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Research paper

Intratumoral cisplatin administration in electrochemotherapy: antitumor effectiveness, sequence dependence and platinum content

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Electrochemotherapy using intratumoral cisplatin administration was tested on EAT tumors in mice. Mice were treated with eight electric pulses (100 μs, 1 Hz, 1040 V) and/or cisplatin (1, 2, 4 and 8 mg/kg). Cisplatin treatment resulted in up to 20 days of tumor growth delay. Electrochemotherapy resulted in tumor cures; local tumor control reached a plateau at 4 mg/kg in 67% of tumor cures. The maximal effect of electrochemotherapy was achieved when cisplatin was injected 5 min before or simultaneously with electric pulses application. Approximately two times more platinum was bound to DNA in electrochemotherapy than in cisplatin treated tumors at all time points tested. Our study shows that electrochemotherapy with intratumoral cisplatin administration is a very effective local treatment of EAT tumors with high curability rate. [© 1998 Lippincott-Raven Publishers.]

Key words: Cisplatin, electrochemotherapy, Ehrlich ascites carcinoma, platinum determination.

Introduction

Electrochemotherapy in the treatment of cancer combines chemotherapy with the application of short intense high voltage electric pulses on the tumor nodule.[1] Application of electric pulses under specific conditions causes a transient increase in cell membrane permeability and thus facilitates the uptake of chemotherapeutic drugs, especially of those that do not freely diffuse into cells.[1] Electrochemotherapy with the nonpermeant chemotherapeutic drug bleomycin has been extensively investigated *in vitro* on cell cultures,[2] *in vivo* on different transplanted and spontaneous tumors in mice and rats,[3–7] and also in clinical trials on head and neck tumors, malignant melanoma, breast adenocarcinoma, and basal cell carcinoma.[8–11] Results of these studies have demonstrated that electrochemotherapy is effective local treatment, inducing partial and complete responses of the tumors.

In addition to bleomycin, other chemotherapeutic drugs like cisplatin, peplomycin, mitomycin C and cyclophosphamide have been tested for their potential application in electrochemotherapy.[12–15] In our previous study on SA-1 fibrosarcoma, B16 melanoma and EAT murine tumor models we demonstrated that electrochemotherapy with i.v. injected cisplatin has good antitumor effect.[15] Prolonged tumor growth delay and increased median survival time of electrochemotherapy treated animals were obtained.[15] Aiming to further improve the antitumor effectiveness of electrochemotherapy with cisplatin, we tested electrochemotherapy with intratumoral injection of cisplatin in our preliminary study on EAT tumors. This combination proved to be even more effective than electrochemotherapy with i.v. injected cisplatin.[12] Injection of cisplatin into the tumor exposes cells to a higher concentration of the drug during electric pulse application, without a prior distribution of the drug in the vascular system, as is the case in systemic therapy. Furthermore, intratumoral cisplatin injection has an advantage over systemic cisplatin injection in that there are lower concentrations of the drug in normal tissues, thus inducing fewer side effects.[16]

The aim of the present study was to elaborate on electrochemotherapy using intratumoral cisplatin injection. Cisplatin dose-response, and the sequencing and timing of cisplatin injection relative to electric pulses application were tested. Tumor response to this

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treatment was followed by tumor growth delay and tumor curability. Furthermore, we measured the platinum content in the tumors and amount of platinum bound to DNA by atomic absorption spectrometry in order to determine whether, in fact, the antitumor effectiveness of electrochemotherapy was a consequence of increased cisplatin concentration in the tumor cells.

Materials and methods

Mice and tumors

CBA mice of both sexes, purchased from the Institute of Pathology, Ljubljana, Slovenia, were used. They were maintained at 24°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. Solid Ehrlich-Lettre ascites carcinoma tumors (EAT; ATCC, Rockville, MD) were induced s.c. in the right flank of the mice by inoculation of 5×10^6 viable tumor cells in 0.1 ml of 0.9% NaCl. Viability of tumor cells was determined using the Trypan dye exclusion test. Tumor cells for inoculation of s.c. tumors were obtained from the ascitic form of the tumors, serially transplanted every 7 days. When the tumors had grown to 6 mm in diameter, the mice were randomly divided into experimental groups containing six to 11 mice and subjected to a specific experimental protocol. Treatment protocols were approved by the Ethical Committee of the Republic of Slovenia no. 27/11/95.

Drug formulation

Cisplatin [*cis*-diamminedichloroplatinum(II); Platinol] was obtained from Bristol Myers Squibb (Vienna, Austria) as a crystalline powder and dissolved in sterile H_2O at a concentration of 1 mg/ml. Further dilutions were made in 0.9% NaCl. Cisplatin doses used in experiments were 1, 2, 4 and 8 mg/kg. For each experiment, a fresh cisplatin solution was prepared.

Treatment protocol

Electrochemotherapy consisted of cisplatin injection followed by the application of electric pulses. Cisplatin was injected intratumorally, i.e. 0.1 ml cisplatin solution was injected slowly into the center of the tumor. Electric pulses were delivered by stainless steel electrodes 8 mm apart (two parallel stainless steel strips, length 35 mm, width 7 mm, with rounded corners). Electrodes were placed at opposite ends of the tumor. Good contact between the electrodes and the skin was assured by means of conductive gel. Eight square-wave electric pulses of 1040 V amplitude, pulse width 100 μs and repetition frequency 1 Hz, given in two perpendicular directions, were generated by electropulsator Jouan GHT 1287 (Jouan, St Herblain, France).[12] The application of electric pulses was performed without anesthesia and except for short-term paresis of the low extremities, lasting a few seconds after the end of application of electric pulses, no other side effects were observed. In cisplatin dose-response experiments, tumors were exposed to electric pulses (1040 V) 10 min after intratumoral cisplatin injection. In the time-response experiments cisplatin solution was injected 40, 20, 10 and 5 min before, simultaneously, and 10 and 20 min after the application of electric pulses. Pertinent control groups were mice without treatment, mice treated with either electric pulses or cisplatin as the sole treatment.

Platinum determination

To determine platinum content in the tumors and amount of platinum bound to DNA, mice were sacrificed at various time points (1, 4, 18, 48, 72 and 96 h) after treatment. Tumors were excised and removed from the overlying skin. For determination of platinum in whole tumors, each tumor was weighed (tumor weights were approximately 100 mg), placed into 15 ml graduated polyethylene tube and digested in 1 ml of 65% nitric acid by incubation at 37°C for at least 2 days to obtain a clear solution. Samples were diluted with water to 10 ml before analysis. For determination of the amount of platinum bound to DNA, each tumor was minced and treated with collagenase (Sigma, St Louis, MO) to obtain a single-cell suspension. DNA was isolated using a modified salting-out method.[17] Platinum content in the samples was measured by electrothermal atomic absorption spectrometry on a Hitachi Z-8270 polarized Zeeman atomic absorption spectrophotometer, adjusted to a wavelength of 265.9 nm.[18]

Treatment evaluation and statistical analysis

The antitumor effectiveness of the treatments was followed by measurement of the tumor diameters, calculation of tumor growth delay and curability of tumors. Tumor growth was followed by the measurement of three mutually orthogonal tumor diameters

(e_1, e_2, e_3) with vernier calipers on each consecutive day. Tumor volumes were calculated by the formula $V = \pi \cdot e_1 \cdot e_2 \cdot e_3/6$. From the tumor volumes, arithmetic means (AM) and (SEM) were calculated for each experimental group. Tumor doubling time (DT) was determined for each individual tumor. Tumor growth delay was calculated for each individual tumor by subtracting the DT of each tumor from the mean DT of the control group and then averaged for each experimental group. The response to treatment was scored as complete when the tumors became unpalpable. Mice that had a complete response 100 days after the treatment were considered cured. The significance of differences between the mean values of the tumor growth delays and platinum concentration of the experimental groups was evaluated by modified *t*-test (Newman-Keuls test) after a one way analysis of variance was performed and fulfilled.

Results

Antitumor effectiveness of electrochemotherapy

Cisplatin dose-response. First, antitumor effectiveness of electrochemotherapy on tumors 6 mm in diameter was evaluated with a 10 min interval between intratumoral injection of cisplatin and application of electric pulses. The DT of untreated tumors was 4.4 ± 0.2 days. Exposure of tumors to electric pulses had a minor, but statistically significant effect on tumor growth inducing a 3.4 ± 0.7 day tumor growth delay. Intratumoral treatment with cisplatin had a good, dose-dependent antitumor effect (Figure 1A). However, when electric pulses were used as a delivery system for cisplatin, the antitumor effectiveness of this combined treatment (electrochemotherapy) was more than additive. In contrast to cisplatin treatment, electrochemotherapy resulted in tumor cures; local tumor control reached plateau at 4 mg/kg with 67% tumor cures (Figure 1B). In those tumors that regrew after electrochemotherapy tumor growth was delayed from 15.8 ± 0.9 days at 1 mg/kg to 45.1 ± 2.4 days at 8 mg/kg cisplatin. Overall, more than four times lower concentrations of the drug were needed for the same antitumor effectiveness (Figure 1A).

Sequencing and timing of cisplatin injection relative to electric pulses application. These experiments were conducted in order to determine the optimal sequence and interval between cisplatin injection and electric pulses application. Cisplatin was injected intratumorally (2 mg/kg) at different intervals: 40, 30, 20, 10 and 5 min before, simultaneously with or 10 and 20 min after application of electric pulses. The antitumor effectiveness of electrochemotherapy was more than additive when cisplatin was injected before or simultaneously with electric pulses, indicating that the drug needs to be present in the tumor when electric pulses are applied (Figure 2). The maximal effect on tumor growth was obtained when cisplatin was injected 5 min before or simultaneously with the application of electric pulses (28.5 ± 4.0 and 28.8 ± 4.4 days tumor growth delay, respectively). The antitumor effectiveness of electrochemotherapy was less pronounced when cisplatin was injected after electric pulses, inducing 15.5 ± 2.0 days at 10 min and 12.0 ± 1.0 days tumor growth delay at 20 min interval (Figure 2A). The optimal sequencing and interval of cisplatin administration and electric pulse applications was also reflected in the data on tumor cures (Figure 2B). Local tumor control was achieved in 40–50% of mice when the interval between cisplatin injection

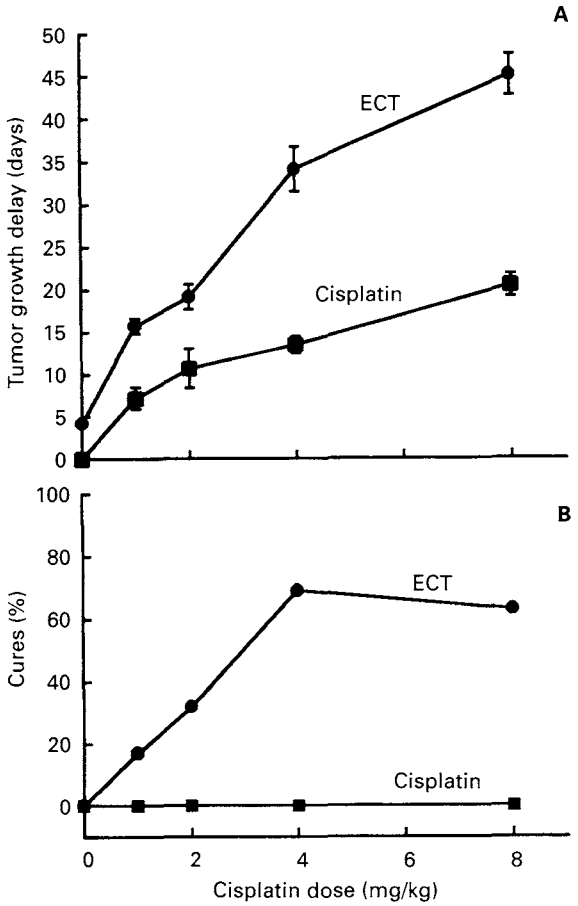

Figure 1. Tumor growth delay (A) and tumor cures (B) of EAT tumors treated with electrochemotherapy (ECT) as well as cisplatin and electric pulses as single treatments. Experimental groups consisted of six to 11 animals.

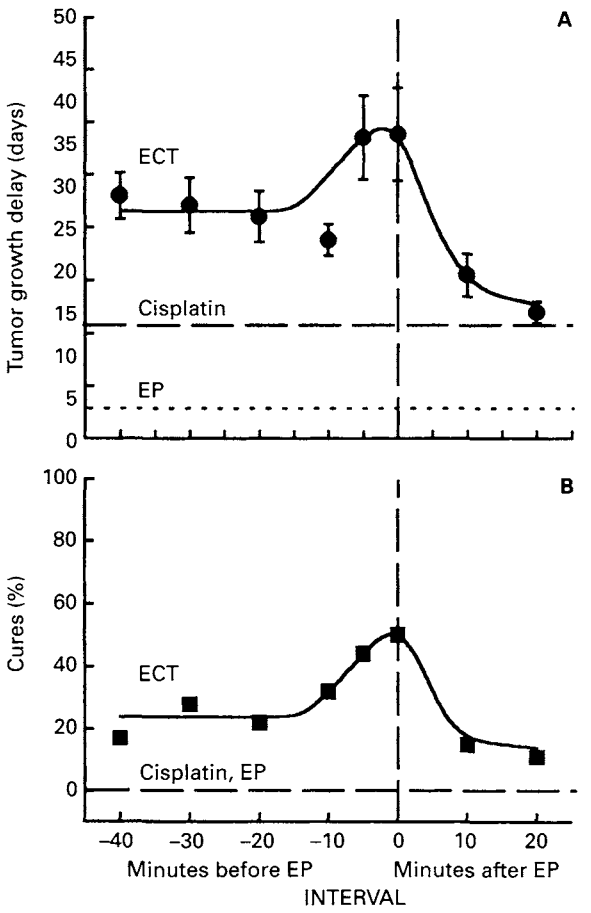

Figure 2. Tumor growth delay (A) and tumor cures (B) of EAT tumors treated with electrochemotherapy (ECT) as a function of the sequence and interval between cisplatin and electric pulses (EP) treatment. The cisplatin dose used was 2 mg/kg. Experimental groups consisted of six to 11 animals.

and the application of electric pulses was less than 5 min.

Analysis of platinum

To determine whether the observed antitumor effectiveness of electrochemotherapy was due to increased cisplatin concentration in the tumor cells, measurements of platinum content in the tumors and amount of platinum bound to DNA were performed at different time points after electrochemotherapy and cisplatin treatment. Tumors were treated with cisplatin (4 mg/kg) 10 min before electric pulse application. At various post-treatment intervals tumors were collected and platinum content determined. In both electrochemotherapy and cisplatin treated mice platinum content decreased quickly, reaching 35% of the

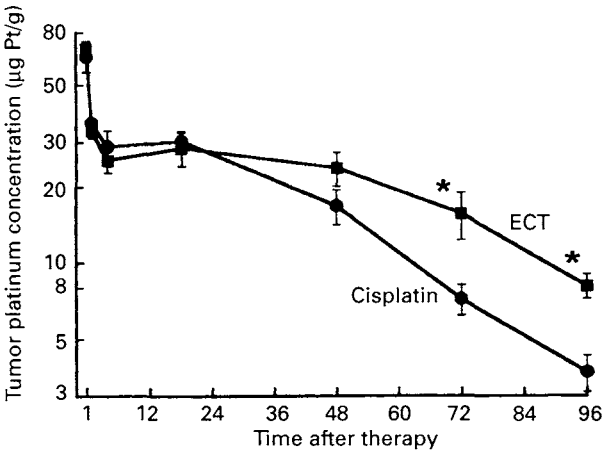

Figure 3. Tumor platinum concentration in electrochemotherapy (ECT) and cisplatin treated tumors. The cisplatin dose used was 4 mg/kg. Time points where the differences between the values are significant are labeled with asterisks. Experimental groups consisted of six to 11 animals.

initial concentration 4 h after the beginning of treatment and remained at this level for up to 48 h (Figure 3). Thereafter, platinum content in the electrochemotherapy treated tumors slowly decreased but remained higher than in cisplatin treated tumors. At 72 and 96 h post-treatment time, an up to two times higher platinum concentration was observed in electrochemotherapy treated tumors.

In contrast, the difference in amount of platinum bound to DNA was observed already 1 h after the treatment. In electrochemotherapy treated tumors 1.5 ± 0.3 ng Pt/μg DNA was measured, while in cisplatin treated tumors 1.3 ± 0.2 ng Pt/μg DNA. This difference was more pronounced at 4 and 18 h post-treatment intervals, where 1.0 ± 0.4 and 1.0 ± 0.2 ng Pt/μg DNA were measured in electrochemotherapy treated tumors in constrast to cisplatin treated tumors where 0.5 ± 0.2 and 0.6 ± 0.1 ng Pt/μg DNA was determined at these time points ($p < 0.05$). Two times higher amount of platinum bound to DNA in electrochemotherapy treated tumors was also measured at 48, 72 and 96 h after the treatment, which is in agreement with measurements of platinum content in the whole tumors.

Discussion

Our study shows that electrochemotherapy with intratumoral cisplatin administration is a very effective local treatment of EAT tumors with high curability rate. Furthermore, we have demonstrated that the amount of platinum bound to DNA and platinum

concentration in whole tumors is higher in electrochemotherapy treated tumors than in cisplatin only treated ones.

In cancer treatment one of the major problems is to deliver a sufficient concentration of the chemotherapeutic drug into the tumor cells for effective cell killing while minimizing drug concentration in normal tissues. In view of this, several attempts at selective tumor drug delivery have been made, including local application of the drugs,[12,16,19,20] targeting by binding of the drugs to tumor-specific antibodies,[21] incorporation of the drugs into liposomes or other vehicles,[19,22-27] or by selectively increasing the permeability of the plasma membrane of tumor cells by employing either chemical (use of detergents)[28] or physical methods (electroporation).[1] In our study we combined two of these approaches: local intratumoral injection of cisplatin and electroporation of tumor cells by the application of electric pulses to the tumor site.

Electroporation of tumors causes a transient increase in plasma membrane permeability, thus facilitating drug delivery into the cells and, consequently, potentiating cell killing.[1] Indeed, as shown in our study, electrochemotherapy with intratumorally injected cisplatin resulted in prolonged tumor growth delay and also tumor cures. Compared to our previous study on electrochemotherapy with i.v. injected cisplatin, where 13% of tumor cures was obtained at the highest dose tested (8 mg/kg cisplatin), the present study shows that 67% of mice were already tumor free at 4 mg/kg (the corresponding dose in humans would be 12.3 mg/m^2) 100 days after treatment.[15] However, electrochemotherapy proved to be an effective treatment regardless of the route of cisplatin administration. For example, electrochemotherapy with i.v. injected cisplatin could be beneficially used in the treatment of patients with cutaneous and s.c. tumor lesions that are on on-going cisplatin chemotherapy. On the other hand, electrochemotherapy with intratumorally injected cisplatin could be used as a treatment modality in patients where repeated surgical procedures and irradiation are difficult to perform, due to the progression of the disease in cutaneous tissue only. In these cases, electrochemotherapy with intratumorally injected cisplatin might be the treatment of choice due to its simplicity, inexpensiveness and shorter treatment procedure which can be done on an out-patient basis.[29]

In our study we also demonstrated that intratumoral cisplatin injection as a single treatment modality had a significant antitumor effect. Tumor growth was greatly delayed and was cisplatin dose dependent. In comparison to our previous study, the antitumor effectiveness of cisplatin as single treatment was better after intratumoral than after i.v. injection.[15] While i.v. injected cisplatin had little or no effect on tumor growth of EAT tumors, intratumorally injected cisplatin had a pronounced antitumor effect inducing up to 20 days tumor growth delay at a 8 mg/kg cisplatin dose. In addition, our results are in accordance with several other studies that have shown the advantage of intratumoral over i.v. cisplatin administration.[19,20,22] A higher tumor concentration of cisplatin and its prolonged retention in the tumor was achieved in comparison to i.v. injected cisplatin.[20,23]

A time dependence study showed the importance of choosing an appropriate sequence and interval of cisplatin injection and electric pulse application. As demonstrated in our previous study, the best interaction is achieved when electric pulses are applied 3 min after i.v. cisplatin injection.[15] In the present study we demonstrated that the maximal antitumor effect is achieved when cisplatin is injected in the shortest interval possible before electric pulses application. Due to the intratumoral injection of cisplatin, a short period of time is necessary for cisplatin to distribute itself within the tumor. However, at longer intervals between electric pulses and cisplatin injection the antitumor effectiveness of electrochemotherapy is decreased, due to the expected washout of cisplatin from the tumor.

Results of the amount of platinum bound to DNA demonstrate that more platinum is bound to DNA in electrochemotherapy treated tumors than in cisplatin treated ones at all time points tested. Therefore, these results cleary demonstrate that *in vivo* application of electric pulses on tumors increases plasma membrane permeability and thus enables binding of cisplatin to its intracellular target, DNA. Measurements of platinum content in the whole tumors revealed that there was no difference in the first 18 h. These results indicate that application of electric pulses does not affect the wash out of cisplatin from the tumors for at least 1 day. This is probably due to the injection volume which was approximately one-half of the tumor mass and therefore most of the cisplatin solution was washed out. However, the observed difference in the platinum content after the second day is the consequence of entrapped platinum in the electroporated tumor cells, since the amount of platinum bound to DNA was higher in the electrochemotherapy treated tumors than in cisplatin treated ones. On day 3 and 4 after the treatment, both cisplatin and electrochemotherapy treated tumors were approximately of the same size. Thus the observed difference in platinum content could not be attributed to the progressive growth of the cisplatin treated tumors.

In conclusion, our study demonstrated that electrochemotherapy with intratumoral cisplatin administration is a very effective local tretment with high curability rate.

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