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Original Paper

Tumour Blood Flow Changes Induced by Application of Electric Pulses

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The effect of electric pulses on tumour blood flow was investigated in the murine fibrosarcoma SA-1. After the application of short intense electric pulses, relative tumour perfusion was measured using an ^{86}RbCl extraction technique. A significant reduction of tumour perfusion (~30% of control) was observed within 1 h following the application of eight electric pulses to the tumour. Thereafter, tumour blood flow slowly recovered, almost reaching the pretreatment level by 24 h. No change in perfusion was induced in the untreated contralateral normal leg muscle. A similar pattern of blood flow reduction was induced when a second set of electric pulses was applied to the tumour following a 24 h interval. The degree of tumour blood flow reduction was dependent upon the number of electric pulses applied, at 1040 V, and less effect was observed if less than eight pulses were applied. A modification of the amplitude of the electric pulses resulted in changes in the direction of tumour blood flow response. Tumour blood flow increased following pulses in the range between 80 and 560 V and decreased at amplitudes higher than 640 V. These results demonstrate that the local application of electric pulses to solid tumours can modify tumour blood flow. Pulses of increased amplitude resulted in the progressive reduction of tumour blood flow with a corresponding increase in tumour cytotoxicity as measured by growth delay. Tumour blood flow reduction by electric pulses could have potential in exploiting modalities mediated by tumour hypoxia, e.g. activation of bioreductive agents. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: SA-1 mouse fibrosarcoma, electric pulses, blood flow, ^{86}RbCl
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INTRODUCTION

Reductions in tumour blood flow can lead to an increase in hypoxia and extracellular acidification which can be exploited, even if transiently, by combination therapy with bioreductive agents [1–3]. Additionally, if blood flow is chronically impaired, a cascade of tumour cell death will occur, due to a lack of nutrients and an accumulation of catabolite products [4, 5].

Many anticancer agents and therapies in current use have been shown to exert their antitumour action, to some extent, as a direct consequence of compromising vascular function; these agents include hyperthermia [6], photodynamic therapy [7], high energy shock waves [8], cytokines, such as tumour necrosis factor-α (TNF-α) [9, 10] and interleukin-1α (IL-1α) [11], and drugs such as hydralazine, serotonin, flavone acetic acid, reviewed in [12], and vinca alkaloids [13]. However, the potential of modifying blood flow in the clinic with many of these agents is limited by several factors including unacceptable systemic toxicity. Nevertheless, studies in experimental systems with such agents have demonstrated that blood flow effects can be exploited to improve therapeutic outcome. There is thus, a need to identify new treatments which can potentially modify tumour blood flow in a clinical setting.

Electric pulses have a broad spectrum of biomedical applications. These applications occur both *in vitro* and *in vivo* and can be used for gene and drug delivery, insertion of

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receptors into plasma membrane, as well as for electrofusion of cells and transdermal drug delivery [14, 15]. Enhanced delivery of chemotherapeutic drugs into tumour cells by the application of short intense direct current electric pulses is termed electrochemotherapy [16]. Electrochemotherapy has been shown to be successful for drugs such as bleomycin and cisplatin that have hampered transport through the plasma membrane [16, 17]. Increased antitumour effectiveness of bleomycin and cisplatin combined with electric pulses has been demonstrated in experimental and clinical studies [16–25].

Tumour cytotoxicity following treatment of solid tumours with electrochemotherapy was proposed to be due to changes in membrane permeability and enhanced drug uptake. *In vitro* studies clearly show that increased drug uptake is predominantly mediated by plasma membrane electroporation [26]. However, following *in vivo* application of electric pulses it is possible that other additional mechanisms may be involved in the observed antitumour effectiveness of electrochemotherapy. One possible mechanism may be changes in tumour blood flow induced by the application of electric pulses. For example, if blood flow is reduced once peak levels of the drug have been achieved within the tumour mass, a more prolonged drug exposure would be obtained.

The current study was undertaken to evaluate the effects of electric pulses on tumour blood flow. The aim of this study was to characterise the time course of tumour blood flow changes in experimental SA-1 tumours in mice and its dependence on the amplitude and number of electric pulses applied and following repetitive applications of electric pulses.

MATERIALS AND METHODS

Mice and tumours

The animals used in the present experiments were male and female A/J mice, purchased from Rudjer Boškovič Institute (Zagreb, Croatia). The mice were maintained at a controlled room temperature (24°C) with a natural day/night light cycle in a conventional animal colony and fed food and water *ad libitum*. The tumour used was fibrosarcoma SA-1 (The Jackson Laboratory, Bar Harbour, Maine, U.S.A.). SA-1 cells were obtained from the ascitic form of the tumours in mice and serially transplanted. Subcutaneous tumours were implanted, under anaesthesia, by injecting 0.1 ml NaCl (0.9%) containing 5×10^5 viable tumour cells under the skin on the rear dorsum. Six to 8 days after implantation, tumours reaching approximately 40 mm^3 in volume (7 mm in diameter) were randomly divided into experimental groups, consisting of at least six mice.

Application of electric pulses

Electric pulses were delivered as previously described [17]. 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 opposite margins of the tumour. Good contact between the electrodes and the overlying skin was assured by means of conductive gel (Parker Laboratories, New York, U.S.A.). Square wave high voltage (direct current) pulses of different amplitudes ranging from 80 (100 V/cm) to 1200 V (1500 V/cm), with pulse width 100 μ sec and repetition frequency 1 Hz were generated by an electropulsator (Jouan GHT 1287; Jouan, France). The number of electric pulses was controlled, 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 sec [27]. When an odd number of electric pulses was applied, the pulses were unevenly distributed into the two directions. For example, when three electric pulses were applied, two were in one direction and the third in the perpendicular direction to the first set of pulses. Treatment with electric pulses was performed without anaesthesia and was well tolerated by the mice.

Measurement of relative blood flow

Relative tissue perfusion was measured using an ^{86}RbCl extraction technique at various time intervals following the application of electric pulses in tumours and normal untreated muscle tissue of the same mouse [28, 29]. Briefly, ^{86}RbCl tissue radioactivity measured 1 min after an intravenous injection, via the tail, was used to calculate relative blood flow as a proportion of cardiac output. A minimum of six mice per group were injected via the tail vein with 185 kBq (37 MBq/ml) ^{86}RbCl (Amersham, Little Chalfont, Buckinghamshire, U.K.) and sacrificed by cervical dislocation 1 min later to allow for circulation of the tracer. Immediately thereafter the tissues were dissected, weighed and counted for ^{86}Rb radioactivity using a gamma counter (Institute Jozef Stefan, Ljubljana, Slovenia). The tails of injected mice were also excised and counted to measure residual activity at the site of injection. The 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 tissue 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/gram or relative uptake (% control).

Assessment of antitumour effect and statistical analysis

Subcutaneous tumour growth was followed by measuring tumour diameter in three orthogonal directions using Vernier caliper (± 0.1 mm) on consecutive days following treatment. Tumour volumes were calculated as described previously [17]. Arithmetic means and standard errors of the mean (SEM) were calculated for each experimental group. Tumour volume doubling time (T_d) was determined from the growth curve for individual tumours. Tumour growth delay was calculated from the mean T_d of experimental groups compared with untreated tumours [17]. Statistical significance was evaluated by a modified *t*-test (Bonferroni *t*-test) after one-way ANOVA was performed and fulfilled. Levels less than 0.05 were taken as indicating significant differences.

RESULTS

Tumour blood flow was evaluated in subcutaneous SA-1 tumours treated with eight electric pulses (1040 V) and in untreated gastrocnemius leg muscle (Figure 1a). A series of eight electric pulses (1040 V) decreased tumour blood flow from 3.7 ± 0.3 to 1.4 ± 0.2% injected ^{86}RbCl (~30% of control) and this reduction was maximal at approximately 1 h, thereafter slowly increasing to almost reach the pretreatment level by 24 h. No changes in blood flow of normal muscle tissue were observed following contralateral tumour treatment, indicating that the local application of electric pulses does not induce systemic changes in blood flow (Figure 1a).

In order to determine whether the changes in tumour blood flow are altered following repeated treatment, tumours were treated with a second set of eight electric pulses 24 h after the first, when the tumour blood flow had almost recovered to the pretreatment level (80–85% of control). A second set of electric pulses induced a similar reduction in tumour blood flow as the first treatment; tumour blood flow was reduced to 30% within 1 h but with some indication of a slower recovery by 24 h to 65% of the pretreatment level (Figure 1b).

To determine the dependence of tumour blood flow changes with respect to the number of electric pulses applied, tumours were treated with between one and 10 electric pulses (1040 V). The relative tumour perfusion was determined 30 min later to denote the time of minimum blood flow. Figure 2 shows that the reduction of tumour blood flow was approximately linearly related to the number of applied electric pulses, for up to eight electric pulses, reaching a minimum value of ~30% of the pretreatment value. A minimum of three electric pulses were necessary to result in significantly reduced tumour blood flow.

Tumour blood flow was also measured after the application of eight electric pulses of differing amplitudes and the

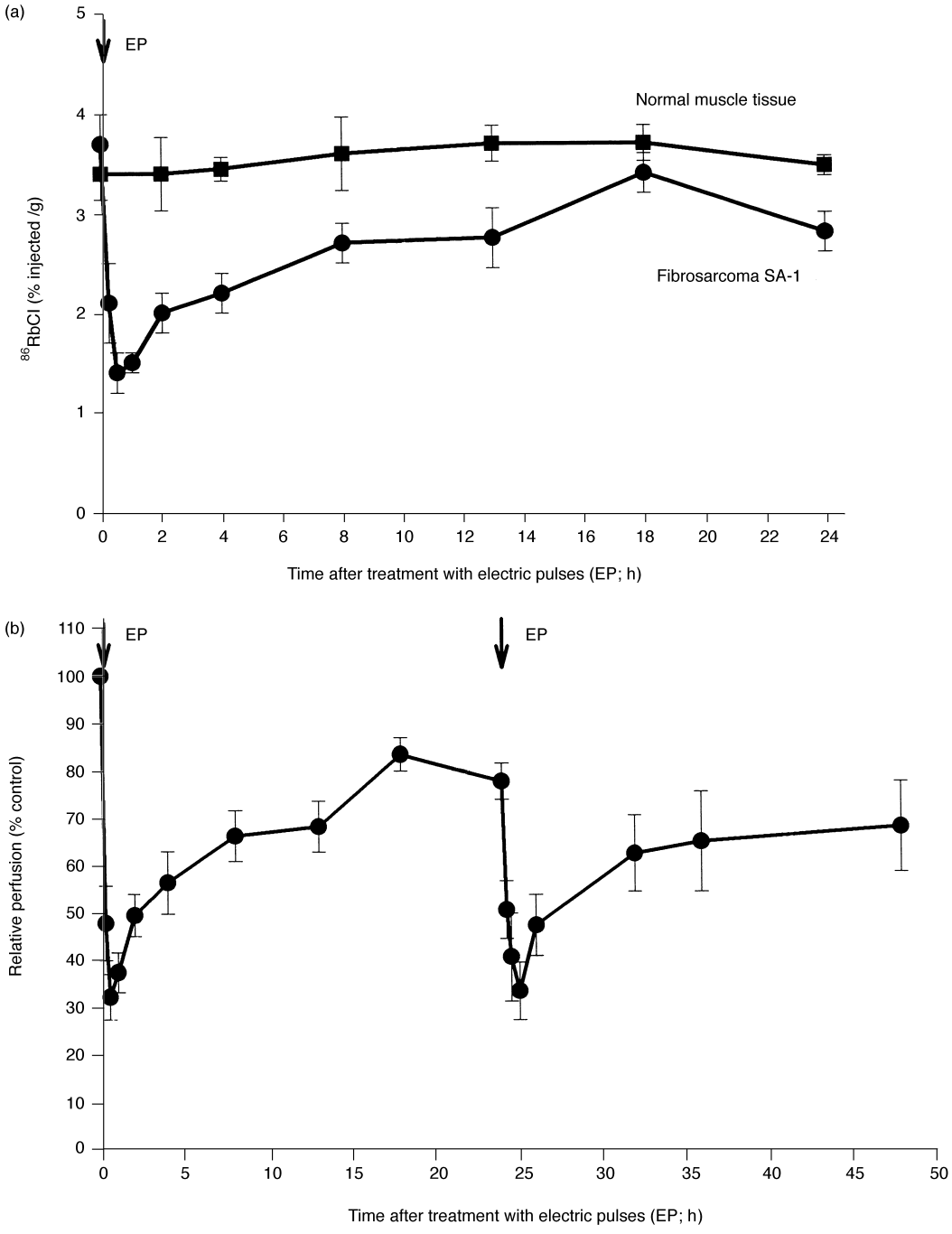

Figure 1. Time course of changes in blood flow measured by ^{86}RbCl extraction of (a) SA-1 tumours and normal muscle tissue treated with eight electric pulses (EP, indicated by arrow 1040 V, pulse width 100 μ sec, repetition frequency 1 Hz and (b) SA-1 tumours following the application of two sets of eight electric pulses (1040 V, pulse width 100 μ sec, repetition frequency 1 Hz). The second set of electric pulses was applied 24 h after the first set. Mean values ± 1 standard error of the mean (SEM) of at least six mice per point.

relative tumour perfusion determined 30 min thereafter (Figure 3a). Tumour blood flow was found to be dependent on the amplitude of the electric pulses, with increased flow, although not statistically significant, in the range between 80 and 560 V, and decreased flow following amplitudes between 640 and 1040 V, reaching a minimum flow of ~30% of control after 1040 V. No further reduction of tumour blood flow was obtained with amplitudes greater than 1040 V.

Tumour growth delay was measured following treatment with eight electric pulses of amplitudes between 80 and 1200 V (Figure 3b). There was an indication from the results that tumour growth may be slightly increased in tumours treated with electric pulses of amplitudes between 80 and 720 V, but delayed in tumours treated with amplitudes between 880 and 1200 V. Tumour growth rate was statistically increased at an electric pulse amplitude of 80 V and decreased at an amplitude of 1200 V.

DISCUSSION

The results of our study demonstrate that the application of electric pulses induces changes of SA-1 tumour blood flow: tumour blood flow is either increased or decreased depending on the pulse amplitude. Following electric pulses of high amplitudes, the reduction of tumour blood flow is rapid with a slow recovery over 24 h following treatment. The extent of the reduction of tumour blood flow is related to both the number and the amplitude (exceeding 640 V) of the electric pulses. In contrast, electric pulses of lower amplitude than 640 V increase tumour blood flow. The lack of response of normal muscle tissue during contralateral electric pulse treatment of tumours, suggests the absence of any systemic change in normal tissue blood flow.

Several tumour blood flow modifying agents have already been identified. Most of them, like hyperthermia [6], photodynamic therapy [7], high energy shock waves [8], TNF-α [9], hydralazine, flavone acetic acid and vinca alkaloids [12, 13], increase tumour hypoxia for several hours by inducing rapid and extreme blood flow shutdown. As a consequence, some of them have been used in combination therapy schemes, either with bioreductive drugs or with other treatment approaches [2, 3]. Many of these treatment combinations have proved to be very effective in experimental studies, although some of the therapies have untoward systemic effects, for example flavone acetic acid (releasing TNF-α) and hydralazine (vascular hypotension), which potentially limit their clinical usefulness. Nevertheless, the search continues for a tumour blood flow modifying agent that would act locally, without systemic toxicity. As demonstrated in this study, one possible treatment could be the application of electric pulses.

The time course of tumour blood flow reduction, induced by the application of high voltage electric pulses, indicates that the effect is rapid and relatively long lived. The reduction of blood flow to ~30% of control occurs quickly with minimum flow lasting for ~1 h with subsequent slow recovery over the following 24 h. The degree of reduction and recovery in blood flow is similar to that observed with agents such as hydralazine and flavone acetic acid [30, 31]. The application of electric pulses may be used to therapeutic advantage by combination with chemotherapeutic agents, bioreductive drugs or hyperthermia. Furthermore, the observation of similar patterns of tumour blood flow changes induced by a second set of electric pulses applied 24 h after the first treatment, suggests that electric pulses could be beneficially used in fractionated regimes. The effect of repeated applications with reduced intervals is proposed for further studies.

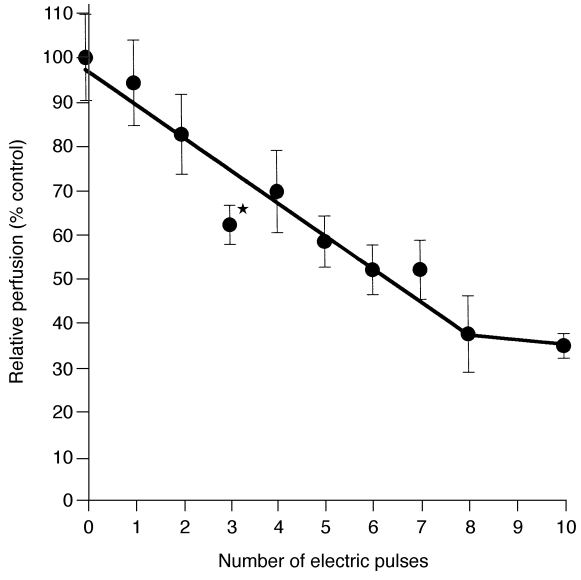

Figure 2. Relative perfusion of SA-1 tumours as a function of the number of electric pulses applied (1040 V, pulse width 100 μsec, repetition frequency 1 Hz). Mean values ±1 standard error of the mean (SEM) of at least six mice per point. *Significantly different (P<0.05) from the values of the controls.

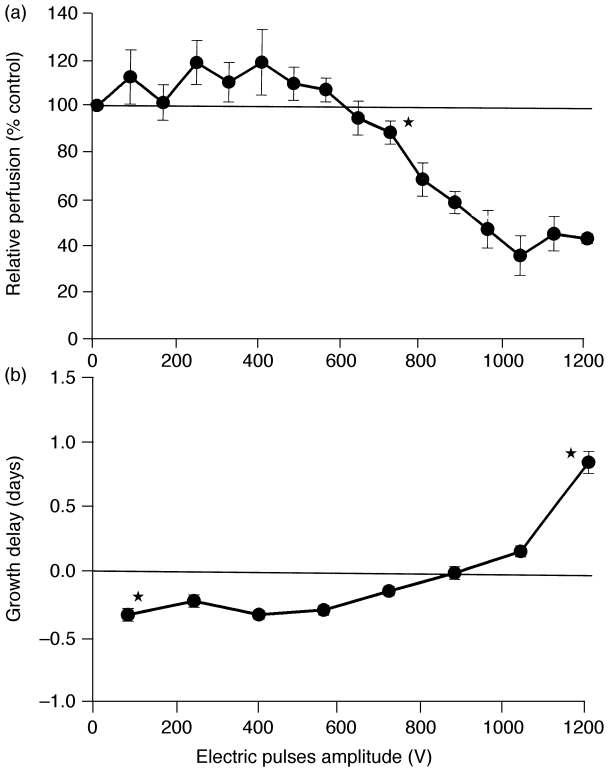

Figure 3. Variations in relative perfusion (a) and measurement of tumour growth delay (b) of SA-1 tumours as a function of the amplitudes of the electric pulses applied (eight electric pulses, pulse width 100 μ sec, repetition frequency 1 Hz). Mean values ±1 standard error of the mean (SEM) of at least six mice per point. *Significantly different (P<0.05) from the values of the controls.

Since electric pulses, as used in this study, are also used in electrochemotherapy to aid drug delivery for some chemotherapeutic drugs, it is possible that direct damage to plasma membrane may be involved in the vascular response to electric pulses. However, the process of cell electroporation is reversible, and up to certain amplitudes of electric pulses it does not affect cell survival [26]. In previous studies it was demonstrated that amplitudes over 720 and 880 V are needed to electroporate cells *in vivo*, at these amplitudes a small reduction in tumour cell survival is observed [17, 32]. Although the percentage of tumour cells directly impaired by electroporation is expected to be low, in the present study it could, to some extent, contribute to the observed small reduction in tumour growth. Therefore, the antitumour effect of high amplitudes of electric pulses may not be the consequence of only a temporary reduction of tumour blood flow, but also due to tumour cell death that has not recovered after electroporation.

The mechanism of the tumour blood flow changes induced by electric pulses is not yet fully understood. A balance between two mechanisms seems likely as implicated by the observed increased blood flow at low amplitudes of electric pulses and decreased blood flow at high amplitudes. As already mentioned in our previous study, during the application of electric pulses to the tumours, areas of much lower electric field intensities exist in the mid-plane between the electrodes and falls further from the centre to the margins of the tumour [27]. Therefore, at intermediate amplitudes, small heterogeneity of the electric field within a solid tumour could elicit increases and decreases in flow in different tumour microregions. However, the effect after pulses with high amplitudes is a significant reduction of tumour blood flow.

It is well documented that the plasma membrane is the primary target of cell electroporation [33]. Therefore, apart from tumour cells, endothelial cells can also be affected by the application of electric pulses. It is already known that electric fields can lead to an increase in intracellular reactive oxygen species and a transient increase in intracellular Ca^{2+}. Both can lead to activation of immediate early response genes regulating cell growth [34]. It is possible that changes in blood flow are dependent upon changes induced in the membrane of endothelial cells within the tumour which could influence coagulation, adhesion, permeability and cell shape.

The application of electric pulses is already used in the treatment of cancer patients with electrochemotherapy [21, 24, 25]. Presently this method is suitable for the treatment of accessible cutaneous tumour nodules. The development of needle electrodes [35, 36] will enable the treatment of deep-seated tumours and facilitate intra-operative treatment of tumours in internal organs and, thus, increase the potential clinical application of such an approach. The fact that such treatment induces blood flow changes within solid tumours indicates that it may well have increased utility if combined with a modality which can exploit the altered tumour environment. For example, at the high amplitudes currently used, the decreased blood flow would induce reduction in oxygenation and pH and such changes could be exploited using agents such as bioreductive drugs.

In conclusion, the results of this study demonstrate that the application of electric pulses to solid tumours induces significant changes in tumour blood flow. This finding may extend the therapeutic use of such pulses which are currently used to improve the intracellular uptake of drugs and DNA within tumour tissue.

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