

Electrochemotherapy: variable anti-tumor effect on different tumor models

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Abstract

Electrochemotherapy is a new approach in the treatment of tumors that takes advantage of the permeabilization of the cell membrane by electric pulses to facilitate the delivery of chemotherapeutic drugs into the cells. According to the procedures described previously, the anti-tumor effectiveness of electrochemotherapy with bleomycin (BLM) was tested on three different murine tumor models, with different biological characteristics, to determine the variability in anti-tumor response. An arrest of growth of fibrosarcoma SA-1, malignant melanoma B-16 and Ehrlich ascites tumor (EAT) was observed in all mice subjected to electrochemotherapy, whereas neither BLM nor electric pulses had an effect on tumor growth when compared with controls. Partial and complete responses were also observed. The best anti-tumor response was observed for the SA-1 tumor model, where tumor growth delay was 31 days and 62% of the animals were free of tumor 100 days after the treatment. Side-effects of electrochemotherapy were demonstrated by body weight loss of the treated animals as well as some animal mortality recorded up to 7 days after the treatment, especially in the animals where tumors were located close to the spine. Our results are in accordance with previous results, and prove that use of high voltage electric pulses is promising for potentiation of BLM anti-tumor effectiveness.

Keywords: Electrochemotherapy; Tumor models

1. Introduction

Electrochemotherapy is a new approach in the treatment of tumors [1,2]. It takes advantage of the permeabilization of the cell membrane by electric pulses to facilitate the delivery of chemotherapeutic drugs into the cells [3,4]. This approach highly potentiates the anti-tumor effectiveness of the cytotoxic drug bleomycin (BLM) [3,4]. The anti-tumor efficacy of electrochemotherapy has been demonstrated *in vitro*, *in vivo* and in clinics [1–7].

BLM is a chemotherapeutic drug used clinically in the treatment of many solid tumors and malignant lymphomas [8]. Its anti-tumor effect is limited by low drug uptake into the tumor cells. Therefore high doses are utilized in clinical protocols, although the presence of only several hundred molecules is needed inside the

cell for BLM cytotoxicity [3]. New approaches are sought to potentiate the effectiveness of BLM, one of these being electrochemotherapy.

Electric pulses (EP) *in vitro* can permeabilize transiently and reversibly cultured cells without loss of cell viability [9]. Such electropermeabilization can facilitate BLM transport into the cells [4]. The *in vivo* anti-tumor effectiveness of electrochemotherapy has been demonstrated and optimized on several animal tumor models [1,2,5,10]. Also, it has been suggested that the anti-tumor response obtained after electrochemotherapy is completed by the natural anti-tumor mechanisms of the organism, most probably immunologic response. Response in immunodeficient mice is less pronounced than in immunocompetent mice, where a high curability rate of the tumors can be achieved [1].

The aim of the study was to repeat electrochemotherapy experiments with BLM according to the procedures described previously and to determine the anti-tumor effectiveness of this therapy on additional tumor models. Three different murine tumor models

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were chosen, differing in biological characteristics, and their response was monitored by following tumor growth delay and complete responses of the tumors.

2. Materials and methods

2.1. Animals

Male A/J and C57Bl/6 mice were purchased from the Rudjer Bošković Institute, Zagreb Croatia and CBA mice were purchased from the Institute of Pathology, University of Ljubljana, Slovenia. Animals were maintained at constant room temperature (24°C) under natural day–night light cycle in a conventional animal colony. Before experiments, animals were subjected to an adaptation period of at least 10 days. Mice in good condition, without signs of fungal or other infection, 10–12 weeks old, were included in experiments.

2.2 Tumors

Three different tumor models were used in the study. Fibrosarcoma SA-1 cells syngeneic to A/J mice and EAT cells syngeneic to CBA mice were obtained from the ascitic form of the tumors. Melanoma B-16 cells syngeneic to C57Bl/6 mice were obtained from *in vitro* cell culture. Cells were grown in EMEM supplemented with 10% FCS, penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹) and gentamycin (11 µg ml⁻¹). Solid subcutaneous tumors, located dorsolaterally in animals, were initiated by the injection of 5×10^5 viable SA-1 cells, 1×10^6 B-16 melanoma cells or 5×10^6 EAT cells. The viability of the cells was determined by Trypan-blue dye exclusion test. When the tumors reached 40 mm³ in volume, mice were marked individually and randomly divided into four groups, consisting of 9–11 mice and subjected to specific experimental protocol on day 0.

2.3. Electrochemotherapy

Bleomycin (Mack, Germany) (BLM) was injected intravenously. Mice were preheated under IR light for a few minutes in order to dilate the tail veins (250 µg BLM dissolved in 0.5 ml physiological saline). Electric pulses were delivered through two stainless steel plate electrodes 8 mm apart (two stainless steel strips, 7 mm in width) and placed at the opposed margins of the tumor. Good contact between electrodes and skin was assured by means of conductive gel. Eight square-wave pulses (amplitude 1050 V, pulse width 100 µs, repetition frequency 1 Hz) were generated by an electropulsator (Jouan GHT 1287, France). In the combined treatment group, mice were treated with electric pulses

3–3.5 min after BLM injection. Mice in the control and electric pulse only groups were injected with physiological saline instead of BLM.

2.4. Assessment of response

Tumor growth was followed by measuring three mutually orthogonal tumor diameters (e_1, e_2, e_3) with a vernier caliper gauge each consecutive day. Tumor volumes were calculated by the formula $\pi e_1 e_2 e_3 / 6$. From the measurements, the mean (AM) and standard errors of the mean (SE) were calculated for each experimental group comprising at least 19 mice, pooled from two separate experiments, including all the necessary control groups. Tumor doubling time (DT) was determined for each individual tumor and tumor growth delay (GD) from the mean DT of experimental groups [11]. The therapeutic response of electrochemotherapy was scored according to WHO guidelines as progressive disease (PD) if tumors increased in size, no change (NC) if the tumors reduced in size less than 50%, partial response (PR) if the tumors reduced more than 50% and complete response (CR) if they became unpalpable. Animals tumor free 100 days after the treatment were termed as cured. The tumor growth curves included only animals with tumor that regrew after the treatment, those animals that were tumor-free 100 days after the treatment were excluded.

Each mouse was also weighed 2–3 times per week. The percentage of body weight loss from pretreatment values was calculated. The maximal weight loss was determined for each mouse 5–6 days after the treatment; this was then averaged for each treatment group. The general condition of the mice was followed throughout the experiments and mortality was recorded.

Statistically the differences between the experimental groups were evaluated by parametric Student's *t*-test, after the *F* test was performed and fulfilled. Levels of *P* less than 0.05 were taken as statistically significant.

3. Results

The anti-tumor effect of electrochemotherapy was tested on three different tumor models SA-1, B-16 melanoma and EAT. The growth rates of the three tumor models were different, the fastest growing tumor being SA-1 with 1.9 days doubling time, then melanoma B-16 with 2.5 days doubling time, and the slowest growing tumor was EAT with 4.6 days doubling time (Fig. 1, Table 1).

Treatment of tumors with BLM only (250 µg intravenously) was ineffective on all three tumor models. Growth delay of the SA-1 tumors was 0.1 days, that of

B-16 melanoma was 0.3 days, and that of EAT was 1.3 days (Fig. 1, Table 1). The BLM treatment dose was well tolerated by the animals, no mortality was observed and body weight loss was not more than 4%.

Electric pulses as single treatment were moderately effective. Tumor growth delay of SA-1 fibrosarcoma was 0.4 days, that of B-16 melanoma was 1.5 days and that of EAT was 2.0 days (Fig. 1, Table 1). The treatment was well tolerated by all animals. Body weight loss was not more than 3%, but two animals died, one bearing SA-1 and one bearing EAT (Table 1). These two animals died in the first set of experiments when the tumors were initiated close to the spine. The SA-1 bearing animal died on day 5, and the EAT bearing animal on day 6. In the second set of experiments tumors were initiated further ventrally and no mortality was recorded.

Electrochemotherapy induced an arrest of tumor growth of all three tumors (Fig. 1). The reduction in tumor volume was detectable already one or two days after the treatment. In the first few days after the treatment tumor regression was accompanied by local oedema, which hindered accuracy of the measurements, but thereafter tumors continued to diminish and also local oedema. Tumor regression was gradual without exulceration, taking 8–10 days before reaching partial or complete response. Transiently, superficial scabs occurred in tumors that responded well, but scabs disappeared progressively in 15–20 days. SA-1 tumors regrew in 20 days after the treatment, B-16 melanoma tumors in 15 days and EAT in 18 days after the treatment. The effectiveness of electrochemother-

apy treatment was reflected in tumor growth delay, being 31 days for SA-1 tumors, 20 days for B-16 melanoma and 21 days for EAT (Table 1). Also the highest percentage of complete responses (79%) was observed for SA-1 tumors, while for B-16 melanoma this was 25% and on EAT 35%. Some of the tumors regrew within 25 days after the treatment, but many not even in 100 days (Fig. 2). The percentage of animals that were without tumors 100 days after the treatment varied, the highest being for SA-1 bearing animals (62%) and lower for the B-16 melanoma and EAT bearing animals (5% and 19% respectively).

Side-effects of electrochemotherapy were evaluated by body weight loss and mortality of the animals. The maximal body weight loss was recorded on days 5 and 6 after the treatment, thereafter the general condition of the animals was improving. Mortality of the animals was recorded up to 7 days after the treatment. In the first set of experiments, when tumors were initiated close to the spine, SA-1 tumor bearing animals lost $21 \pm 2\%$ of body weight and four of them died as a result of the treatment. In the second set of experiments when the tumors were initiated further ventrally, no mortality was recorded and body weight loss was only $14 \pm 1\%$. Similar results were obtained with EAT bearing animals where in the first set of experiments two animals died as a consequence of the treatment and they lost $11 \pm 2\%$ body weight; in the second set of experiments when the tumors were initiated further ventrally no mortality was recorded and animals lost approximately the same body weight ($15 \pm 2\%$). In C57Bl/6 mice B-16 melanoma was initiated in both

Table 1
Summary of the electrochemotherapy anti-tumor effect on three different tumor models, SA-1, B-16 melanoma and EAT

| Tumor type, experimental groups | <i>n</i> | DT ^a (days) | Growth delay ^b (days) | Response to treatment ^c | Long-term survivors ^d (<i>n</i> , %) |
|---------------------------------|----------|------------------------|----------------------------------|------------------------------------|--|
| <i>SA-1</i> | | | | | |
| Control | 31 | 1.9 ± 0.5 | – | – | 0 |
| BLM | 30 | 2.0 ± 0.5 | 0.1 ± 0.02 | PD | 0 |
| Electric pulses | 26 | 2.3 ± 0.5 | 0.4 ± 0.02 | PD | 0 |
| Combination | 29 | 32.9 ± 11.4 | 31.0 ± 3.8 | PR/CR | 18 (62) |
| <i>B-16</i> | | | | | |
| Control | 20 | 2.5 ± 0.4 | – | – | 0 |
| BLM | 20 | 2.8 ± 0.6 | 0.3 ± 0.2 | PD | 0 |
| Electric pulses | 20 | 4.0 ± 1.0 | 1.5 ± 0.3 | PD | 0 |
| Combination | 20 | 22.8 ± 4.2 | 20.3 ± 1.1 | PR/CR | 1 (5) |
| <i>EAT</i> | | | | | |
| Control | 21 | 4.6 ± 1.7 | – | – | 0 |
| BLM | 19 | 5.9 ± 1.7 | 1.3 ± 0.5 | PD | 0 |
| Electric pulses | 19 | 6.6 ± 2.8 | 2.0 ± 0.7 | PD | 0 |
| Combination | 21 | 25.5 ± 4.2 | 20.9 ± 1.0 | PR/CR | 4 (19) |

^a DT tumor doubling time (AM ± SD).

^b Growth delay (AM ± SD).

^c Response to treatment was evaluated according to WHO guidelines; PD progressive disease, PR partial response, CR complete response.

^d Long-term survivors (cures) were determined 100 days after the treatment.

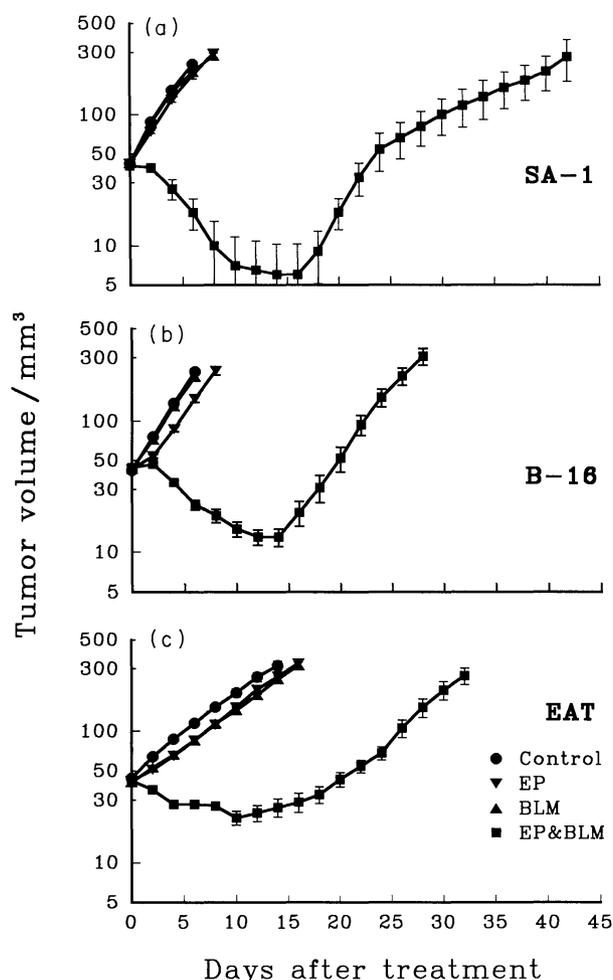


Fig. 1. Effect of electrochemotherapy on growth of SA-1 (a), B-16 melanoma (b), and EAT (c) subcutaneous tumors. Animals bearing 40 mm³ tumors were treated with BLM (250 μ g) intravenously and/or with 8 electric pulses (1050 V, 1 Hz, 100 μ s) 3–3.5 min after BLM treatment. The growth curves include animals in which tumors regrew; those that were tumor-free 100 days after the treatment were excluded.

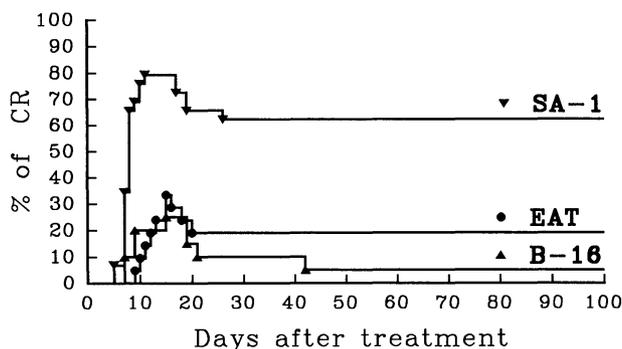


Fig. 2. Percentage of complete responses in SA-1, B-16 melanoma and EAT bearing animals after electrochemotherapy.

sets of experiments further ventrally, therefore no mortality and only $3.0 \pm 1.6\%$ body weight loss was recorded.

4. Discussion

This study shows that electrochemotherapy with BLM is an effective anti-tumor agent for the three murine tumor models tested in the three different syngeneic mice strains: fibrosarcoma SA-1 in A/J mice, malignant melanoma B-16 in C57Bl/6 mice, and EAT in CBA mice. A variable anti-tumor effect was observed, the best effect being for SA-1 tumors where 62% of the animals were free of tumor 100 days after the treatment. Electrochemotherapy was less effective on the B-16 melanoma and EAT.

Our results are in accordance with previously published results and extend them to other murine models in various mice strains [1,2,12]. Significant tumor growth delay was observed with electrochemotherapy on all three murine tumor models, ranging from 31 days on SA-1 tumors to 20 days on B-16 melanoma and 21 day on EAT. All tumors treated with electrochemotherapy responded partially or completely for at least 20 days, while the anti-tumor effect of BLM or electric pulses as single treatment was moderate or negligible, resulting at the most in just a brief transient slow down in tumor growth. In most cases, although electrochemotherapy was very effective, tumors regrew and eventually killed the animals. Only for SA-1 tumors was a substantial curability rate achieved, since animals were free of tumors 100 days after the treatment.

Therapy with electric pulses was performed with the same experimental setting as described previously [1,2,12], eight square-wave pulses, amplitude 1050 V, pulse width 100 μ s, frequency 1 Hz. Therapy with BLM was performed 3.0–3.5 min before therapy with electric pulses [12,13]. Injection of BLM was intravenous into the tail vein, and not into the orbital sinus as previously reported [12,13]. This modification of the experimental procedure might affect the anti-tumor response, because it requires preheating of the animals, which may influence the pharmacokinetics of BLM. This aspect must be clarified in further experiments since the timing of drug administration is very important for the effectiveness of electrochemotherapy. Nevertheless, preliminary experiments on skin damage indicate that a lag time of less than 3 min between BLM and EP therapy is less effective. The BLM dose chosen in our experiments was 250 μ g per animal, although it was demonstrated that for effective tumor control 100, 50 or even 10 μ g per animal are sufficient [1,2,12,13]. Nevertheless, this BLM dose did not induce any mortality of the animals and did not affect animal body weight.

Side-effects of electrochemotherapy have been reported by Mir et al. [1]. In this study too, in the first set of experiments electrochemotherapy resulted in early mortality of a few animals up to 7 days after the treatment. This was observed only when tumors were initiated close to the spine. In the second set of experiments, when the tumors were initiated further ventrally, no mortality due to the treatment was recorded. As some early deaths were also observed in mice bearing tumors close to the spine and treated only by the electric pulses, it is conceivable that mortality could be due mainly to damage of the spine by electric pulses rather than due to the combination of BLM and the electric pulses. Nevertheless, this is a minor point because in animals larger than mice, electrochemotherapy is a true local treatment and not a regional treatment as in the case of mice.

The importance of immune response in the anti-tumor effectiveness of electrochemotherapy was discussed and published. Eradication of the last clonogenic cell in the organism is possible only in immunocompetent hosts [1,2], and stimulation of anti-tumor mechanisms of the organism increases the effectiveness of electrochemotherapy [12,13]. The immune reaction associated with tumor regression was demonstrated also by the infiltration of immunocompetent cells in tumor tissue after electrochemotherapy and the presence of a peritumoral oedema in the most responsive tumor models [1]. Peritumoral oedema was observed also for SA-1 tumors, which were the most responsive to electrochemotherapy in this study. For B-16 melanoma and EAT tumors less oedema was observed. This might correlate with the immunogenicity of these tumors. SA-1 is highly immunogenic [14], while B-16 melanoma [15] and EAT [16] are moderately immunogenic. Immunogenicity of the tumors can also be correlated with the anti-tumor effectiveness of electrochemotherapy, since electrochemotherapy resulted in a high curability rate for immunogenic tumor (SA-1), and in a low curability rate for moderately immunogenic tumors (B-16, EAT).

In conclusion, this study shows that electrochemotherapy is effective in the treatment of subcutaneous tumors. Results of this study are in accordance with previously reported data, and support them, demon-

strating the anti-tumor effectiveness of electrochemotherapy on additional murine tumor models and various mice strains. A variable response to electrochemotherapy was observed, which reveals on the importance of the host's anti-tumor mechanisms for eradication of all clonogenic tumor cells.

Acknowledgement

The work was supported by the Ministry of Science and Technology of the Republic of Slovenia, contract no. P3-5252-0302.

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