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Changing electrode orientation improves the efficacy of electrochemotherapy of solid tumors in mice

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Abstract

Electrochemotherapy is a new and promising approach in treatment of tumors employing locally applied high voltage d.c. electric pulses in combination with chemotherapeutic drugs. Increased permeability of the plasma membrane, induced by electric pulses, enables the chemotherapeutic drugs to enter into the cytosol, thus potentiating their anti-tumor effectiveness. In most of the studies published until now, trains of pulses were delivered via percutaneous parallel plate electrodes, so that the tumor was situated in between the electrodes. In our study the train of electric pulses was divided into two trains, the second one oriented perpendicularly to the first one. This changing of the electrode orientation resulted in improved anti-tumor efficacy of the electrochemotherapy: prolonged tumor growth delay and higher percentage of short and long term complete responses of the tumors. In this paper we also suggest a possible explanation for the observed effect, based on the knowledge of electric field distribution in the tissue and induced transmembrane potential.

Keywords: Electrochemotherapy; Bleomycin; Membrane permeability; Solid tumour

1. Introduction

One of the ways to increase the cytotoxicity of chemotherapeutic drugs is to potentiate drug delivery into the cells and tissues. This is the principle of electrochemotherapy, which employs increased permeability of the plasma membrane by high voltage d.c. electric pulses. This nonselective plasma membrane permeabilization facilitates entry of chemotherapeutic drugs into the cells, which can then act on their intracellular targets [1–4]. The anti-tumor effectiveness of electrochemotherapy was extensively developed on tumor models in mice and also demonstrated in clinical trials [5–10].

Most of the studies on electrochemotherapy with bleomycin (BLM) were done with "electric field intensities" from 1300–1500 V cm^{-1} with pulse width 100 μs and frequency 1 Hz [5,7,9,10]. These experiments were performed according to the results of the first experimental study by Mir et al., where they investigated the optimization of electrochemotherapy [1,7,11]. Their conclusions were that the anti-tumor effectiveness of electrochemotherapy with BLM is BLM dose and electric field intensity dependent. Also, they demonstrated that there is no major difference in the long term cures after three or eight electric pulses used in electrochemotherapy treatment. Nevertheless, most of the studies were done using a train of eight electric pulses in one direction. However, according to our observations many of the tumors after successful treatment, and substantial periods of partial or complete response, regrew in the margins where there was no contact with the electrodes. Therefore, we presumed that in those areas of the tumors some clonogenic tumor cells must have remained that were sub-optimally permeabilized and therefore survived and eventually regrew into the tumor.

The aim of our study was to determine how changing of the electrode orientation influences tumor growth delay and number of complete responses after electrochemotherapy with BLM. The train of eight electric pulses used for cell plasma membrane permeabilization was divided into

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Table 1
Anti-tumor effect of electrochemotherapy with BLM on EAT tumors

Experimental groups	n	Doubling time [a] /days	Growth delay [b] /days	Response to treatment [c]	Long term CR [d] n (%)
Control	34	4.4 ± 0.2	–	–	0
BLM 100 μg	29	6.2 ± 0.3	1.6 ± 0.3	PD	0
EP [e] 4 pulses	7	4.6 ± 0.2	0.7 ± 0.2	PD	0
EP 8 pulses	27	6.8 ± 0.4	2.4 ± 0.3	PD	0
EP 4 + 4 pulses	15	6.0 ± 0.4	2.0 ± 0.3	PD	0
ECT [f] with 4 pulses	10	25.0 ± 1.6	21.1 ± 1.6	NC	0
ECT with 8 pulses	34	28.0 ± 1.5	23.5 ± 1.5	NC/PR/CR	5 (15%)
ECT with 4 + 4 pulses	20	33.8 ± 2.1	29.9 ± 2.1	PR/CR	7 (35%)

[a] Tumor doubling time (AM ± SE). [b] Tumor growth delay (AM ± SE). [c] Reesponse to treatment was evaluated according to WHO guidelines; PD, progressive disease; NC, no change; PR, partial response; CR, complete response. [d] Long term CR (cures) were determined 100 days after the treatment. [e] EP, electric pulses. [f] ECT, electrochemotherapy.

two trains, the second one given perpendicularly with respect to the first one. Also, possible explanations based on the contemporary knowledge of the phenomenon and electric field distribution in the tissue are proposed.

2. Materials and methods

2.1. Animals

In the experiments inbred CBA mice of both sexes were used. Mice were purchased from the Institute of Pathology, University of Ljubljana, Slovenia and were maintained at constant room temperature (24 °C) with a 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 fungal or other infection, 10–12 weeks old, were included in experiments.

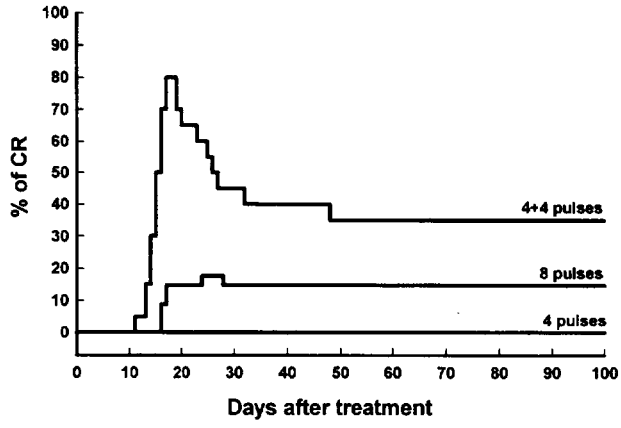

Fig. 1. Percentage of complete responses (CR) in EAT bearing mice after electrochemotherapy. Mice bearing approximately 40 mm^3 tumors were treated with BLM (100 μg) intravenously and/or with 4, 8 or 4+4 electric pulses (1040 V, 1 Hz, 100 μs, electrode distance 8 mm) 3–3.5 min after BLM treatment.

2.2. Tumors

As a tumor model, Ehrlich ascites tumor (EAT) syngeneic to CBA mice was used. Before each experiment the tumor was grown intraperitoneally as ascites, and cells were harvested, washed and resuspended in 0.9% NaCl for implantation. The viability of the cells was determined by Trypan-blue dye exclusion test. Solid subcutaneous tumors, transplanted dorsolaterally, were initiated by injection of 5×10^6 EAT cells. When the tumors reached approximately 40 mm^3 in volume, mice were randomly divided into experimental groups, consisting of 7–10 mice, and subjected to a specific experimental protocol on day 0.

2.3. Electrochemotherapy

Bleomycin (Mack, Germany) (BLM) was injected intravenously into the lateral tail vein of the mice (100 μg BLM dissolved in 0.5 ml 0.9% NaCl). In order to dilate the veins mice were pre-heated under IR light for a few minutes.

Electric pulses were delivered by two parallel stainless steel plate electrodes 8 mm apart (two stainless steel strips, 7 mm in width with rounded tips) and placed at the opposed margins of the tumor. Good contact between the electrodes and skin was ensured by means of conductive gel. Square-wave high voltage d.c. pulses (amplitude 1040 V, pulse width 100 μs, repetition frequency 1 Hz) were generated by electropulsator Jouan GHT 1287 (France). The amplitude and electrode distance is given as pulse amplitude/electrode distance (V cm^{-1}) (in most reports referred as "electric field intensity") to enable comparison of the results and easier presentation of the results. Tumors were treated either with 4, 8 or 4 + 4 pulses in train. In the treatment groups that were treated with 4 or 8 pulses the train of pulses was delivered in one direction through the tumor. In the 4 + 4 pulses treated group two trains of 4 pulses were delivered. The second train of 4 pulses was

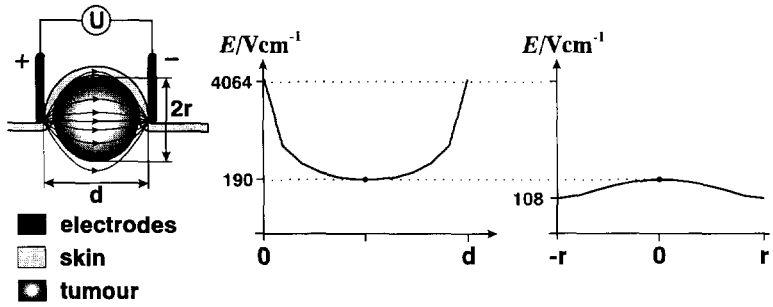

Fig. 2. Electric field (E) distribution between the electrodes (approximated by point sources). The distance between the electrodes ($d = 2r$) is 8 mm, voltage applied (U) 1040 V and conductivity 0.27 S m^{-1} (average value obtained from available literature). The electric field intensity in the middle plane between the electrodes drops towards the tumor margins to 57% of that in the center. A logarithmic plot is used since electric field falls off very rapidly with increasing distance from the electrodes.

delivered perpendicularly with respect to the first one with a time interval of 1 s between the two trains of pulses.

In the combined treatment group, tumors were treated with electric pulses 3–3.5 min after BLM injection. Mice in the control and electric pulses groups were injected with 0.9% NaCl instead of BLM.

2.4. Assessment of response

Tumor growth was followed by measuring three mutually orthogonal tumor diameters with vernier caliper on each consecutive day. Tumor volumes were calculated by the formula for the volume of an ellipsoid [9]. For each individual tumor doubling time (DT) and tumor growth delay (GD) from the DT of each individual tumor in experimental groups minus mean DT of control group were calculated. From the values, the mean (AM) and standard errors of the mean (SE) were calculated for each experimental group of mice, including all the necessary control groups. 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. Tumors that did not regrow after 100 days were termed long term CR. Animals with long term CR were not included in GD calculations.

The significance of the differences between the mean values of the DT and GD of the experimental groups was evaluated by modified t-test with Bonferroni adjustment after one-way analysis of variance had been completed.

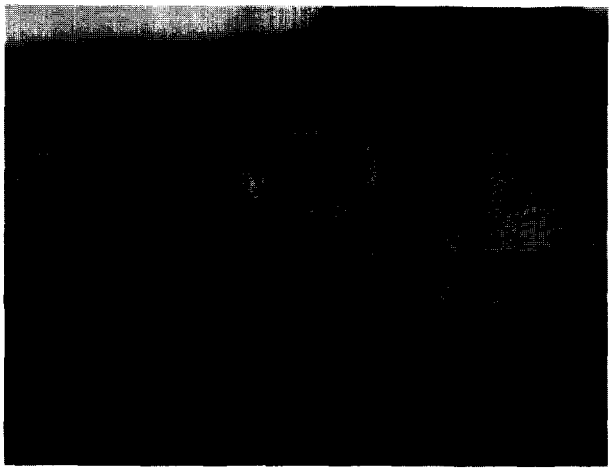

Fig. 3. Macroscopic appearance of EAT tumor 10 days after electrochemotherapy treatment (BLM, 100 μg; electric pulses, 1040 V, 1 Hz, 100 μs; electrode distance, 8 mm). Treatment was performed with the train of 8 pulses in one direction. According to the shape of the tumor it is evident that the tumor regrew from the area where there was no contact with the electrodes (arrows).

3. Results

Electrochemotherapy effectiveness with 4 or 4 + 4 electric pulses was compared to electrochemotherapy with 8 electric pulses used for electropermeabilization. Electrochemotherapy with 4 pulses was equally effective compared to electrochemotherapy with 8 electric pulses measured by GD (Table 1). However, the difference in antitumor effectiveness was shown in the percentage of CR; therapy with 4 pulses did not result in any short term or long term CR of the tumors (Fig. 1). In contrast, therapy with 8 pulses resulted in 18% short term CR and in 15% long term CR. Therefore, comparison of these two therapy protocols indicates that therapy with 8 electric pulses in the train is favorable to therapy with 4 electric pulses, because it results in sterilization of all clonogenic tumor cells in some tumors.

According to our observation that many tumors after a substantial period of PR regrew in the areas where there was no contact with the electrodes, we delivered electric pulses in two perpendicular directions in order to cover the whole tumor mass more effectively. Therefore, the train of 8 pulses was divided into two trains of 4 pulses (4 + 4), the second one given perpendicularly with respect to the first one. The results clearly show that more clonogenic

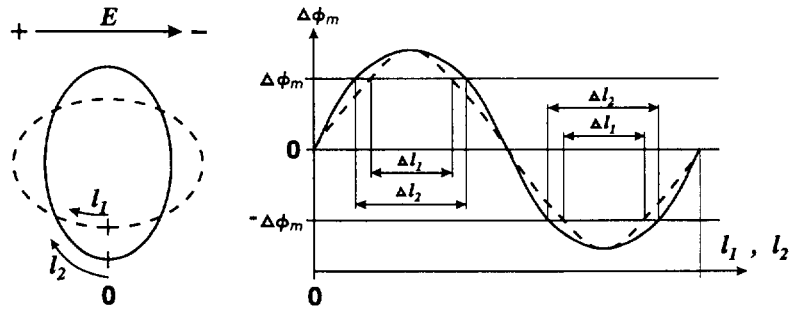

Fig. 4. The effective area (Δl_1, Δl_2) of the membrane where induced transmembrane potential ($\Delta\phi_m$) exceeds a threshold value ($\Delta\phi_m$) (arbitrarily drawn) is different in an ellipsoid cell oriented in parallel or perpendicularly to the external electric field (E). The size of the area (Δl) with increased permeability can be as much as 3–4-times bigger on a single pole of the cell (in the case of an ellipsoid with 2:3 main diameter ratio) when oriented perpendicularly to the electric field as compared to parallel orientation.

cells are sterilized by changing the electrode orientation (4 + 4 pulses) than after therapy with 8 electric pulses in one train and in one direction (Fig. 1, Table 1). This is demonstrated by prolonged GD of the 4 + 4 pulses treated group compared to 8 pulses treated group ($P < 0.001$). In addition, electrochemotherapy with 4 + 4 pulses resulted in a high percentage of short term CR (80%) which occurred 3–4 days earlier than in the 8 pulses treated group (Fig. 1). Furthermore, a higher percentage of long term CR (35%, $P < 0.001$) was observed.

4. Discussion

Our results clearly show that electrochemotherapy performed with 8 pulses in the train is more efficient in comparison to 4 electric pulses, which could be expected according to the quantitative and qualitative description of the electroporation given by Rols and Tessie [12]. However, changing of the electrode orientation for electropermeabilization, hence delivering 4 pulses to the tumor in one direction and another 4 perpendicular to the first ones, yielded markedly improved efficiency of electrochemotherapy.

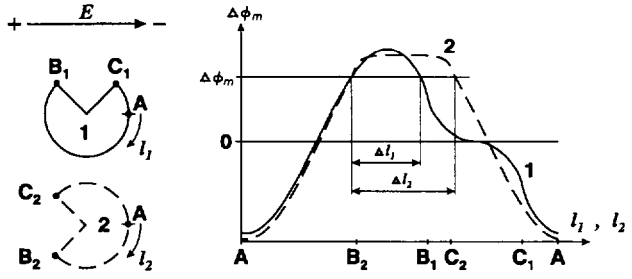

Fig. 5. Effective area (δl_1, Δl_2) of the membrane where induced transmembrane potential ($\Delta\phi_m$) exceeds a threshold value ($\Delta\phi_m$) (arbitrarily drawn) depends on electric field (E) orientation to the cell in the case where shape irregularities are present.

There are two possible explanations for the results obtained. The first explanation we find on the macroscopic level, considering the electric field distribution in the tissue (Fig. 2). After the breakdown of the stratum corneum, which occurs almost instantly after the first electric pulse is applied, the tissue between the electrodes can be considered homogeneous in the first approximation. Although the electrodes used are parallel plates, for theoretical consideration of the current density distribution and electric field intensity determination, it is more realistic to consider that the tumor is placed in the electric field generated by two point sources. By taking into account such geometry it can be clearly demonstrated that the electric field is strongly inhomogeneous, i.e. decreasing from the electrodes towards the center – the middle plane between the electrodes. The electric field further decreases when moving in the middle plane between electrodes outwards from the line connecting both electrodes. Thus, the cells in the center of the tumor and especially those on the tumor edge between the electrodes may experience an electric field of sub-threshold levels (Fig. 2). A short numerical calculation performed (using a software package based on a boundary element method [13]) showed a strong inhomogeneity of the electric field in the observed axis. On the electrodes (two point sources, diameter 1 mm) separated by 8 mm a voltage of 1040 V was applied (as in the experimental protocol). The "space" between the electrodes was assigned 0.27 S m^{-1} conductivity, an average value for tumor tissue obtained from the available literature [14–16]. Distribution of the electric potential was calculated in the areas of interest by solving a Laplace equation in two dimensions. Electric field intensity was then calculated, yielding the values of 4064 V cm^{-1} on the surface of the electrodes and only 190 V cm^{-1} in the center of the tumor. The electric field intensity is further decreased towards the periphery of the tumor in the midplane to 108 V cm^{-1} (i.e. 57% of the center midplane value). Obtained values for the electric field, although not accurate owing to

calculation only in two-dimensional space and not accounting for heterogeneous conductivity of the tissue, show that the induced maximal transmembrane potential varies from 3048 mV in the cells in the immediate vicinity of the electrodes, to 143 mV in the cells in the center of the tumor, to only 81 mV in the cells on the margins of the tumor, which are not in contact with the electrodes. The above values for induced transmembrane potential due to external electric field E are estimated based on the formula:

$$\max \Delta\phi_m = \max(1.5rE \cos \theta) \quad (1)$$

where $\Delta\phi_m$ is the induced transmembrane potential, r is the cell's half-diameter (5 μm), and θ is the angle between the electric field vector and the vector normal to the surface of the cell.

Since the electropermeabilization is a threshold phenomenon, meaning that a certain threshold critical value of the transmembrane potential has to be exceeded, some of the cells may be exposed to an electric field of insufficient intensity to induce permeabilization. We have observed on several occasions in our experimental work that tumors were eradicated except at both edges in the mid-plane between electrodes (Fig. 3). A strong inhomogeneity of electric field in the tissue was shown and had already been suggested by other authors [17] and depends primarily on the distance between the electrodes and their shape.

The second possible explanation may be found on the microscopic level, observing a single cell in an external electric field. Permeabilization of the cell plasma membrane first occurs on the poles of the cell facing the anode and cathode, where the maximal transmembrane potential is also induced [18]. An increase in membrane permeability occurs when the induced transmembrane potential exceeds a threshold value ($\Delta\phi_m$), which varies from one cell line to another [19]. For spherical cell Rols and Tessie [12] calculated an effective area where increase in plasma membrane permeability is possible. However, since cells are not ideally spherical, the effective area of the membrane where an increase in membrane permeability can occur depends on cell orientation in the electric field (Fig. 4). Effective area varies also owing to other irregularities in the cell shape (deviations from spherical and ellipsoid shape) (Fig. 5). In order to obtain a quantitative description of the induced transmembrane potential owing to an external electric field software based on the boundary element method (BEASY) was used. Elliptically and nonregularly shaped cells were placed in a homogenous electric field of different orientations with respect to the cells' main axis. The results obtained are presented in Figs. 4 and 5. In both figures an arbitrary threshold value for transmembrane potential $\Delta\phi_m$ is indicated in order to demonstrate the difference in effective area where an increase in membrane permeability can occur. The effective areas Δl_1 and Δl_2 are indicated in the figures, clearly demonstrating the differences in size depending on cell orientation in the external electric field. Microscopic observation of histological sections revealed that the cells in the tumor we used are approximately and on average spherical. However, it was possible to find cells where the two main diameters were in a ratio of 3 to 2 (which was also the ratio of diameters we used for the calculations presented in Fig. 4).

If we take into account the fact that each of the clonogenic cells in the tumor has to be destroyed in order to achieve a cure, then changing of the electrode orientation, as our results show, may improve the outcome of electrochemotherapy. The two possible explanations which we offer in the discussion are not mutually exclusive. The effectiveness of electrochemotherapy as a consequence of electric field inhomogeneity and cell shape deviations from the spherical can be improved either by increasing the voltage applied (which is not always possible or/and acceptable) or by changing the electrode orientation, as we clearly demonstrated in our experiments.

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