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Anti-tumor effectiveness of electrochemotherapy with bleomycin is increased by TNF-α on SA-1 tumors in mice

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Abstract

With the aim to increase anti-tumor effectiveness of electrochemotherapy, adjuvant immunotherapy with tumor necrosis factor-α (TNF-α) was tested on tumors in mice. Increased anti-tumor effectiveness on SA-1 tumors was observed after combining TNF-α, injected either intratumorally or peritumorally, with electrochemotherapy using suboptimal dose of bleomycin (BLM). The increased anti-tumor effectiveness was neither the result of potentiated anti-tumor effectiveness of TNF-α due to exposure of tumors to electric pulses, nor due to interaction with BLM. Therefore, the effect of adjuvant TNF-α treatment might be immunomodulatory, augmenting the anti-tumor activity of electrochemotherapy, and possibly adding a systemic component to the localized electrochemotherapy treatment. © 1997 Elsevier Science Ireland Ltd.

Keywords: Electrochemotherapy; Electroporation; Bleomycin; Tumor necrosis factor-α; Sarcoma experimental

1. Introduction

One of the ways to increase cytotoxicity of chemotherapeutic drugs is to potentiate drug delivery into cells and tissues. This principle is employed in electrochemotherapy where exposure of cells and tissues to short intense electric pulses increases chemotherapeutic drug delivery [1]. Electric pulses transiently and reversibly increase plasma membrane permeability, enabling poorly and non-permeant drugs like bleomycin (BLM) to diffuse into the cells and to reach their intracellular targets [2,3]. BLM is a non-permeant, but potent cytotoxic molecule when introduced into the cell, therefore electropermeabilization of cells and tissues by electric pulses allows BLM to enter the cell and fully exert its cytotoxic potential. Increased BLM uptake by electropermeabilization was demonstrated in the studies using radiolabelled ^{57}Co-BLM [4,5].

Electrochemotherapy with chemotherapeutic drugs BLM and cisplatin (CDDP) has already been elaborated in preclinical studies [2,6–10], demonstrating that a very low dose of chemotherapeutic drugs is needed for effective local tumor control. These pre-

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clinical data on electrochemotherapy were confirmed in the first clinical trials on malignant melanoma, squamous cell carcinoma and basal cell carcinoma [11–14]. The results of these clinical studies demonstrated that electrochemotherapy with BLM and CDDP offers an approach to treatment of cutaneous and subcutaneous lesions with high response rate. Although electrochemotherapy with BLM or CDDP often leads to complete regression of tumors, many tumors regrow because not all tumor cells have been eradicated. Therefore, new ways are sought to further increase anti-tumor effectiveness of electrochemotherapy.

One of the ways to increase anti-tumor effectiveness of chemotherapeutic drugs is adjuvant immunotherapy with biological response modifiers. Both T-lymphocytes as well as monocytes-stimulating agents are used as adjuvant immunotherapy. Following this idea preclinical studies combining electrochemotherapy with BLM and interleukin-2 (IL-2) have already been performed. Stimulation of T-cells by systemic injection of IL-2 resulted in an increased cure rate in mice [15]. Furthermore, systemic antitumor immunity mediated by $CD4^+$ and $CD8^+$ T-cells was observed after adjuvant therapy with IL-2-secreting cells [16]. In mice bearing two tumors, electrochemotherapy of only one tumor induced cure of the untreated, contralaterally established tumor of the same origin [16].

However, studies with monocytes-activating agents, with the aim to further increase antitumor effectiveness of electrochemotherapy and adding a systemic component to the localized electrochemotherapy treatment, have not been performed. Therefore, this study was undertaken in order to test the hypothesis that adjuvant treatment with monocytes-stimulating biological response modifier TNF-α can increase anti-tumor effectiveness of electrochemotherapy with BLM on SA-1 tumors in mice.

2. Materials and methods

2.1. Tumor necrosis factor

TNF-α used in the study was prepared by LEK d.d., Ljubljana in collaboration with National Institute of Chemistry, Slovenia. Synthetic TNF gene (British Biotechnology, UK) was subcloned into E. coli expression vector pCYTEXP1, modified to enable constitutive expression. In shake culture or small laboratory fermentor an expression level of about 20% was usually achieved. From crude bacterial lysate TNF-α was isolated by ammonium sulfate precipitation followed by sequential application of DEAE Sepharose and Phenyl Superose columns (Pharmacia Biotech, Sweden). The final protein was >99% pure as determined by silver stained SDS-PAGE. Endotoxin level estimated by LAL test (Funakoshi Co. Ltd., Japan) was below 0.1 ng/mg. Cytotoxicity was determined on murine L929 cell line in the presence of actinomycin D. Specific activity of TNF-α was 2×10^7 U/mg. The protein was stored at a concentration of 1.0 mg/ml in PBS at and –80°C in plastic vials. For each experiment, fresh solution of TNF-α was prepared.

2.2. In vitro assay for sensitivity of SA-1 cells toTNF-α and electric pulses

Fibrosarcoma SA-1 cells (The Jackson Laboratory, Bar Harbor, ME) were grown as a monolayer in Eagle minimal essential medium (EMEM) (Sigma, USA) supplemented with 10% fetal calf serum (FCS) (Sigma, USA). Cells were routinely subcultured twice a week and were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Sensitivity of SA-1 tumor cells to continuous exposure to TNF-α as well as sensitivity to combined treatment with TNF-α and electric pulses was determined by MTT assay [17].

To determine the sensitivity of SA-1 cells to continuous exposure to TNF-α, the cells were plated in 96 microwell plates (1000 cells in 100 ml EMEM with 10% FCS per well) (Costar, USA) and incubated for 18 h in a humidified atmosphere with 5% CO_2 at 37°C. After 18 h incubation period the medium was removed and replaced with 100 μl EMEM containing different TNF-α concentrations ranging from 5×10^3 to 1×10^6 U/ml. A particular TNF-α concentration was added to eight wells. After 2 days, 100 μg of MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide, Sigma, USA) (20 μl of 5 mg/ml PBS solution) was added to each well and further incubated for 4 h. The medium was then removed and the formazan crystals were dissolved in 100 μl

of dimethyl sulfoxide (Sigma, USA). The plates were shaken for 99 s and the absorbance of the resulting solution was measured at 540 nm using microplate reader (Anthos, Austria). The survival of cells treated with different TNF-α concentrations was presented as percentage of the absorbance of untreated control cells. The experiment was repeated twice.

The sensitivity of SA-1 cells to combined treatment with TNF-α and electric pulses was determined as described previously [2]. Briefly, exponentially growing cells were trypsinized, washed three times and resuspended in EMEM supplemented with 0.5 mM $CaCl_2$ at a population density of 2.2×10^7 cells/ml. A quantity of 90 μl of cell suspension was mixed with 10 μl of different TNF-α concentrations ranging from 1×10^3 to 5×10^6 U/ml. Half of this mixture was placed between two stainless steel electrodes 2 mm apart and subjected to eight square wave electric pulses, with a pulse length 100 μs, amplitude 200 V and a repetition frequency 1 Hz. Pulses were generated by electropulsator Jouan GHT 1287 (Jouan, France). After pulsing, cells were incubated for 5 min at room temperature and plated in 96 microwell plates (1000 cells per well, six wells per each TNF-α concentration). The other half of the cell suspension was treated as the first half, except for the electric pulses treatment. After 3 days the MTT assay was performed. The survival curve of cells treated by combination of electric pulses and TNF-α was normalized to electric pulses treatment alone. The experiment was repeated twice.

2.3. Mice

In the experiments, an inbred strain of A/J mice of both sexes was used, purchased from Rudjer Bošković Institute, Zagreb, Croatia. The mice were maintained at a constant room temperature (22°C) and natural day/night light cycle, in conventional animal colony. Before experiments, the mice were subjected to an adaptation period of at least 10 days. Mice weighing 20–22 g, in good condition, without fungal or other infections, 10–12 weeks old, were included in experiments.

2.4. Tumors

Fibrosarcoma SA-1 cells (The Jackson Laboratory, Bar Harbor, ME) syngeneic to A/J mice cells were obtained from ascitic form of the tumors in mice, serially transplanted every 7 days. Solid subcutaneous tumors, located dorsolaterally in mice, were initiated by an injection of 5×10^5 viable SA-1 cells. The viability of cells, determined by Trypan-blue dye exclusion test, was over 95%. Six to 8 days after transplantation, when the tumors reached approximately 40 mm^3 in volume, the mice were randomly divided into experimental groups, consisting of 810 mice each, and subjected to specific experimental protocol on day 0.

2.5. TNF-α treatment

For the experiments in vivo, TNF-α was prepared in phosphate buffered saline. Different routes of TNF-α application were used, i.e. intratumoral and peritumoral. Intratumorally, TNF-α was injected into the center of the tumor (0.1 ml) and the needle was withdrawn slowly in order to avoid leakage of the solution. Peritumoral TNF-α injection was split into two portions (0.05 ml) and injected subcutaneously approximately 5 mm from the tumor margin, on two opposite sides of the tumor.

2.6. Bleomycin treatment

Bleomycin (BLM) (Mack, Germany) was dissolved in 0.9% NaCl solution at a concentration of 2 μg/ml. BLM was injected intravenously in bolus into the lateral tail vein of the mice at a dose of 0.05 mg/kg in 0.5 ml of 0.9% NaCl solution (approximately 1 μg per animal). BLM solution was prepared fresh for the daily injections.

2.7. Application of electric pulses

Electric pulses were delivered by two flat parallel stainless-steel electrodes 8 mm apart from each other (two stainless-steel strips, length 35 mm, width 7 mm, with rounded corners) and placed at the opposite margins of the tumor. Good contact between the electrodes and the skin was assured by means of conductive gel. Eight square-wave electric pulses of 1040 V amplitude, pulse width 100 μs and repetition frequency 1 Hz were generated by electropulsator Jouan GHT 1287 (Jouan, France). The application

of electric pulses was performed without anesthesia and was well tolerated by the mice.

2.8. Study design

To determine whether TNF-α can potentiate the anti-tumor effectiveness of electrochemotherapy, intratumoral and peritumoral application of TNF-α was combined with electrochemotherapy. Peritumoral application of TNF-α was chosen in order to avoid the interaction of TNF-α with the applied electric pulses, and to determine possible immunomodulatory action of TNF-α. In the electrochemotherapy protocol, mice were exposed to electric pulses (1040 V) 3 min after intravenous BLM injection (0.05 mg/kg). In combined treatment of electrochemotherapy with intratumoral injection of TNF-α (5×10^4 U per mouse), TNF-α was injected 5 min before the exposure of tumors to electric pulses. In combined treatment of electrochemotherapy with peritumoral TNF-α injection (5×10^4 U per mouse), TNF-α was injected 60 min after the exposure of tumors to electric pulses. The pertinent control groups consisted of groups treated with TNF-α, electric pulses, BLM and combinations of TNF-α, electric pulses and BLM (Table 1). Anti-tumor effectiveness was followed by measurement of the tumor diameters, calculation of tumor growth delay and following the survival of mice.

2.9. Response evaluation and statistical analysis

Tumor growth was followed by measuring three mutually orthogonal tumor diameters (e_1, e_2, e_3) with vernier caliper on each consecutive day. Tumor volumes were calculated by the formula $\pi e_1 e_2 e_3/6$. From the measurements, arithmetic means (AM) and standard error of the means (SE) were calculated for each experimental group. Tumor doubling time (DT) was determined for each individual tumor, and tumor growth delay (GD) from the mean DT of experimental groups was calculated [2]. The response to treatment was scored as complete response when the tumors became unpalpable. Mice that were in complete response 100 days after the treatment were considered as cured.

The significance of differences between the mean values of the DT and GD of the experimental groups was evaluated by modified *t*-test (Newman–Keuls test) after one-way analysis of variance was performed and fulfilled. Survival curves were plotted using Kaplan-Meier's method.

Table 1

Anti-tumor effect of electrochemotherapy with BLM: potentiation of anti-tumor effectiveness with TNF-α

Group	*n*	DT[a]	GD (cont.)[b]	Significance (*P*)	Cures, *n* (%)[c]
Control	8	2.0 ± 0.1		0	
EP 1300 V/cm[d]	8	2.6 ± 0.2	0.6 ± 0.2	0.8	0
BLM 0.05 mg/kg i.v.	8	2.0 ± 0.1	0.0 ± 0.1	1.0	0
TNF-α i.t.[e]	8	3.0 ± 0.2	0.0 ± 0.2	0.8	0
TNF-α i.t. + EP	9	5.7 ± 0.6	3.7 ± 0.6	0.006	0
TNF-α i.t. + BLM	8	3.9 ± 0.3	1.9 ± 0.3	0.5	0
EP + BLM	9	7.6 ± 1.4	5.6 ± 1.4	0.0005	0
TNF-α i.t. + EP + BLM	9	17.1 ± 1.2	15.1 ± 1.2	0.0001	3 (33)
TNF-α p.t.[e]	8	3.1 ± 0.2	1.1 ± 0.2	0.9	0
TNF-α p.t. + EP	10	4.1 ± 0.2	2.1 ± 0.2	0.5	0
TNF-α p.t. + BLM	8	2.9 ± 0.1	0.9 ± 0.1	0.9	0
EP + BLM	9	7.6 ± 1.4	5.6 ± 1.4	0.0005	0
TNF-α p.t. + EP + BLM	8	16.8 ± 2.5	14.8 ± 2.5	0.0001	2 (25)

[a]Tumor doubling time (AM ± SE).
[b]Tumor growth delay compared to control (AM ± SE).
[c]Cures: complete responses 100 days after the treatment.
[d]Electric pulses; electrode distance 8 mm.
[e]TNF-α treatment with 5×10^4 U; intratumorally (i.t.) 5 min before EP, peritumorally (p.t.) 60 min after EP.

3. Results

3.1. Sensitivity of SA-1 cells to TNF-α and combined treatment with electric pulses

In vitro sensitivity of sa-1 cells to tnf-α was tested after continuous exposure of sa-1 cells to tnf-α at concentrations ranging from 5×10^3 to 1×10^6 u/ml by means of mtt assay. None of the concentrations of tnf-α used affected cell survival when tnf-α was present throughout the incubation period (Fig. 1).

The exposure of cells to eight electric pulses, amplitude 200 V (electrode distance 2 mm) at the frequency 1 Hz, pulse duration 100 μs, did not significantly affect the SA-1 cells survival (surviving fraction = 0.92 ± 0.1). To determine whether the exposure of cells to electric pulses can affect the cytotoxicity of TNF-α, the cells in suspension containing TNF-α at concentrations ranging from 5×10^3 to 5×10^6 U/ml were exposed to electric pulses. TNF-α was present during exposure of cells to electric pulses and 5 min thereafter. In the whole range of TNF-α concentrations the electric pulses had no effect on TNF-α cytotoxicity (Fig. 1).

3.2. Anti-tumor effect of electrochemotherapy combined with TNF-α

To determine whether TNF-α can potentiate the anti-tumor effectiveness of electrochemotherapy,

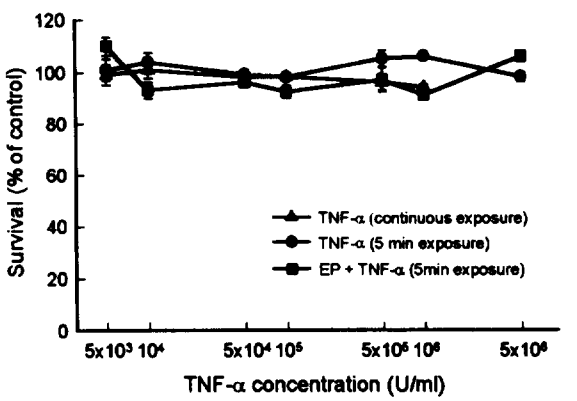

Fig. 1. The effect of TNF-α alone or in combination with electric pulses (EP) on survival of SA-1 cells. Survival of cells was determined by means of MTT assay. Survival curve of SA-1 cells treated with combination of TNF-α and electric pulses was normalized to electric pulses treatment alone. The data represent AM ± SE.

TNF-α was combined with electrochemotherapy using suboptimal, very low dose of BLM. We tested intratumoral TNF-α application 5 min before electrochemotherapy as well as peritumoral application of TNF-α 60 min after electrochemotherapy. Peritumoral TNF-α application was tested in order to avoid the interaction of TNF-α with the applied electric pulses, and to determine possible immunomodulatory action of TNF-α.

Intratumoral, as well as peritumoral application of TNF-α exerted a moderate anti-tumor effect on SA-1 tumors (Table 1). Treatment with BLM as well as the treatment with electric pulses as single treatment failed to produce any anti-tumor effect. Also, there was no interaction between TNF-α and BLM treatments. Exposure of tumors to electric pulses after intratumoral application of TNF-α had moderate but statistically significant anti-tumor effect. In contrast, less anti-tumor effect was observed when TNF-α was injected peritumorally 60 min after application of electric pulses (Table 1). However, the combined treatment with BLM and electric pulses (electrochemotherapy) had a pronounced anti-tumor effect, inducing 5.6 days tumor growth delay, even though a very low dose of BLM was used (0.05 mg/kg) (Table 1). As demonstrated in previous studies higher BLM doses result in better anti-tumor effectiveness, and may also induce tumor cures.

Adjuvant treatment with TNF-α potentiated anti-tumor effectiveness of electrochemotherapy. Combined treatment with intratumoral TNF-α application 5 min before electrochemotherapy resulted in 15.1 days tumor growth delay. Also, peritumoral TNF-α application 60 min after electrochemotherapy resulted in similar tumor growth delay (Table 1). Specifically, the interaction between TNF-α injected either intratumorally 5 min before electrochemotherapy or peritumorally 60 min after electrochemotherapy was more than additive. In addition, combined treatment of TNF-α and electrochemotherapy resulted in curability of the tumors, whereas electrochemotherapy itself did not result in any tumor cures. The combined treatment of TNF-α and electrochemotherapy resulted in 33% cures after intratumoral TNF-α application and in 25% cures after peritumoral TNF-α application (Table 1). Increased anti-tumor effectiveness of combined treatment of TNF-α and electrochemotherapy was reflected also in prolongation of median survival

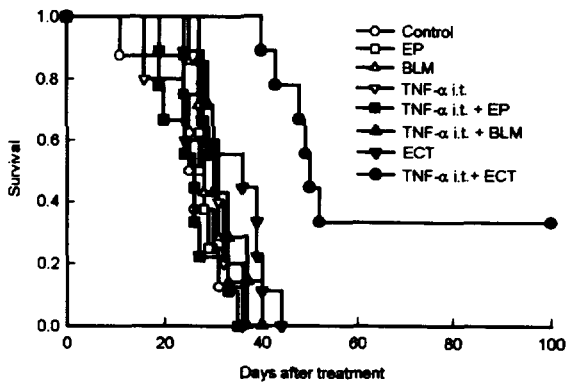

Fig. 2. The survival of SA-1-bearing mice after electrochemotherapy (ECT) combined with TNF-α injected intratumorally 5 min before electrochemotherapy. Mice bearing 40 mm^3 subcutaneous SA-1 tumors were treated with 5×10^4 U TNF-α intratumorally, 2 min later with 0.05 mg/kg BLM intravenously and/or with eight electric pulses (EP) 3 min thereafter (EP amplitude, 1040 V; repetition frequency, 1 Hz; pulse width, 100 μs; electrode distance, 8 mm).

times of mice ($P < 0.001$). Compared to electrochemotherapy group where median survival time was 32.5 days, combined treatment of intratumoral TNF-α application and electrochemotherapy resulted in 50.0 days median survival time, and 60.5 days after combined treatment of peritumoral TNF-α application and electrochemotherapy (Figs. 2 and 3).

4. Discussion

This study shows that adjuvant immunotherapy with TNF-α increases anti-tumor effectiveness of electrochemotherapy with BLM. We found a prolonged tumor growth delay and increased curability of SA-1 tumors in mice after combined treatment with TNF-α and electrochemotherapy, using a very low dose of BLM.

First, we observed interaction in anti-tumor effectiveness after combined treatment of tumors with TNF-α and electric pulses. An additive interaction of the two treatment modalities was observed. However, this interaction cannot be attributed to the potentiated cytotoxicity of TNF-α after exposure of tumor cells to electric pulses. Our in vitro study demonstrated that neither TNF-α nor electric pulses or combined treatment were cytotoxic to SA-1 tumor cells. In vivo, particularly for sarcomas, it was demonstrated that vascular effects of TNF-α have an important role in anti-tumor effectiveness [18]. In addition, the exposure of tumors to electric pulses can lead to temporal arrest of tumor vascular perfusion (unpublished observations). Therefore, the tumor growth delay which was observed after combined treatment with TNF-α and electric pulses could be attributed to the vascular effect of TNF-α [19,20] and/or electric pulses.

Second, no interaction in anti-tumor effectiveness was observed after combined treatment of tumors with TNF-α and BLM. However, in vitro study demonstrated that BLM cytotoxicity could be potentiated by TNF-α. At a maximum BLM dose (3 U/ml), BLM cytotoxicity was increased by 31% in the presence of 100 U of TNF-α [21]. In our experiments, we did not observe increased anti-tumor effectiveness of BLM treatment with TNF-α application, as demonstrated by tumor growth delay (Table 1), probably due to the very low BLM dose used.

Third, the interaction between electrochemotherapy and TNF-α was more than additive; TNF-α greatly potentiated anti-tumor effectiveness of electrochemotherapy as demonstrated by prolonged tumor growth delay and high curability of the tumor. Apart from the vascular effect of TNF-α in combination with electric pulses, this potentiation might be due to indirect antitumor effects of TNF-α,

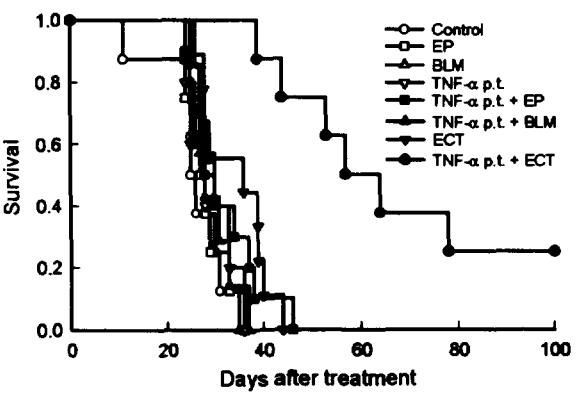

Fig. 3. The survival of SA-1-bearing mice after electrochemotherapy (ECT) combined with TNF-α injected peritumorally 60 min after electrochemotherapy. Mice bearing 40 mm subcutaneous SA-1 tumors were treated with 0.05 mg/kg BLM intravenously and/or with eight electric pulses (EP) 3 min thereafter (EP amplitude, 1040 V; repetition frequency, 1 Hz; pulse width, 100 μs; electrode distance, 8 mm). Sixty min after electrochemotherapy TNF-α (5×10^4 U) was injected peritumorally.

particularly to the immunomodulatory activity of TNF-α [18,22]. Significant contribution of TNF-α to anti-tumor effectiveness of electrochemotherapy was observed either when TNF-α was injected intratumorally 5 min before or peritumorally 60 min after electrochemotherapy. The data presented are even more encouraging since the observed anti-tumor effectiveness of electrochemotherapy in this study was moderate. The BLM dose used in electrochemotherapy was 0.05 mg/kg (approx. 1 μg per mouse), which is a suboptimal dose, and therefore does not have very good anti-tumor effect. Preclinical studies demonstrated that doses higher than 0.05 mg/kg BLM can induce better anti-tumor effect and tumor cures [1,9]. However, the results of this study demonstrate that after adjuvant immunotherapy with TNF-α, even at such a low BLM dose used in electrochemotherapy, a significant prolongation of tumor growth delay was achieved, leading also to curability of the tumors. To determine the therapeutic index and the degree of the interaction between adjuvant immunotherapy with TNF and electrochemotherapy with bleomycin, extensive studies are needed. However, already with this protocol based on suboptimal dose of bleomycin used for electrochemotherapy and only one TNF-α dose tested, the results of the study indicate that there is a synergistic interaction between the two treatments.

Immunomodulatory activity of TNF-α is known to be the activation of various classes of leukocytes, particularly the activation of monocytes/macrophages [22,23]. TNF-α is both the activator and the key mediator of macrophages and natural killer cells tumoricidal action [22]. In addition, TNF-α acts as a second signal for T-cell activation [24]. The role of T-cells in obtaining tumor cures after electrochemotherapy was already demonstrated in previous studies [15,16]. Studies on adjuvant immunotherapy with IL-2 demonstrated that tumor curability is increased by adjuvant therapy with IL-2 or by IL-2 secreting cells [15,16]. In addition to increased localized anti-tumor effect, a systemic response was observed as well, leading to tumor regression at distant sites where no electrochemotherapy was performed [16]. Therefore, the observed potentiation of anti-tumor effectiveness of electrochemotherapy with TNF-α is likely due to the various immunomodulatory activities of TNF-α and also the possible mechanism involved in eradicating tumor cells after electrochemotherapy. This makes TNF-α a good candidate for adjuvant immunotherapy, capable of potentiating the localized anti-tumor effects of electrochemotherapy.

In conclusion, adjuvant immunotherapy with TNF-α results in increased anti-tumor effectiveness of electrochemotherapy with BLM. The potentiated anti-tumor effectiveness is neither the result of potentiated anti-tumor effectiveness of TNF-α due to exposure to electric pulses nor the interaction with BLM. Therefore, the effect of adjuvant TNF-α treatment might be immunomodulatory, augmenting the anti-tumor activity of electrochemotherapy, and possibly adding a systemic component to the localized electrochemotherapy treatment. Based on the promising clinical data on electrochemotherapy, adjuvant immunotherapy can further promote clinical use of electrochemotherapy.

Acknowledgements

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