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Effectiveness of tumor electrochemotherapy as a function of electric pulse strength and duration

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Abstract

The aim of this study was to evaluate the effectiveness of electrochemotherapy (ECT) as a function of various combinations of pulse strength and duration. C57Bl mice bearing LLC tumors were injected i.p. with bleomycin (BLM) at doses 5 mg/kg in 0.2 ml of physiological saline. Thirty minutes later, tumors were positioned between plate electrodes and were pulsed with eight-square wave electric pulses with an individual pulse strength of 900, 1100, 1300 or 1500 V/cm and duration of 0.1, 0.25, 0.5 or 1 ms. Effectiveness of ECT was estimated by measuring inhibition of tumor growth and by estimating extent of necrosis in histological slices of the treated tumors. At pulse strength of 900 V/cm and duration of 0.1 ms, electrochemotherapy was ineffective. Noticeable inhibition of tumor growth (threshold of ECT) was obtained when pulse duration at this field strength was increased up to 0.25 ms. Further increase of pulse strength and/or duration resulted in progressive enhancement of antitumor effects. Using tumor doubling time (DT) as a criteria, we showed that the same efficacy of ECT could be achieved using various pairs of values for pulse strength and duration. Largest antitumor efficacy of ECT was obtained at pulse strength of 1500 V/cm and duration of 1 ms. These pulse conditions applied alone neither significantly suppressed tumor growth nor induced noticeable side effects of the surrounding tissues. The results of this study thus suggest that the effectiveness of electrochemotherapy can be enhanced (in comparison to widely accepted conditions of electrochemotherapy—8 pulses of 1300 V/cm, 0.1 ms) if 1500-V/cm, 1-ms electric pulses are used. Our study also implicates that other pulse conditions could be found for this enhanced ECT.
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1. Introduction

When the cell in suspension or in tissue is exposed to sufficiently strong and long lasting electric field pulse(s) the permeability of the cell membrane temporarily increases providing access of exogenous molecules into the cytosol of the cell. The phenomenon is known as electroporation or electropermeabilization [1]. It has been successfully exploited for different biomedical applications in vivo [2]. The most promising between these applications are DNA electrotransfer into tissues for gene therapy [3–5] and antitumor electrochemotherapy [6–9]. Antitumor electrochemotherapy (ECT) is much more progressed and currently entered clinical trials. The treatment consists of the injection of highly cytotoxic drug (like bleomycin or cysplatin) followed by delivery of high voltage electric pulses to the tumor. The key point of ECT is that tissue permeabilization allows local targeting of cytotoxic drugs into the cells of the tumor exposed to electric field. This enables one to obtain effective antitumor responses with highly reduced doses of cytotoxic drugs.

Effectiveness of ECT basically depends on two main requirements of the treatment: (i) effective distribution of the highly cytotoxic drug (at appropriate doses) in the tumor and (ii) permeabilization of the vast majority of the tumor cells. Since in ECT trials most usually bleomycin, a hydrophilic drug, is used, it is assumed that the drug injected systemically or intratumorally prior to delivery of electric pulses distributes freely in the volume of tumor tissue. To our knowledge, dynamics and pattern of this distribution in whole tumor are

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still unknown. Nevertheless, effectiveness of ECT is dose dependent [6,8,10], which clearly shows that appropriate amounts of the drug are needed for the effective electrochemotherapy.

The second requirement has been an object of more intense studies. In vitro cell permeabilization depends on many parameters, but all above, on the parameters of electric pulses, that is: shape, strength, duration, number and frequency at which the pulses are delivered [11–16]. In vitro, it was established that high percentage of permeabilized cells, with minor percentage of killed cells, can be obtained using eight-square wave pulses with individual pulse strength of 1300 V/cm, duration of 0.1 ms [17,18]. These pulses were also effective in vivo conditions to permeabilize cells in tumor tissues [6,19,20]. Consequently, already in the first experiments on in vivo electrochemotherapy, high responses of the treatment were obtained [6]. These findings suggest that majority of the tumor cells in tissue were permeabilized by the pulses and destroyed by bleomycin internalized into the cells. Therefore, eight square-wave pulses of 1300 V/cm, 0.1 ms were widely accepted in the world and applied for electrochemotherapy of many tumor models in preclinical as well as in clinical trials [6–9,21–24].

In spite of these reports, there is a lack of more systemic studies on the effectiveness of ECT in dependence of various parameters of electric pulses. This study was thus designed to investigate the effectiveness of ECT in dependence of various combinations of electric pulse strength and duration. Along this, we also aimed to determine relation of pulse strength and pulse duration resulting in the same efficacy of electrochemotherapy.

2. Materials and methods

2.1. Animals and tumor model

Eight- to twelve-week-old female C57Bl mice (Institute of Immunology, Vilnius, Lithuania) were employed in the experiments. Animals were maintained at steady room temperature (22 °C) with natural day–night cycle. To obtain solid tumors, the tumor tissue was removed from the mice bearing Lewis Lung carcinoma (LLC) tumors and minced with a pair of scissors after addition of sterile 0.9% NaCl with ratio 5 ml to 1 g of tumor tissue. Then 0.2 ml of the suspension was injected subcutaneously in the right hind limb of the experimental mice. When tumors reached approximately 200–400 mm^3 in volume, the mice were divided into experimental groups (at least six mice in each group) and subjected to specific treatment.

2.2. Electrochemotherapy

Bleomycin (BLM) (Nippon Kayaky, Japan) in 0.2 ml was administered by intraperitoneal injection 30 min before delivery of electric pulses. Treated mice received 5 mg/kg of the drug. Electric pulses were delivered using distance-adjustable plate (two flat stainless steel plates, 8 mm in width, 20 mm in length) electrodes. The electrodes were placed at the opposite sides of the tumor and the distance of the plates was adjusted according the size of the tumor. To reach good contact between the skin and the electrodes, the fur was clipped off from the tumor area, and conductive gel was used. Square-wave electric pulses were generated by an electroporator constructed and manufactured in our laboratory. The tumors were pulsed with eight-square wave electric pulses of variable strength (900, 1100, 1300 and 1500 V/cm) and duration (0.1, 0.25, 0.5 and 1 ms) delivered at 1 Hz. Shape, voltage and duration of each individual pulse were controlled by means of storage oscilloscope (C8-13, Russia). ECT treatment was performed without anesthesia and was well tolerated by the mice. Mice were sacrificed either 14 days after the treatment or 2 days after tumor in mouse doubled in volume.

2.3. Assessment of response and treatment of the data

The tumor's longest diameter (a) and the next perpendicularly longest diameter (b) were measured with a caliper every second day, starting from the experimental day '0'. The tumor volume (V) was calculated by the formula $V=ab^2\pi/6$ [25]. From the measurements, tumor doubling time (DT) was determined for each individual mice. From the values, the mean and standard errors of the mean of tumor volume and DT were calculated. Similarly, extent of tumor necrosis (see bellow) in individual groups was expressed as a mean±standard error of the mean. To evaluate statistical difference between experimental groups, two-tailed Student's t-test for paired values was used. Statistical difference was taken to be significant when $p<0.05$.

Experimentally defined DT in dependence of pulse duration for each pulse strength (900, 1100, 1300 and 1500 V/cm) were fitted to a two parameter logarithmic curve $DT(\tau)=a+b\times\ln(\tau)$, where τ is pulse duration, a and b are parameters that determine shape of the logarithmic curve. Thereby, four fitted curves exposing tumor doubling time as the functions of pulse duration were obtained. The line crossing these curves in parallel to τ axis gave us at least four pulse strength–duration values defining the same tumor doubling time, and consequently the same efficiency of ECT. By plotting these values on strength–duration coordinates and fitting them to a three parameter exponential curve $E(\tau)=a+b\times e^{-c\tau}$ (E is pulse strength, τ is pulse duration, a, b and c are parameters that determine shape of the exponential curve), the set of strength–duration relations, which define the same DT, was determined. All fits were obtained by least-squares nonlinear regression using Sigma Plot 6.10 (SPSS, Richmond, CA, USA).

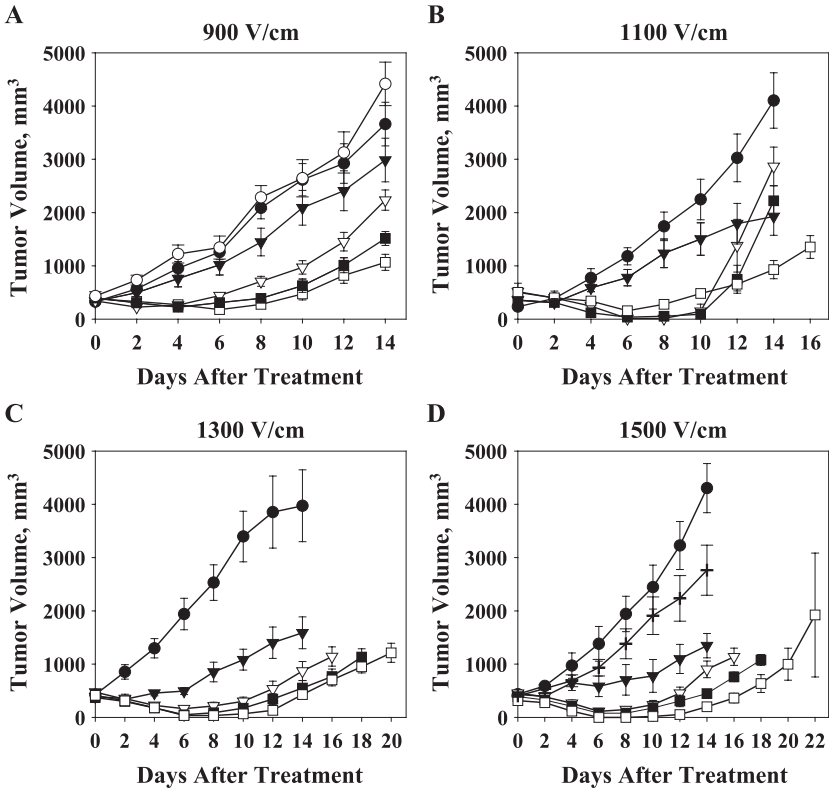

Fig. 1. Lewis Lung Carcinoma growth curves of control tumors (●), of the tumors treated with bleomycin, 5 mg/kg (○), electric pulses (+) and electrochemotherapy at various parameters of electric pulse strength: 900 V/cm (A), 1100 V/cm (B), 1300 V/cm (C), 1500 V/cm (D) and pulse duration: 0.1 ms (▼), 0.25 ms (▽), 0.5 ms (■), 1 ms (□). Each point in the figure represents the mean±S.E.M.

2.4. Histological analysis

Tumors were excised 3 days after the treatment. Specimens were fixed immediately in 4% buffered neutral formalin for 24 h. The tissue was processed for dehydration in an alcohol of rising concentration for 24 h in each. Then the tumor tissue was immersed in two portions of a clearing agent for 1.5 h in each followed by immersion in liquid paraffin for 1.5 h in one portion and for 24 h in another. After embedding the tumor tissue in paraffin, slices of 4 microns thickness were prepared using Reichert-Jung microtome (Germany). The slices were stained by standard hematoxylin and eosin method. For each experimental condition, four mice were treated, and from each mouse five tumor slices were prepared. Histological preparations were examined under microscope (PZO, Poland) equipped with a digital photo camera (Canon, NY, USA). From each slice, five images were taken from random places. In overall, extent of tumor necrosis after specific treatment was determined using 100 photographs.

3. Results

3.1. Evaluation of effectiveness of ECT in dependence of pulse strength and duration

The main objective of this study was to evaluate effectiveness of electrochemotherapy on Lewis Lung Carcinoma tumors in dependence of pulse strength and duration. To this end, four sets of experiments using various combinations of strength (900, 1100, 1300 or 1500 V/cm) and duration (0.1, 0.25, 0.5 or 1 ms) were performed. The effectiveness of ECT expressed as changes of tumor volume and doubling times (DT) in dependence on these pulse parameters are shown in Fig. 1 and Table 1.

Table 1
Tumor doubling times (DT) resulting from electrochemotherapy using various parameters of electric pulse strength (900, 1100, 1300 and 1500 V/cm) and pulse duration (0.1, 0.25, 0.5 and 1 ms)

Control	Pulse duration and strength (V/cm)	0.1 ms	0.25 ms	0.5 ms	1 ms
3.1±0.4	900	3.6±0.6	8.3±0.3	11.3±0.4	12.4±0.8
3.4±0.6	1100	6.3±1	13.2±1	12.7±0.7	15±0.5
3.7±0.5	1300	10±1.8	14.4±0.8	16.3±0.8	17.3±0.7
2.9±0.5	1500	9.7±2	14.7±0.4	17±0.6	19.2±0.8

DT is expressed in days as mean±S.E.M.

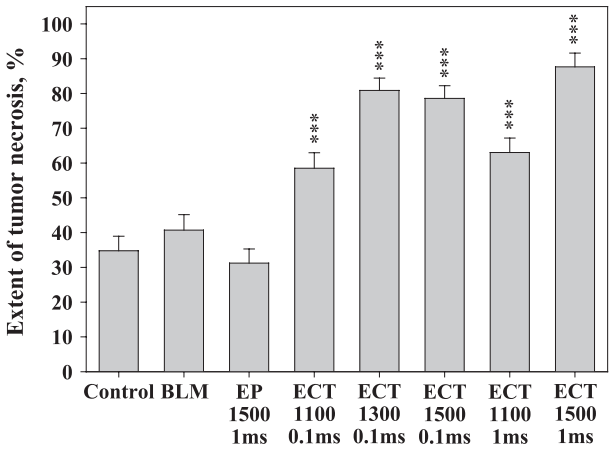

Fig. 2. Extent of necrosis after treatments with bleomycin (BLM), electric pulses (EP) and electrochemotherapy using various parameters of electric pulse strength and duration. Each column in the figure represents the mean±S.E.M. All groups marked with three asterisks are statistically different (p<0.001; t-test) from the controls.

As a positive control to electrochemotherapy groups, treatment of tumors was performed with bleomycin alone at a dose of 5 mg/kg (a dose used throughout in our experiments) (Fig. 1, panel A). This treatment did not inhibit tumor growth as compared with controls (p>0.05): DT at this condition was 3.7±0.6 while in control group it was 3.1±0.4. As another positive control, treatment was performed using electric pulses only. The most intense conditions of the pulses (strength—1500 V/cm, duration—1 ms) were tested (Fig. 1, panel D). It was found that this treatment was also ineffective (p>0.05). DT at this condition was 2.3±0.3 while in controls it was 2.9±0.5.

ECT treatment using lowest parameters of pulse strength and duration (900 V/cm, 0.1 ms) was also ineffective (p>0.05). Inhibition of tumor growth by ECT started to be significant (p<0.05) when, at the same pulse strength, the pulse duration was increased up to 0.25 ms (Fig. 1, panel A; Table 1). Further increase in pulse duration up to 1 ms resulted in progressive increase of antitumor effects. Similar tendencies were also determined for pulse strengths of 1100 V/cm (Fig. 1, panel B). However, at this strength, ECT was effective (p<0.05) already at 0.1-ms pulse duration with further progressive increase of the effectiveness at 0.25, 0.5, 1 ms (Table 1). A clear shift to longer DTs was obtained using 1300-V/cm pulse strengths (Fig. 1, panel C; Table 1). Additionally, at 1300 V/cm, using longer duration of the pulses (starting from 0.5 ms), short-term complete responses of the treatment were observed in several cases. Largest antitumor effects of electrochemotherapy were obtained with 1500-V/cm strength pulses (Fig. 1, panel D; Table 1). At this strength using 1-ms pulses, 1-week complete responses were observed in four cases out of six. Overall, at higher pulse strengths and/or longer pulse duration, a clear shift to prolonged inhibition of tumor growth was obtained (Fig. 1, Table 1).

3.2. Extent of necrosis

The extent of tumor necrosis was determined in controls, in the group treated with bleomycin (5 mg/kg), in the group treated with electric pulses (1500 V/cm, 1 ms) and in electrochemotherapy groups using various parameters of pulse strength and duration (Fig. 2). The treatment with bleomycin alone or with the electric pulses did not result in significant increase of tumor necrosis as compared to the controls. However, in all electrochemotherapy groups, the extent of necrosis was increased significantly (p<0.05). In electrochemotherapy groups at 0.1-ms pulse duration, extent of the necrosis increased from 59% to 81% and 79% for pulse strength 1100, 1300 and 1500 V/cm, respectively. Largest extent of tumor necrosis was obtained at 1500-V/cm and 1-ms pulse conditions. Even though there was no statistical significance, these pulses compared to widely accepted pulse conditions (1300 V/cm, 0.1 ms) [6–9] resulted in an increase of tumor necrosis from 81% to 88% (Fig. 2).

3.3. The relation of pulse strength and duration

In order to find out the relation of pulse strength and duration resulting in the same effectiveness of ECT, tumor doubling time (DT) as a function of pulse duration was determined for each pulse strength (Fig 3). The relation of pulse strength and duration resulting in the same effectiveness of ECT was evaluated for DT being in the range between 9 and 12 days (Fig. 4). Finding pulse strength–duration values for various DTs and fitting them to a three parameter exponential curve $E(\tau)=a+b\times e^{-c\tau}$, we found a pulse strength–duration curve that crosses the point of interest: 1300 V/cm and 0.1 ms, i.e. the pair of values for pulse strength and duration widely used by other authors [6–9]. The DT at which such pulse strength–duration curve was obtained was 9.35 days (Fig 4). This allowed us to find out

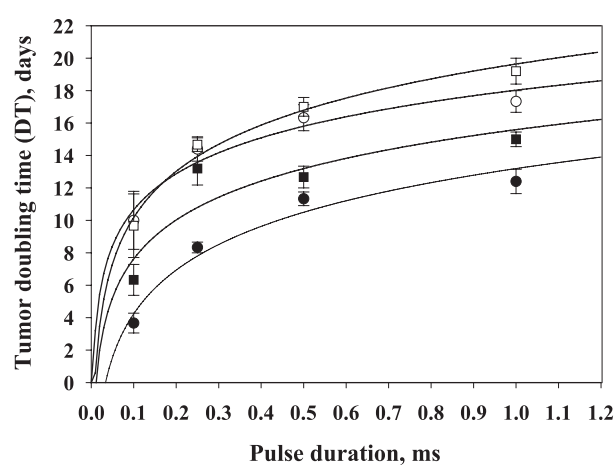

Fig. 3. The dependence of tumor doubling time on electric pulse duration of electrochemotherapy at various pulse strengths 900 V/cm (●), 1100 V/cm (■), 1300 V/cm (○), 1500 V/cm (□). Each point in the figure represents the mean±S.E.M.

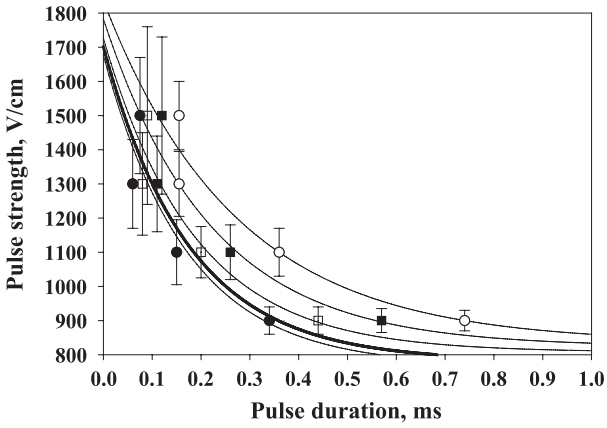

Fig. 4. The relationship of pulse strength and pulse duration giving the same effectiveness of electrochemotherapy expressed as the same tumor doubling time (DT): 9 days (●), 9.35 days (bold line), 10 days (□), 11 days (■), 12 days (○).

pairs of pulse strength and pulse duration giving the same antitumor effectiveness of ECT as with the pulse parameters widely accepted in the world (eight electric pulses with pulse strength of 1300 V/cm, duration of 0.1 ms and pulse repetition frequency of 1 Hz). For example, the same efficacy of ECT as that obtained at 1300 V/cm and 0.1 ms (DT=9.35) can be obtained using pulse strength–duration pairs such as: 900 V/cm and 0.36 ms, 1000 V/cm and 0.25 ms, 1100 V/cm and 0.18 ms, 1200 V/cm 0.14 ms. Similarly, other pairs of pulse strength and duration resulting in larger or lower, but the same effectiveness of ECT can be found.

4. Discussion

Effectiveness of ECT directly depends on the number of tumor cells that were exposed to over-threshold electric field and consequently destroyed by the bleomycin internalized into permeabilized cells. In this study, we investigated the effectiveness of ECT in dependence of various combinations of electric pulse strength and duration.

Using lowest conditions of electric pulses applied in this study (pulse strength of 900 V/cm and duration 0.1 ms), we found that ECT treatment was ineffective (Fig. 1, panel A; Table 1). In terms of electropermeabilization, however, it does not refer that under these pulse conditions, any tumor cell was permeabilized. It does only imply that the number of malignant cells in the tumor that were permeabilized and destroyed by ECT was not sufficient to detect suppression of the tumor growth. According to our histological data, this number is less than 60% (Fig. 2; compare with column 4 when pulses of 1100 V/cm, 0.1 ms were used).

In our experiments, tumors started to be significantly suppressed by ECT when at the same 900-V/cm pulse strength, the duration was increased up to 0.25 ms (Fig. 1, panel A). These findings show that, in the range of our parameters tested, the minimal electrical pulse strength and duration needed to evoke significant inhibition of tumor growth by ECT are 900 V/cm and 0.25 ms. Consequently, it could be concluded that under these electric pulses parameters, the number of cells that undergone permeabilization and internalized bleomycin was sufficient to obtain significant (threshold of ECT) antitumor effect. Using stronger or/and longer pulses inhibition of tumor growth was progressively more pronounced: consequently, larger number of cells in the tumor was permeabilized and destroyed by ECT (Fig. 1, Table 1). Similar result is also seen from histological data: increase in pulse strength from 1100 to 1500 V/cm, as well as increase in pulse duration from 0.1 to 1 ms defined larger extent of tumor necrosis (Fig. 2).

Several groups have tested effectiveness of ECT in dependence of pulse strength [6,8,10,19,26]. It was found that using eight 100-μs duration pulses, long lasting effects of ECT are obtained when pulses are stronger than 900 V/cm [6,10,19]. This is in agreement with our data, however, our results stress that at these pulse conditions (900 V/cm, 0.1 ms), the ECT on LLC tumor is ineffective. Increase of pulse strength from 1300 to 1500 V/cm resulted in some, however, insignificant increase of antitumor effects [6,8,26]. This is again in agreement with our results: at any pulse duration, increase of pulse strength from 1300 to 1500 V/cm did not result in significant increase of tumour DT (Table 1). However, at any pulse strength, increase of pulse duration from 0.1 to 1 ms did result in significantly ($p<0.05$) larger DTs (Table 1).

The relation between electric field pulse strength and duration defining the same effectiveness of ECT is seen in Fig. 4. Assuming that distribution of bleomycin within the tumors is similar, Fig. 4 also shows quantitative dependence of pulse strength and duration needed to permeabilize approximately the same fraction of tumor cells in the tissue. For example, in our study using eight-square wave electric pulses of pulse strength of 1300 V/cm and duration of 0.1 ms, an extent of necrosis after ECT was 81% (Fig. 2). Approximately the same fraction of cells permeabilized and destroyed by ECT could also be achieved using other combinations of pulse strengths and duration: 1200 V/cm and 0.14 ms, 1100 V/cm and 0.18 ms, 1000 V/cm and 0.25 ms, and 900 V/cm and 0.36 ms (Fig. 4). The choice of the combination depends on the equipment available as well as on the intentions tissue permeabilization is to be achieved for. For example, if larger molecules of the cytotoxic drug were used in ECT, the combination of pulse strength and duration would be preferable for longer pulses, as it is suggested from in vitro studies [13]. Similarly, for smaller molecules, stronger and shorter pulses would be more suitable [13]. It was shown that long pulses are preferable if DNA is to be delivered into tissues [3]. Provided that permeabilization is achieved, long pulses additionally facilitate DNA transfer into the cells through electrophoretic forces [27,28].

The largest antitumor effect of ECT in our study was obtained with eight pulses of 1500-V/cm strength and 1-ms duration (Fig. 1, panel D). In four mice out of one, 1-week

complete responses were obtained. These results suggest that the vast majority of cells in the tumor was permeabilized and destroyed by ECT. One could assume that most of the cells were destroyed by the effect of the electric pulses alone through irreversible permeabilization of the cells. However, tumor growth in the group that was treated with eight electric pulses of 1500-V/cm strength and 1-ms duration in the absence of bleomycin was similar as in control group (Fig. 1, panel D). In addition, our histological data show that at these pulse conditions, necrotic areas were similar as in control tumors (Fig. 2). Therefore, it could be concluded that even if some cells undergone irreversible permeabilization using 1500 V/cm pulses, its number was not sufficient to induce significant tumor inhibition.

The ultimate goal of electrochemotherapy is to destroy 100% of tumor cells or close to it. In ECT, this could be achieved only if permeabilization of this fraction of cells in the tumor is achieved (in fact, to achieve permeabilization of 100% of tumor may not be absolutely necessary since immune system contributes to elimination of some viable tumor cells unaffected by ECT [6,29]). In vitro using eight rectangular pulses of 0.1 ms 100% of cells or very close to it are permeabilized at 1300–1500 V/cm [17,19]. Similar pulse strength required to permeabilize and destroy 100% of cells is obtained in vitro studies where cells were exposed to electric pulses in the presence of the bleomycin [30]. Permeabilization of cells in tumor tissue starts at lower pulse strengths than the permeabilization of the same line of the cells in suspension [19]. This effect could be mainly associated with the different arrangement of cells in tissue and in suspension. Indeed, while cells in suspension are dispersed homogeneously leaving enough space for conductive medium between the cells, the cells in the tumor are of highly irregular shapes, sizes and are very closely compacted to each other, at least in case of Lewis Lung carcinoma. Because of these conditions, the electric field inside the tumor is highly inhomogeneous and the field strength inside various points of the tissue is different, irrespectively of the same voltage at the electrodes placed at the opposite sides of the tumor. From one side, this implies that some fraction of cells in tumor due to this inhomogeneity could be permeabilized at lower strength than the same fraction of the cells in suspension. However, on the other hand, to obtain permeabilization of 100% of the cells in the tumor, strengths of the field should be higher, as was in our case on LLC tumors. Indeed, in vitro it was established that using eight 1300-V/cm, 0.1-ms pulses in the presence of bleomycin, approximately 100% of the cells are killed [30], whereas our results show that ECT at these pulse conditions resulted in only 81% of tumor necrosis. Using 1500-V/cm, 1-ms pulses extent of necrosis was 88%, showing that most probably more cells were permeabilized and destroyed by the bleomycin internalized (Fig. 2).

The recurrences of the tumors after ECT with eight electric pulses at 1500 V/cm and 1-ms duration may be associated with a small fraction of surviving cells. Surviving cells in turn may reflect not only that these cells were unpermeabilized, but also that bleomycin was not at sufficient amounts around these cells. Because of the irregularities of tumor tissue, chaotic vasculature, microcirculation, as well as transvascular and interstitial transport, the distribution of the drugs in the tumors may be inhomogeneous [31]. This shows that homogenous distribution of the cytotoxic drug at sufficient doses in the tumors is also critical for effective ECT. Assuming that distribution of bleomycin within the tumor tissue is more homogenous if the tissue is exposed to the drug for longer times, we used i.p. injection of bleomycin instead of i.v. injection. Our preliminary studies (data not shown) showed that for effective ECT, such route of bleomycin injection requires higher doses to be used and longer delay between the injection and the pulse delivery.

It could be assumed that some cells were not killed even permeabilization of these cells was achieved. In that case, any method that facilitates distribution of the drug in the tumor would also enhance efficacy of the treatment. One may suppose that electric pulses along permeabilization of the cells may also facilitate distribution of the drug in the tumor. Similar phenomenon is discussed for DNA electrotransfer in the murine muscles [28]. If the electric pulses permeabilize the barriers for the distribution of the bioactive molecules in tissues, the more intense pulses may do it better.

Increased efficacy of ECT using 1500-V/cm and 1-ms pulses also could be associated with increased inhibition of blood perfusion in the tumor after the application of the pulses [32,33]. These blood perfusion changes are supposed to occur due to permeabilization of endothelial cells of blood vessels that may lead to the destruction of those vessels if the bleomycin is present [34,35]. One may suppose that the effect of ECT on blood vessels is stronger if stronger and longer electric pulses (e.g. 1500 V/cm, 1 ms) are used. Consequently, additional toxic impact through the infarction of the malignant cells is given that ultimately may enhance antitumor effect.

In conclusion, a set of pairs of values for electric pulse strength and duration giving the same tumor growth inhibition (quantitatively expressed as tumor doubling time) was determined. These findings show that the same (not necessary largest) efficacy of ECT could be achieved by using different combinations of pulse strength and duration. The largest antitumor efficacy of ECT in our study was obtained using 1500-V/cm strength pulses of 1-ms duration. These pulses in the absence of bleomycin neither were toxic to adjacent tissues nor inhibited tumor growth. The results of this study thus suggest that the effectiveness of electrochemotherapy can be enhanced (in comparison to widely accepted conditions of electrochemotherapy—eight pulses of 1300 V/cm, 0.1 ms) if 1500-V/cm, 1-ms electric pulses are used. Our study also implicates that other pulse conditions could be found for this enhanced ECT. Further investigations are needed. If the results are confirmed on other tumor models as well as by using cisplatin, i.e. another potential drug for ECT, these results may have direct implications for clinical ECT trials.

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