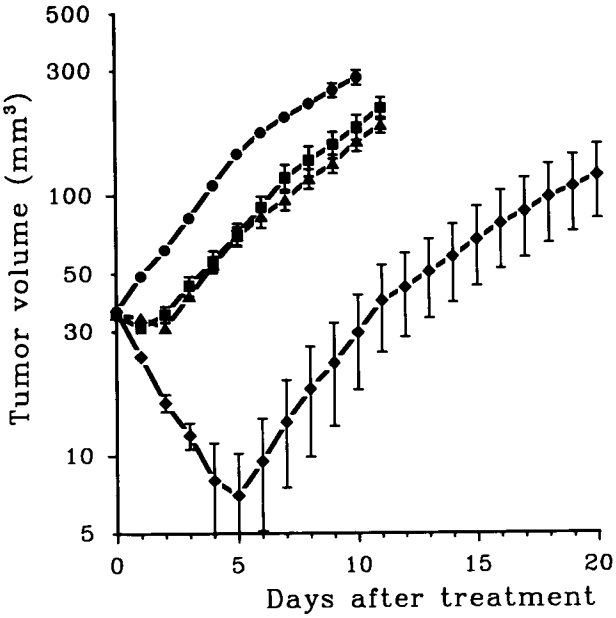


Figure 2. Anti-tumor effect of split-dose TNF treatment (0.5×10^5 U on day 0 1 h before electrotherapy (ET) and 1.5×10^5 U 24 h thereafter) combined with ET (0.6 mA for 60 min). ●, Control; ■, ET; ▲, TNF peritumoral; ◆, combined ET and TNF peritumoral.

proved significantly the survival rate of these animals, without side effects ($p = 0.001$) (Figure 3). Forty per cent of the animals were tumor free 100 days after the treatment, with mean survival time 63.4 ± 8.7 days; all animals were dead within 50 days in the control, TNF- and electrotherapy-treated groups.

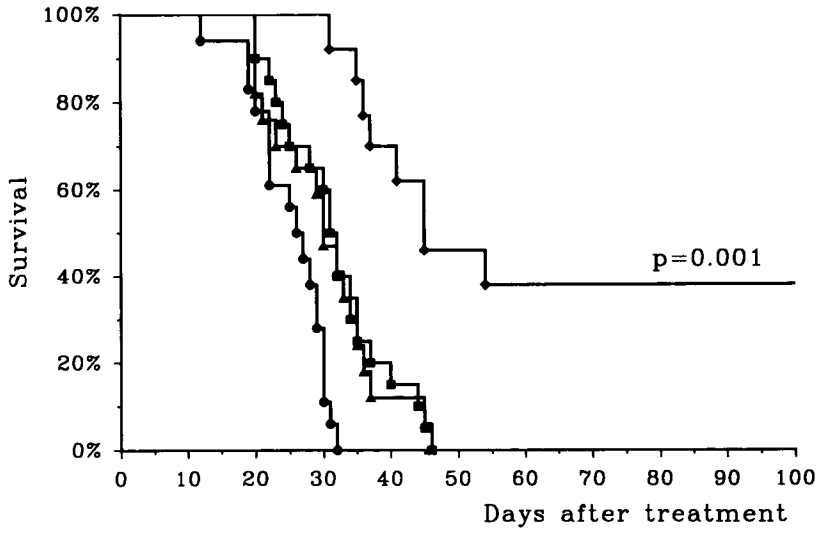
The effect of different treatment modalities on tumor tissue was determined 2 days after the treatment when the tumor reduction was noticeable in all treated groups. Histologically the type of necrosis was similar in all cases. Lesions consisted of a central coalescent totally necrotic area surrounded by a zone of tumor cells displaying pyknosis and karyorexis in different phases. The combined treatment of electrotherapy with split TNF treatment resulted in massive tumor destruction (70%), compared with a much smaller extent of necrosis following single treatment either with TNF or electrotherapy (control, 10%; electrotherapy, 15%; and TNF, 40%).

Discussion

This study has shown that combined treatment of transplantable fibrosarcoma tumors with TNF and electrotherapy is effective in the control of local tumor growth. Evidence that TNF and electrotherapy interact in anti-tumor effectiveness is presented in synergistic tumor growth delay achieved irrespective of TNF timing in the treatment protocol. In fact, treatment with TNF in a split dose, immediately before and 24 h after electrotherapy, induced even increased tumor growth delay and a high percentage of long-term survivors.

TNF, a biological response modifier, has raised great hopes as a possible anti-tumor agent, but because of severe side effects its use in clinics is hampered. One way to increase its effectiveness and reduce side effects is to apply TNF at the tumor site. Intratumoral and regional therapy with TNF are currently being tested.[5,6,8] In our study we injected TNF peritumorally, i.e. subcutaneously in the vicinity of the tumor, without damage to tumor capsule.

Figure 3. Effect of combined treatment with TNF and electrotherapy (ET) on survival of SA-1 tumor-bearing mice. Tumors were treated with TNF 1 h before ET (0.5×10^5 U) and 24 h after ET with 1.5×10^5 U. Electrotherapy was performed with 0.6 mA for 60 min. Survival was plotted by the Kaplan–Meier method. ●, Control; ■, ET; ▲, TNF peritumoral; ◆, combined ET and TNF peritumoral.



This route of application proved to be effective in our previous study.[23] Peritumoral application was equally or even more effective than intravenous application, with less side effects.[24] In addition, lower serum TNF levels in mice were detectable after peritumoral than after intravenous application.[24] Nevertheless, in all settings high doses of TNF are needed for effective antitumor therapy. In our previous study we demonstrated that with higher doses side effects, but not survival rate, increase.[25] Therefore new treatment combinations are being tested, to obtain good results with lower TNF doses.

One of the treatment modalities that may increase the effectiveness of TNF is electrotherapy. Its effect is locoregional on tumor growth, with no detectable side effects.[11,13,14] The mechanisms underlying electrotherapy are probably multiple: biochemical reactions in the vicinity of the electrodes and influences of electric current directly on tumor cells. Among biochemical reactions are changes in pH and changes in ion composition in the extracellular matrix, all of which exert an influence on cell growth and survival.[22,26] Direct current can modulate cell growth directly by enhancing or suppressing cell division.[27,28] For the tumor cells used in this study, it was established that direct current suppresses cell proliferation *in vitro*.[18] Therefore this might be a possible mechanism for anti-tumor effect *in vivo* as well. In order to avoid biochemical and mechanical intrusion into the tumor, in our experiments electrodes were placed subcutaneously outside the tumor. In previous studies it was established that virtually the same anti-tumor effect is achieved as with electrodes inserted directly into the tumor mass.[22] In this study an electric current with moderate anti-tumor effect was chosen, although with higher currents better results can be achieved.

In combined treatment, anti-tumor mechanisms of both treatment modalities interact; this is documented by histology, tumor growth delay and prolonged survival of animals. *In vivo* TNF exerts its anti-tumor action through modulation of immune functions[2] and vascular endothelium.[3,4] TNF is able to modify hemostatic properties of vascular endothelium in such a way that genesis of thrombi is facilitated[29] and is also cytotoxic to vascular endothelium cells.[2] All these effects result in vascular occlusion, diminished tissue perfusion and necrosis. In peritumoral TNF treatment, a cytotoxic effect on tumor cells is also possible, because for cytotoxicity *in vivo*, high TNF concentrations are necessary.[23,29] Electrotherapy can also affect vasculature of the tumors, by induction of microcapillary leakage.[30] Therefore this might be the mutual anti-tumor mechanism with synergistic anti-tumor effectiveness in TNF and electrotherapy. This is demonstrated also by massive destruction (70%) of the tumor 2 days after the combined treatment. The mechanism of more than additive effect in split-dose TNF treatment in combination with electrotherapy is unknown. It is possible that 24 h after electrotherapy the tumor is sufficiently damaged, so that the anti-tumor mechanisms of the organism, stimulated by TNF, can eradicate the remaining tumor cells effectively.

This study shows that TNF and electrotherapy, as local treatments, interact in anti-tumor effectiveness on fibrosarcoma in mice. By combining priming TNF treatment with TNF treatment after electrotherapy, effective tumor control was achieved, including a high survival rate. Whether locoregional tumor treatment with TNF and electrotherapy is feasible in the clinic remains to be investigated.

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