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Tumor Blood Flow Modifying Effect of Electrochemotherapy with Bleomycin

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Abstract. *Background: Electrochemotherapy combines administration of the chemotherapeutic drug, followed by application of electric pulses in order to increase drug delivery into the cells. The aim of this study was to determine the tumor blood flow modifying effect of electrochemotherapy with bleomycin and correlate it with its antitumor effectiveness and extent of tumor necrosis. Materials and methods: Electrochemotherapy of SA-1 subcutaneous tumors in A/J mice was performed by application of electric pulses to the tumors, following administration of bleomycin, and antitumor effectiveness determined by tumor growth delay and tumor cures as well as extent of tumor necrosis. Tumor blood modifying effect of therapy was evaluated by Patent blue staining technique and ^{86}RbCl extraction technique. Results: A good correlation of the two methods evaluating tumor blood flow, Patent blue staining and the established ^{86}RbCl extraction technique was found (r=0.944). Electrochemotherapy resulted in complete and permanent shut down of tumor blood flow within 12 hours, which lasted for at least 5 days. The results on tumor blood flow reduction correlated well with the good antitumor effectiveness of electrochemotherapy and with the extent of the necrosis in the tumors. Conclusions: The results indicate that Patent blue staining technique is a simple and reliable method for estimation of tumor blood flow and that antitumor effectiveness of electrochemotherapy with bleomycin could be partly attributed to its tumor blood modifying and anti-vascular effect.*

In electrochemotherapy, electric pulses are used as a means of increasing chemotherapeutic drug delivery into the cells [1]. Exposure of cells to electric pulses under specific conditions, increases plasma membrane permeability, temporally and reversibly, without affecting cell viability [2,3]. This technique, termed electroporation, has been used for insertion of various molecules into the cells, including chemotherapeutic drugs and genes [1,4,5]. Among several chemotherapeutic drugs tested for potentiation of their cytotoxicity by electroporation, bleomycin and cisplatin have been found to be very suitable, due to their limited transport through the plasma membrane [6,7]. The increased antitumor effectiveness of bleomycin and cisplatin, combined with electric pulses has been demonstrated in experimental and clinical studies [6-16].

In addition to increased drug delivery into the cells, electric pulses were found to exert tumor blood modifying effect. In our previous studies we demonstrated that application of electric pulses to the tumors, reduces tumor blood flow, which was dependent on the amplitude of the applied electric pulses [17,18]. Electric pulses, as used in our standard in vivo experimental conditions in electrochemotherapy, reduced tumor blood flow up to 30% of the pre-treatment value. This reduction of tumor blood flow could contribute to the antitumor effectiveness of electrochemotherapy, by prolonging exposure of tumor cells to the chemotherapeutic drug [19,20].

The measurements of tumor blood flow changes after application of electric pulses were made by reliable, but laborious techniques such as ^{86}RbCl extraction technique and contrast enhanced magnetic resonance imaging [17,18]. In search for an easier approach, which would be simple, quick and indicate of tumor blood flow changes, tumor staining with Patent blue was introduced. In addition, we examined the tumor blood flow modifying effect of electrochemotherapy with bleomycin, which has not been yet evaluated.

Therefore, the aim of our study was to determine the tumor blood flow modifying effect of electric pulses by

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Patent blue staining, and the results obtained with this technique were correlated with the measurements obtained by the ^{86}RbCl extraction technique. In addition, tumor blood flow modification was evaluated on tumors treated by electrochemotherapy with bleomycin and was correlated with antitumor effectiveness and extent of tumor necrosis, after electrochemotherapy.

Materials and Methods

Animals and tumors. A/J mice of both sexes, purchased from the Institute Rudjer Boskovic, Zagreb, Croatia, were used. They were maintained at 22°C with a natural day/night light cycle in a conventional animal colony. The mice were 8-12 weeks old at the beginning of the experiments. The tumor used was fibrosarcoma SA-1 (The Jackson Laboratory, Bar Harbour, ME). SA-1 cells were obtained from the ascitic form of the tumors in mice, which were serially transplanted twice per week. Subcutaneous tumors were implanted, by injecting 0.1 ml NaCl (0.9%) containing 5×10^5 viable tumor cells under the skin on the rear dorsum. Six to 8 days after implantation, tumors reached approximately 40 mm^3 in volume (6 mm in diameter) and mice were randomly divided into experimental groups, consisting of at least 6 mice. Experiments were repeated twice.

Application of electric pulses. Electric pulses were delivered as previously described [7]. Briefly, the applicator consisted of two flat parallel electrodes 8 mm apart (two stainless-steel strips, width 7 mm, with rounded corners). Electrodes were placed percutaneously at the opposing margins of the tumor. Good contact between the electrodes and the overlying skin was assured by means of conductive gel (Parker Laboratories, inc., N.Y., USA). Square wave high voltage (direct current) pulses of 1040 V (1300 V/cm - voltage to distance ratio), with pulse width 100 ms at repetition frequency 1 Hz were generated by electropulsator Jouan GHT 1287 (Jouan, France). The number of electric pulses applied was ranging from 1 to 10 during each treatment. When more than one electric pulse was applied, the number of electric pulses was divided into two sets, the second set oriented perpendicularly to the first, with a time interval of 1 second in order to cover the whole tumor with sufficiently high electric field [21-23]. Treatment with electric pulses was performed without anaesthesia and was well tolerated by the mice. Treatment protocols were approved by the Medical Ethical Committee of the Ministry of Health of the Republic of Slovenia No. 326-07-269/97.

Electrochemotherapy. Electrochemotherapy consisted of bleomycin (BLM) injection followed by the application of 8 electric pulses. BLM (Bleomycin, Mack, Germany) was dissolved in phosphate buffered saline and the dose of 100 μg/mouse in 0.2 ml volume was injected into lateral tail vein of the preheated mice. BLM solution was prepared freshly for daily injections. The interval between the BLM injection and application of electric pulses was 3 minutes. Pertinent control groups were including untreated, electric pulses only, and bleomycin only treated tumors. Antitumor effectiveness of electrochemotherapy was assessed by measurements of tumor diameters in three orthogonal directions using Vernier calliper on consecutive days following treatment. Arithmetic mean and standard error of the mean was calculated for each experimental group. Tumor doubling time was calculated from the growth curve of individual tumors. Tumor growth delay was calculated from the mean tumor doubling time of the experimental groups compared to untreated tumors [7].

Measurement of relative tumor blood flow. Relative tumor perfusion was measured by ^{86}RbCl extraction technique at various time points following application of electric pulses [17,24,25]. Briefly, ^{86}RbCl tissue radioactivity measured 1 minute after an intravenous injection, via the tail, was used to calculate relative blood flow as a proportion of cardiac output. A minimum of 6 mice per group were injected via the tail vein with 185 kBq (37 MBq/ml) ^{86}RbCl (Amersham PLC, Little Chalfont, Bucks., UK) and sacrificed by cervical dislocation following 1 minute to allow for circulation of the tracer. Immediately thereafter the tumors were dissected, weighed and counted for ^{86}Rb radioactivity by gamma counter (Institute Jozef Stefan, Ljubljana, Slovenia). The tails of injected mice were also removed and counted to measure residual activity at the site of injection. Results were rejected if the tail counts were greater than 10% of the activity of the injected solution. Relative tissue perfusion was calculated as follows; radioactivity in the tumors was expressed as a percentage of the total activity injected (minus that remaining in the tail) per gram. This gives a measure of perfusion as a function of cardiac output and is expressed as % injected activity per gram or relative uptake (% control).

Assessment of tumor staining by Patent blue. Patent blue (Byk Gulden, Switzerland) was used to estimate tumor perfusion. Patent blue (1.25%) diluted in 0.2 ml physiological saline was injected into tail vein of animals after they were subjected to specific treatment. After the dye was evenly distributed through the tissue for approximately 1 minute, animals were sacrificed and tumors were carefully dissected. Tumors were cut along their largest diameter and the percentage of stained versus non-stained cross-section was immediately estimated visually by two persons. The mean of both estimations was used as an indicator of tumor perfusion. The results of the experiments were pooled together and were presented as mean and standard error of the mean for each experimental group [26].

Histology of tumors. Tumour histology was performed on the same tumors as were used for the Patent blue staining. The specimen were fixed in 10% buffered neutral formalin. One tissue block cut through the largest diameter of the tumor was embedded in paraffin and stained with haematoxylin-eosin by standard method. Slides of 6 tumors per group were examined in a blind fashion. Tumor necrosis was determined in a whole mount tumor section through the largest tumor diameter [19].

Statistical analysis. Statistical significance was evaluated by modified t-test (Bonferroni t-test) after one-way ANOVA was performed and fulfilled. Pearson correlation coefficient was calculated to determine the correlation between Patent blue staining method and ^{86}RbCl extraction technique. Sigmastat statistical software (SPSS inc.) was used for statistical calculations.

Results

Correlation between ^{86}RbCl extraction and Patent blue staining technique. To determine the correlation between ^{86}RbCl extraction technique and Patent blue staining of the tumors, two sets of experiments were conducted evaluating effect of electric pulses application on tumor blood flow. Each method measures different parameter, ^{86}RbCl extraction technique measures flow of plasma through the tumor, whereas the Patent blue staining measures the collection of the dye in vascular and interstitial space.

In the first set of experiments the changes in tumor

blood flow were determined by both methods. A series of 8 electric pulses (1040V) that were applied to the tumors decreased tumor blood flow to approximately 30% of the pre-treatment value (Figure 1). This reduction, as measured by the two techniques, was maximal at about 30-60 minutes, thereafter slowly increasing to almost reaching pre-treatment level by about 24 hours. The data obtained by the two techniques correlated ($r=0.944$; $p<0.001$).

In the second set of experiments dependence of tumor blood flow changes with respect to the number of electric pulses applied was established. To denote the time of the minimum blood flow, tumor blood flow changes were determined 30 minutes after application of electric pulses (1040V), by measurement of relative tumor blood flow by ^{86}RbCl extraction technique and tumor staining by Patent blue. The reduction of tumor blood flow was dependent on the number of applied electric pulses, reaching the minimum value of approximately 30% of the pre-treatment value at 8 and 10 electric pulses applied (Figure 2). Again, a very good result correlation was obtained by the two techniques ($r=0.962$; $p<0.001$).

These results demonstrate that Patent blue staining of the SA-1 tumors is reliable technique for estimation of tumor blood flow, is simple and quick, and provides similar information as the ^{86}RbCl extraction technique.

Antitumor effectiveness, tumor blood flow changes and extent of necrosis in electrochemotherapy treated tumors. Since the Patent blue staining provided good estimation of blood flow changes, this method was used for evaluation of blood flow changes in tumors treated with electrochemotherapy and pertinent control groups.

Electrochemotherapy with bleomycin was found to have very good antitumor effect. When using the bleomycin dose of 100 μg/mouse 70% of electrochemotherapy treated animals were tumour free 100 days after the treatment. In contrast, treatment with electric pulses and bleomycin as single treatment induced only moderate tumor growth delay inspite of considerable blood flow reduction after application of electric pulses (Table I, Figure 1,2).

Evaluation of tumor blood flow in the tumors treated by electrochemotherapy demonstrated that tumor perfusion was steadily decreasing up to 12 hours after the treatment, reaching almost complete shut down of tumor perfusion. These tumors did not restore blood flow up to 5 days after the treatment (Figure 3). Application of electric pulses only induced the same changes in tumor perfusion as demonstrated in previous experiments (Figure 1,3). However, bleomycin treatment alone did not induce changes in tumor blood flow; tumor staining after bleomycin treatment was not significantly different compared to control. The tumors in the control group were stained up to 80% after 5 days.

Data on extent on tumor necrosis after electrochemotherapy with bleomycin correlated well with data on tumor blood flow changes, however with few hours delay (Figure 4). Extensive necrosis of tumors treated with electrochemotherapy was observed already 24 h after treatment and tumors remained fully necrotic thereafter. Also, application of electric pulses induced destruction of the tumors, extent of necrosis was transient up to 55%, and correlated with tumor blood flow changes and moderate antitumor effectiveness (Figure 4, Table I). The treatment with bleomycin did not result in tumor necrosis being in acomparable range as that in the untreated tumors.

Table I. *Tumor growth after electrochemotherapy with bleomycin.*

Group	n	DT/days (average ± SE)	P compared to DT of control	GD/days (average ± SE)	Cures
Control	20	1.8±0.05			0 %
Bleomycin 100μg/mouse	20	1.9±0.1	> 0.05	0.1 ± 0.1	0 %
Electric pulses	17	3.1 ± 0.2	< 0.05	1.3±0.2	0 %
Electrochemotherapy	17	34.5 ± 2.9	< 0.05	32.7±2.9	70 %

Discussion

This study shows that measurement of tumor blood flow changes can be adequately measured by Patent blue staining technique as with ^{86}RbCl extraction technique. We also found that electrochemotherapy with bleomycin completely and permanently shuts down tumor blood flow, and that this observation correlates well with antitumor effectiveness, as well as with the extent of tumor necrosis.

Results of this study show that Patent blue staining of the tumors correlate with measurements of relative blood perfusion measured by ^{86}RbCl extraction technique. The finding was confirmed when transient changes in tumor blood perfusion were evaluated after application of electric pulses, as well in measurements when tumors were exposed to different number of electric pulses. Although the two techniques evaluate different parameters results were very similar. Namely, Patent blue staining shows distribution of the dye in the vascular and interstitial space, whereas ^{86}RbCl extraction technique measures flow of plasma in the tumors. Since the ^{86}RbCl extraction technique is a time consuming and laborious technique [24,25], staining of tumours with Patent blue could be used as an estimate of the tumor blood flow changes, predominantly in the situations when a quick and simple technique is desired. Patent blue is a non-toxic dye that is used in lymphography, therefore there is no limitation for its use in patients.

In electrochemotherapy, electric pulses are used as a

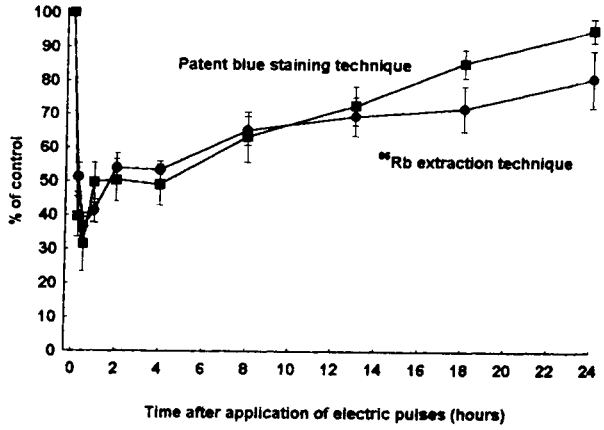

Figure 1. *Time course of changes in tumor blood flow after application of 8 electric pulses (1040 V, pulse width 100 μs, repetition frequency 1 Hz). Tumor blood flow changes were measured by ^{86}RbCl extraction of SA-1 tumors and by tumor staining by Patent blue. Mean values ± standard error of the mean of at least 6 mice per point.*

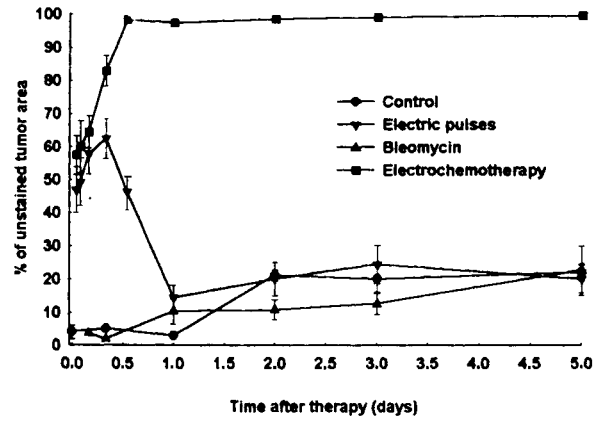

Figure 3. *Time course of changes in tumor blood flow after electrochemotherapy with bleomycin, measured by Patent blue staining. Eight electric pulses were applied to the tumor (1040 V, pulse width 100 μs, repetition frequency 1 Hz) 3 minutes after intravenous injection of 100 μg of bleomycin. Mean values ± standard error of the mean of at least 6 mice per point.*

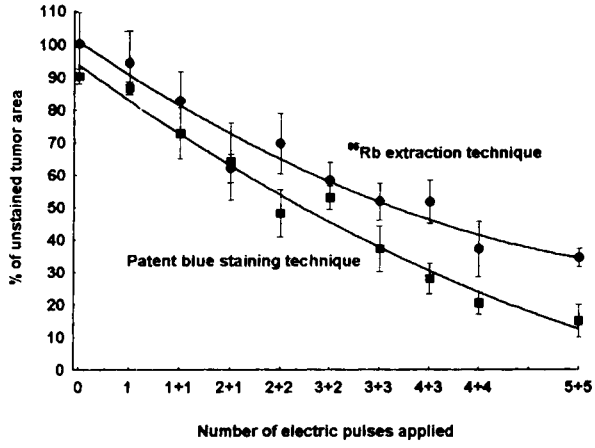

Figure 2. *Tumor blood flow changes as a function of number of electric pulses applied (1050 V, pulse width 100 μs, repetition frequency 1 Hz). Tumor blood flow changes were measured by ^{86}RbCl extraction of SA-1 tumors and by tumor staining by Patent blue. Mean values ± standard error of the mean of at least 6 mice per point.*

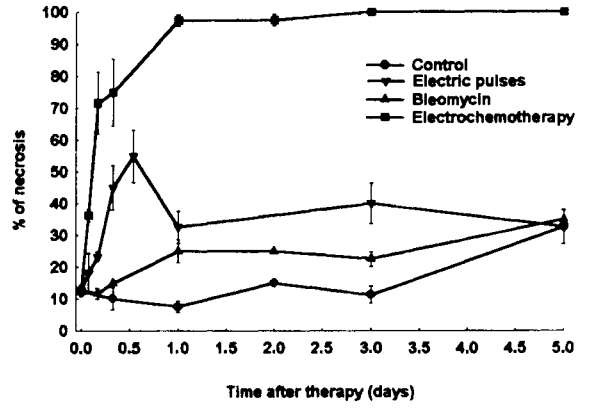

Figure 4. *Time course of changes in tumor necrosis after electrochemotherapy with bleomycin. Eight electric pulses were applied to the tumour (1050 V, pulse width 100 μs, repetition frequency 1 Hz) 3 minutes after intravenous injection of 100 μg of bleomycin. Mean values ± standard error of the mean of at least 6 mice per point.*

means to deliver sufficient amount of chemotherapeutic drug into the cells for effective cytotoxic action [1]. This method is aimed at chemotherapeutic drugs that have hampered transport through the plasma membrane, though once inside the cell they are highly cytotoxic. This mechanism has been demonstrated to be effective for the chemotherapeutic drugs, bleomycin as well as cisplatin, in different tumor models [6,7,9]. However, our recent studies have demonstrated, that application of electric pulses to the tumors can modify tumor blood flow [17,18]. As demonstrated in this study, application of electric pulses that are used in electrochemotherapy, results in substantial reduction of tumor blood flow, which is restored within 24 hours. This effect may contribute to the

antitumor effectiveness of electrochemotherapy, by entrapment of the drug within the tumor, and thus by prolonging its antitumor action. As demonstrated in this study, blood flow in the tumors treated by electrochemotherapy with 100 μg bleomycin is abrogated within 12 hours after the treatment and is not restored thereafter. The results of antitumor effectiveness of electrochemotherapy with bleomycin and extent of tumor necrosis correlate well with the tumor blood flow modifying effect.

The quick and dramatic effect of electrochemotherapy can be attributed to the high cytotoxicity of bleomycin after electroporation of tumor cells. However, to some extent the antitumor effectiveness may be ascribed also to cytotoxic effect of electrochemotherapy to endothelial cells in tumor blood vessels. Bleomycin, after electroporation of endothelial cells, may severely damage the vasculature of the tumors and consequently induce a cascade of tumor cell death by abrogating oxygen supply of the cells. This phenomenon, described as vascular targeted therapy, recently gained high interest and has been exploited in several studies [27]. Abnormal physiology of the tumor blood vessels has been used as a target, to obtain antitumor effect [28]. Several drugs have been used till now, such as combrestatin A4 and others [27]. However, further studies are needed to determine sensitivity of the endothelial cells to electrochemotherapy and to establish the differential sensitivity between the tumor and endothelial cells. As our preliminary study shows, endothelial cells are at least as sensitive as tumor cells to electrochemotherapy with bleomycin (unpublished data).

Therefore, when analysing antitumor effectiveness of electrochemotherapy, several mechanisms can be identified; electroporation that facilitates drug accumulation in the cells [19]; reduced tumor blood flow after application of electric pulses only that prolongs exposure of the cells to the drug [17], immune system that increases antitumor effectiveness of electrochemotherapy [29,30] and as demonstrated in this study, effectiveness of electrochemotherapy with bleomycin could be partly attributed to its tumor blood modifying and anti-vascular effect.

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