Urol Res (2000) 28:391 – 397

ORIGINAL PAPER

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Potentiation of effects of anticancer agents by local electric pulses in murine bladder cancer

Received: 28 August 1999 / Accepted: 27 July 2000

Abstract Electrochemotherapy is a novel cancer treatment in which electric pulses (EP) are used as a means of delivering anticancer agents to the cytoplasm of cancer cells (electroporation). The present study evaluates whether electrochemotherapy has in vitro and in vivo anticancer effects in murine bladder cancer. Using mouse bladder tumor cells (MBT-2 cells), in vitro electrochemotherapy was performed by applying EP to the cell suspension immediately after the addition of anticancer agents. The cytotoxicity of adriamycin (ADM), bleomycin (BLM) and cis-diamminedichloroplatinum(II) (CDDP) was determined by measuring succinate dehydrogenase (SD) activity in both electroporated and non-electroporated cells. In addition, intracellular concentrations of these anticancer agents were also measured. In the in vivo study, tumor-bearing C3H/He mice were treated with an intraperitoneal injection of anticancer agents followed by a local delivery of EP at the tumor site. Then, tumor growth rate (TGR) was determined and compared to that in the sham-treated control group, the EP-only group and the drug-only group. The in vitro study showed that, with electroporation, the cytotoxicity of BLM in electroporated cells was increased by as much as 95.7-fold compared to that of non-electroporated MBT-2 cells; CDDP showed only an increase of 1.8-fold and ADM showed no increase. After electroporation, the intracellular concentration of BLM, CDDP and ADM showed an increase of 120-, 1.7- and 0.8-fold, respectively. In electrochemotherapy for in vivo growing tumors, the potentiation of the antitumor effect was most prominent when combined with BLM, only slightly with CDDP, and totally absent with ADM. It is clear from in vitro and in vivo studies that, in a murine bladder tumor, the anticancer effect of BLM can be considerably potentiated by applying EP. Thus, BLM seems to be the most suitable anticancer agent for electrochemotherapy of bladder cancer.

Key words Electroporation · Electrochemotherapy · Anticancer agent · Murine bladder cancer

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Introduction

When a cell is exposed to high voltage and short electric pulses (EP), cell permeability can be increased transiently without causing permanent damage to the cell. This phenomenon, termed electroporation or electropermeabilization, has been used to insert exogenous molecules and gene materials into living cells [18, 27]. Recently, the technique of electroporation was also applied to a potential cancer treatment termed electrochemotherapy [3, 6–9, 15–18, 21, 22, 25]. The therapy uses EP as a means of delivering anticancer agents directly to the cytoplasm of the tumor cells.

Although electrochemotherapy is still far from being practicable for clinical cancers, a considerable antitumor effect has been demonstrated in animals bearing tumors when EP were applied at the tumor site after administration of anticancer agents. With regard to urogenital tumors, we have shown that EP can potentiate the effect of bleomycin (BLM) on in vivo growing mouse bladder tumor [29]. These studies have suggested that, in a given tumor, the therapeutic efficacy of electrochemotherapy may depend on the type and dosage of anticancer agents as well as on the intensity of applied EP.

In the present study, while focusing on electrochemotherapy of bladder cancer, we conducted an in vitro comparison of cytotoxicity and intracellular drug concentration between electroporated and non-electroporated murine bladder tumor cells with various anticancer agents. We extended this study to animals and evaluated antitumor effects of electrochemotherapy by changing

the types and dosage of anticancer agents. In addition, the effects of pulse intensity were also studied in both in vitro and in vivo situations.

Materials and methods

Murine bladder tumor

Murine bladder tumor MBT-2, induced by *N*-4-5-nitro-2-furyl-thiazolyl formamide (FANFT), was used in this study. This tumor is a poorly differentiated transitional cell carcinoma and has retained its histological characteristics during serial transplantation in syngeneic mice.

Electrical device

EP were delivered from a square-wave impulse generator (T820; BTX, San Diego, Calif., USA). The applied EP were monitored with an oscilloscope (Optimizer; BTX) connected to the generator in order to check actual voltage, duration and shape of pulses.

In vitro studies

MBT-2 cells were suspended in RPMI1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS; Gibco) at a final concentration of 1×10^6 cells/ml. The single cell suspension of MBT-2 was transferred to a 24-well plate (Falcon). Electroporation was carried out in the well plate using a specially designed pair of electrodes (Unique Medical, Iwanuma, Japan). This pulse-delivering apparatus for suspended cells consists of ring and core electrodes. The ring electrode can be fitted into the inner margin of the well, and the core electrode is placed at its center. Each electrode was coated with gold to prevent heat production. EP were applied under the following condition: eight pulses of 100 μs duration at a frequency of 1 Hz and the desired intensity, which ranged from 200 to 1600 V/cm.

As for anticancer agents, adriamycin (ADM), BLM and cis-diamminedichloroplatinum(II) (CDDP) were used. These anticancer agents were dissolved in the same medium used for the cell suspension. In vitro electrochemotherapy was performed by delivering EP to the cell suspension immediately after adding various doses of anticancer agents. The cells were treated with EP alone, anticancer agents alone and EP plus anticancer agents (electrochemotherapy). Then, the cells were incubated for 3 days at 37 °C in a humidified atmosphere (95% air/5% CO_2) in order to determine the effects of these treatments on cellular viability.

Cellular viability was assessed by measuring the amount of succinate dehydrogenase (SD) in the cells, since SD activity is known to be correlated linearly with the number of viable cells (SDI test) [2, 12]. Briefly, 0.2 ml of 0.4% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) solution, 0.2 ml of 10^{-1} M succinate sodium and 1.6 ml of phosphate buffered saline (PBS) were added to each well. The cells were then incubated for 4 h. The bottom, obtained by centrifugation at 3000 rpm for 10 min, was solubilized by 2 ml of dimethylsulfoxide (DMSO). The SD activity of the cells was determined by measuring optical density (OD) at 540 nm of the above solution using a double beam spectrophotometer (Nihon Bunko Kogyo, Tokyo, Japan). The SD activity after each treatment was expressed as a percentage of the SD activity of non-treated cells. Thus, this relative SD activity was defined as $(b/a) \times 100$, where a and b are the OD of non-treated and treated cells, respectively.

In electroporated and non-electroporated cells, intracellular concentrations of anticancer agents were also evaluated. ADM (6 μg/ml), BLM (30 μg/ml) and CDDP (24.9 μg/ml) were added to a cell suspension containing 20 mM HEPES. The mixture of cell suspension and each agent was incubated for 30 min after applying EP. Then, the intracellular concentration was measured. ADM: the mixture of cell suspension and [^{14}C]-ADM (Amersham) was incubated in a vibratile water bath at 37 °C for 30 min. After washing the mixture three times, the cytoplasmic concentration of [^{14}C]-ADM was measured using a liquid scintillation counter [1, 11]. BLM: the concentration of BLM was determined according to the bioassay procedure of Ohnuma et al. [19]. *Bacillus subtilis* ATCC 6633 was incubated with BLM-treated cells in the modified Mueller–Hinton broth at 37 °C for 18 h following 24 h of pre-culture at 4 °C. The same incubation was performed with various concentrations of standard BLM solution. A standard curve was obtained by plotting diameters of clear zones produced by BLM against its concentration. The BLM concentration of the sample was measured using this standard curve. CDDP: the amount of intracellular platinum was determined by means of atomic absorption spectroscopy.

All in vitro experiments were performed in triplicate and each group was repeated at least twice.

In vivo studies

Female C3H/He mice (6–8 weeks old), weighing 20–22 g, were used. MBT-2 cells (1×10^6 cells) were injected subcutaneously into the thigh of each mouse. After the tumor volume had reached the desired level (about 500 mg), the tumor-bearing mice were randomly divided into the following four groups: a sham-treated control group (D−E−), a group receiving anticancer agents only (D + E−), a group receiving EP only (D–E +) and a group receiving both treatments (D + E +). Each group consisted of at least five animals.

Under pentobarbital anesthesia (40 mg/kg), EP were applied at the tumor site using a tweezer electrode, which can sandwich the tumor. The contact was ensured by electrocardiogram (ECG) paste. A run of EP consisted of eight pulses of 100 μs and of various intensities of electric field (600 and 1000 V/cm) at a frequency of 1 Hz. Anticancer agents were administered intraperitoneally 30 min before applying EP. Based on the mouse lethal dose (LD_{50}) for each anticancer agent, two kinds of dosage were used for this in vivo study, i.e., one-sixth of the LD_{50} (L dosage) and one-third of the LD_{50} (H dosage), which corresponded to the maximum tolerance dosage for mice [24]. Thus, L and H dosages were 5.9 and 11.7 mg/kg for ADM, 50.5 and 101.0 mg/kg for BLM, and 8.9 and 4.5 mg/kg for CDDP, respectively.

The tumor's longest diameter (a) and the second diameter (b) perpendicular to (a) were measured by using a caliper on the day of treatment and every 2 days thereafter. The relative tumor volume (RTV) was calculated by the formula $ab^2/2$. In order to assess the antitumor effects of treatment, tumor growth rate (TGR) was determined from RTV_{Dn}/RTV_{D0}, where RTV_{D0} is an initial RTV and RTV_{Dn} is the RTV on the nth day after treatment.

Statistical analyses

In vitro SD activity and intracellular concentrations of anticancer agents were tested for significance using the Mann–Whitney U-test. In vivo TGR was tested for significance between groups by a one-way analysis of variance (ANOVA).

Results

In vitro study

The viability of pulsed cells at different field strengths was determined. The SD activity after EP treatment was represented as a percentage of non-pulsed cells' SD activity and related to electric field intensity (Fig. 1). When the intensity of the electric field was increased from 200 to 1000 V/cm, the SD activity of pulsed cells maintained the same level as that of non-pulsed cells. However, at

field intensities from 1200 to 1600 V/cm, the SD activity was significantly decreased as the field intensity was increased. Thus, up to 1000 V/cm, EP alone did not show lethal effects.

The cytotoxicity of anticancer agents in electroporated cells was compared to that in non-electroporated cells. In this study, the field intensity of EP was fixed at 600 V/cm, which alone caused no damage to the cells. The cytotoxicity of BLM was considerably potentiated by applying EP (Fig. 2a). Compared to the non-pulsed cells, the SD activity of pulsed cells (BLM + EP) was significantly lower at the BLM concentrations of 5, 10, 50, 100, 500 and 1000 μg/ml. Approximately at 50–100 μg/ml BLM, EP induced a maximum augmentation of cytotoxicity. This potential effect was estimated by the value: IC_{50} (50% inhibition of SD activity) without EP/IC_{50} with EP, and was shown to be 95.7. CDDP (0.1–100 μg/ml) decreased the SD activity of non-pulsed cells, dose-dependently. This dose–toxicity curve was slightly shifted to the left by EP (Fig. 2b). In pulsed cells, the SD activity was significantly lower than that of non-pulsed cells at the concentration of 1.0, 5.0 and 10 μg/ml. The EP-induced potentiation of cytotoxicity (IC_{50} without EP/IC_{50} with EP) was only 1.8. With regard to ADM (0.1–100 μg/ml), there was no significant difference between the SD activity of non-pulsed and pulsed cells at each concentration (Fig. 2c). The potentiation effect was 0.8.

When the field intensity of EP was varied (200–1000 V/cm), the cytotoxic effect of anticancer agents was briefly examined. The concentration of anticancer agents used for this study was 1.0 μg/ml for ADM, 50 μg/ml for BLM and 1 μg/ml for CDDP. In combination with BLM, 400 V/cm EP abruptly lowered the SD activity

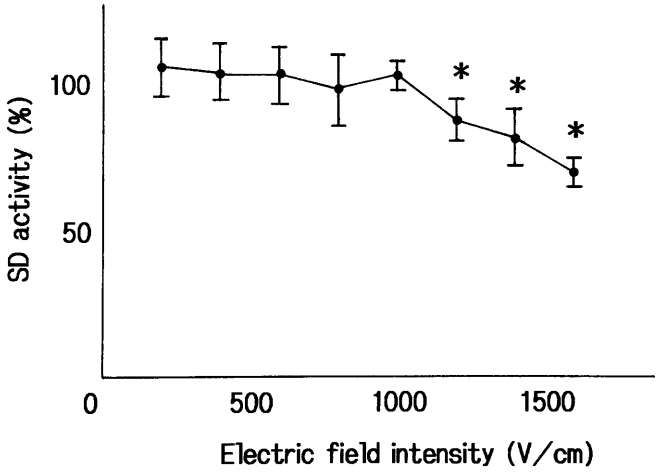

Fig. 1 Determination of cytotoxicity of electric pulses alone at different field intensities. Cellular viability was assessed by succinate dehydrogenase (SD) activity of the cells. Marked reduction of the SD activity was recognized only when the field intensity increased up to 1200 V/cm. Each point equals mean ± SEM, $n = 6$; $^*P < 0.01$

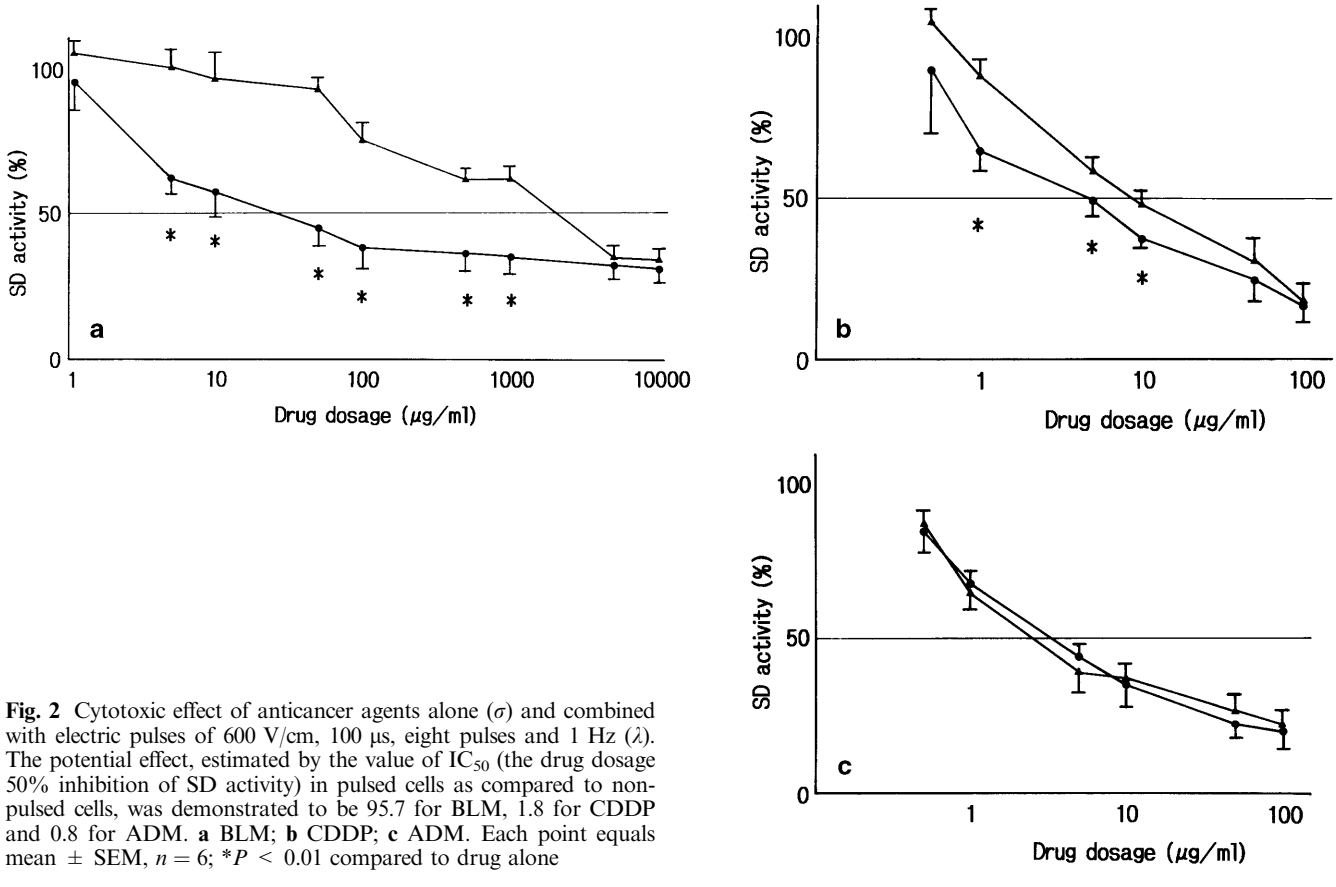

Fig. 2 Cytotoxic effect of anticancer agents alone (σ) and combined with electric pulses of 600 V/cm, 100 μs, eight pulses and 1 Hz (λ). The potential effect, estimated by the value of IC_{50} (the drug dosage 50% inhibition of SD activity) in pulsed cells as compared to non-pulsed cells, was demonstrated to be 95.7 for BLM, 1.8 for CDDP and 0.8 for ADM. **a** BLM; **b** CDDP; **c** ADM. Each point equals mean ± SEM, $n = 6$; $^*P < 0.01$ compared to drug alone

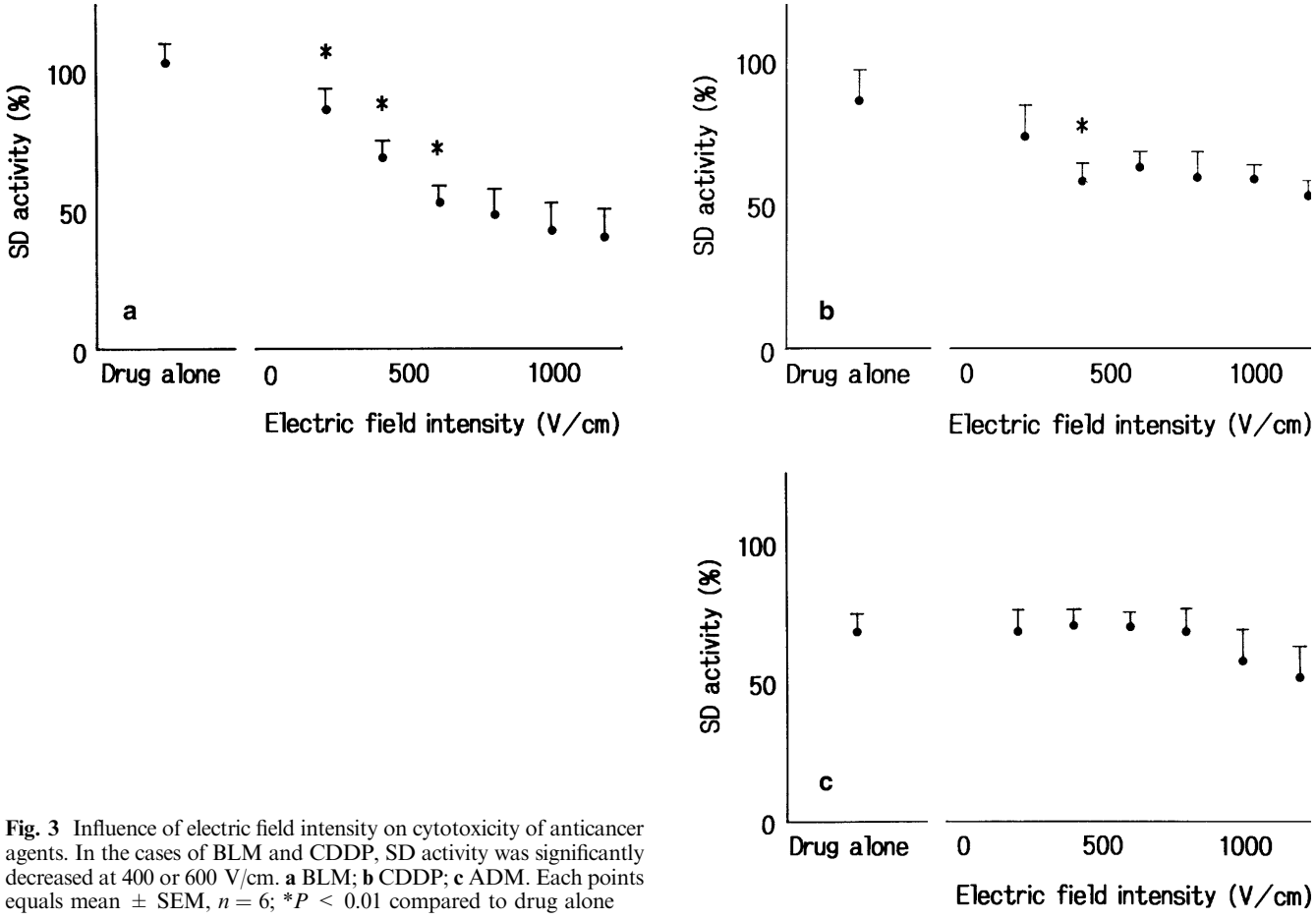

Fig. 3 Influence of electric field intensity on cytotoxicity of anticancer agents. In the cases of BLM and CDDP, SD activity was significantly decreased at 400 or 600 V/cm. **a** BLM; **b** CDDP; **c** ADM. Each points equals mean ± SEM, $n = 6$; $^*P < 0.01$ compared to drug alone

(Fig. 3a). At 600 V/cm, the SD activity was decreased a little, but this decrease was not significant. A further increase in field intensity did not lower the SD activity of pulsed cells. Thus, at the field intensity of 400 V/cm, the cytotoxicity of BLM became a plateau. In the cells treated with CDDP and EP, the SD activity was significantly lower than in those treated with CDDP alone at 400 V/cm. At a field intensity of more than 600 V/cm, the cytotoxicity of CDDP was not potentiated by EP (Fig. 3b). In the case of ADM, its cytotoxicity was never enhanced by EP, even though the field intensity was increased to 1000 V/cm (Fig. 3c).

Intracellular concentration of each drug in pulsed cells was compared to that in non-pulsed cells (Table 1). The electrical parameters of pulses for this study were 600 V/cm, 100 μs, eight pulses and 1 Hz. In electroporated cells, the intracellular concentrations of BLM were increased by as much as 120-fold, whereas CDDP showed an increase of only 1.7-fold. With regard to ADM, the intracellular concentration was not significantly increased by electroporation.

Table 1 Intracellular concentration of anticancer agents after applying EP (600 V/cm, 100 μs, eight pulses and 1 Hz)

Anticancer agent	Intracellular concentration	
	Control	After EP
BLM (μg/10^8 cells)	0.36 ± 0.14	43.2 ± 14.2*
CDDP (μg/10^8 cells)	2.14 ± 0.57	3.55 ± 0.74*
ADM (cpm/10^6 cells)	71,692 ± 12,450	88,181 ± 9200

$^*P < 0.01$

In vivo antitumor effect of electrochemotherapy

The influence of EP on tumor growth was assessed at two different field intensities, i.e., 600 and 1000 V/cm. In each of the groups that received either 600 V/cm EP or 1000 V/cm EP at the tumor site, TGR showed no significant difference compared to the TGR of the control group. Thus, both intensities of EP had no antitumor effect. In the following experiments, using a fixed electrical field intensity (600 V/cm), the antitumor effect of electrochemotherapy was examined.

When 50.5 mg/kg BLM (L dosage) was given to tumor-bearing mice, TGR of this group (D+E−) was the same as that of the control group (D−E−). In the D+E+ group (electrochemotherapy), TGR was significantly lower than that of the BLM alone group (D+E−), indicating that at this dosage of BLM, EP augmented the antitumor effect of BLM to a great extent (Fig. 4a). However, 101 mg/kg BLM (H dosage) alone

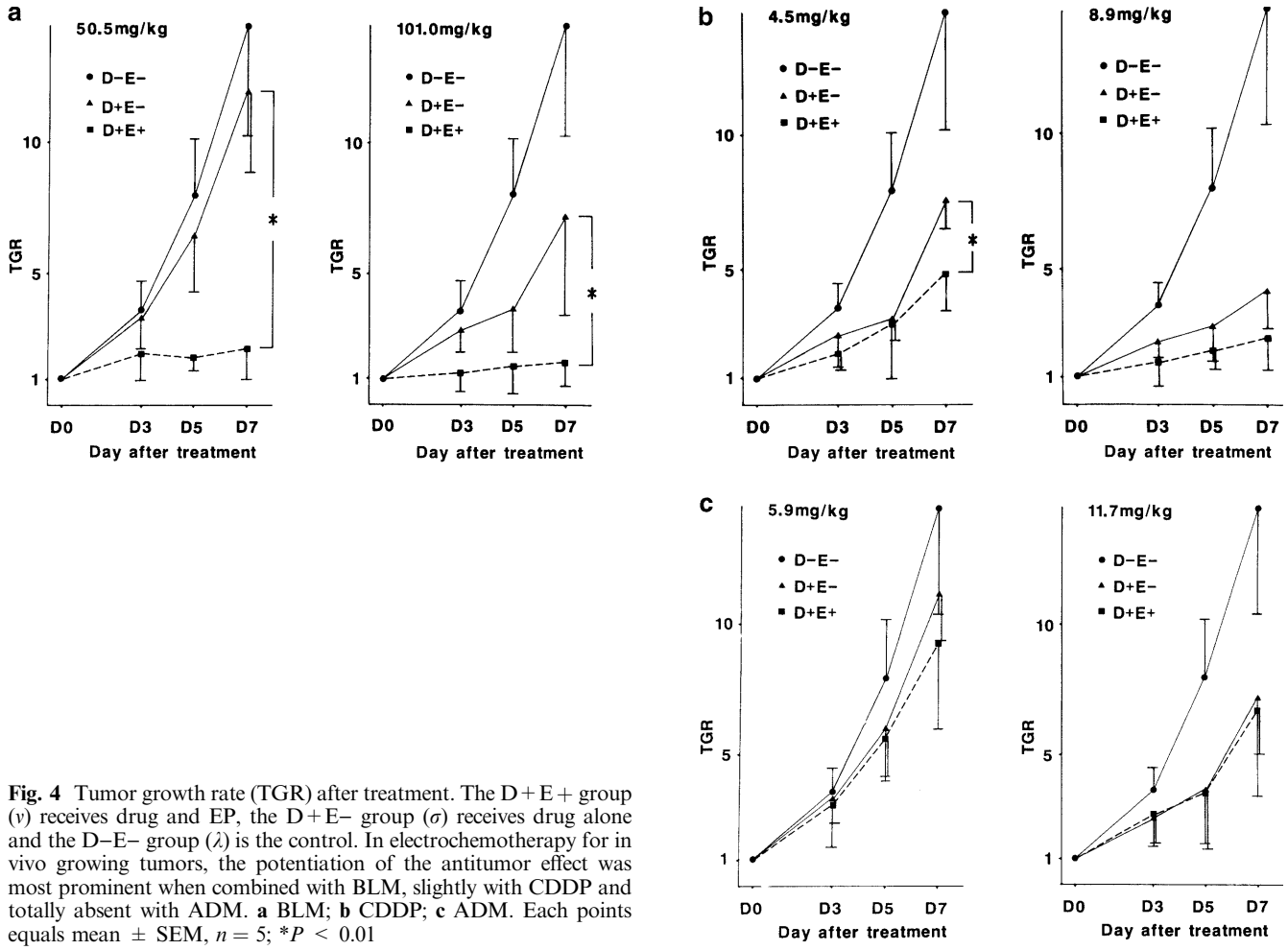

Fig. 4 Tumor growth rate (TGR) after treatment. The D+E+ group (v) receives drug and EP, the D+E− group (σ) receives drug alone and the D−E− group (λ) is the control. In electrochemotherapy for in vivo growing tumors, the potentiation of the antitumor effect was most prominent when combined with BLM, slightly with CDDP and totally absent with ADM. **a** BLM; **b** CDDP; **c** ADM. Each points equals mean ± SEM, $n = 5$; $^*P < 0.01$

showed a considerable antitumor effect. Although there was a significant difference in TGR between the D+E− and D+E+ groups, the augmentation of antitumor effects by EP was not prominent. The L dosage of CDDP (4.5 mg/kg) also showed an antitumor effect. Electrochemotherapy using the L dosage of CDDP showed a significant but small potentiation of antitumor effect (Fig. 4b). When the H dosage of CDDP (8.9 mg/kg) was administered, the antitumor effect of the drug itself became so strong that there was no significant difference in TGR between the D+E+ and D+E− group. The L dosage of ADM (5.9 mg/kg) alone as well as electrochemotherapy using this dosage of ADM did not affect the tumor growth. Similar to the cases of BLM and CDDP, the H dosage of ADM (11.7 mg/kg) inhibited the tumor growth. At the H dosage of ADM, there was no significant difference in TGR between the D+E+ and D+E− group (Fig. 4c).

In addition, electric field intensity was raised to 1000 V/cm, and the same in vivo study was performed. However, the results still reflected the above relationship with the type and dosage of anticancer agents. With regard to the adverse effects of these treatments, the H dosage of each anticancer agent significantly reduced the mouse body weight by 10–20%. EP did not affect the tumor weights of the mice. The skin on the surface of the tumorous lesion appeared edematous in all animals immediately after the application of EP. This change was most prominent when 1000 V/cm EP was applied. Heat burn was never noticed. No mice died during the experiment.

Discussion

The present study investigates the possibility of electrochemotherapy for the treatment of bladder cancer. This new therapy aims to potentiate the cytotoxicity of anticancer agents by electroporation. It is, therefore, important to determine the optimal conditions for EP and anticancer agents under which EP alone or drug alone has a minimal toxicity, but anticancer effects can be greatly augmented when EP and drug are combined.

With regard to the combined anticancer agents, several studies [3, 6, 13, 15, 21, 22] have demonstrated the effectiveness of electrochemotherapy with various types anticancer agents. However, these studies concern the in vitro cytotoxicity alone, and the results cannot be transferred directly to animals bearing tumors. In this respect, we systematically evaluate the effect of EP on

the in vitro and in vivo cytotoxicity of three chemotherapeutic agents. Our results show that in both the in vitro and in vivo situations, potentiation of the cytocidal effect on murine bladder cancer cells (MBT-2) is most prominent when combined with BLM, only slight with CDDP and totally absent with ADM.

The anticancer agents that were employed in the present study are targeted to DNA. To manifest their antineoplastic effects, these agents need to be taken into the cancer cells and their cytocidal effects are positively correlated with their intracellular concentrations [1, 5, 23, 26]. Therefore, this study compares intracellular concentration of anticancer agents in electroporated MBT-2 cells to that of non-electroporated cells. The results indicate that, with electroporation, BLM, CDDP and ADM show an increase of 120-, 1.7- and 0.8-fold, respectively, which is consistent with the increased cytotoxicity of the above agents after electroporation. Thus, the effectiveness of electrochemotherapy seems to be dependent on the increase in intracellular concentration of anticancer agents due to EP.

Although electroporation is supposed to be theoretically capable of delivering all types of anticancer agents to the cytoplasm of tumor cells [22], the present study demonstrates that BLM shows much higher intracellular concentration as compared to CDDP and ADM. This difference in cytoplasmic concentration may be related to the drug uptake mechanisms of intact cells. BLM is a water-soluble substance and known to enter the intact cells by slow passive diffusion [26]. Because of its slow diffusion, the cell membrane is thought to constitute a barrier for BLM's uptake. Therefore, only a little amount of the external BLM enters the intact cells [23]. When the membrane barrier for BLM is removed transiently by electroporation, BLM can cross the membrane freely and consequently the intracellular concentration of BLM would be increased considerably.

If the drug crosses the plasma membrane by an active transport mechanism or rapidly diffuses through the membrane due to its lipophilic nature, the membrane crossing is a step only weakly limiting the access to its intracellular target. The previous study [5] suggests that CDDP may be taken up into the cell by some form of active transport. This may account for our result that intracellular concentration of CDDP is increased only 1.7-fold after electroporation. However, Melvik et al. [14] demonstrated that in cultured human NHIK 3025 cells, CDDP uptake increased by 3-fold relative to non-electroporated cells after applying EP. This difference in the intracellular concentration could be attributed to different transport mechanisms of CDDP or different sensitivities to EP according to cell type.

In the case of ADM, this agent was shown to cross the plasma membrane due to active and passive transport systems [10, 27], indicating that ADM accumulates in high amounts in intact cells. It is, therefore, assumed that the intracellular concentration of ADM is already increased before electroporation. This is substantiated from the result that no differences were observed between the non-electroporated and electroporated cells in terms of its intracellular concentration.

As to the optimal electric conditions, we evaluated the cytotoxic effect of EP by changing field intensity. The results show that EP with a field intensity ranging from 200 to 1000 V/cm never affect viability of MBT-2 cells. However, the cellular viability is decreased at a field intensities from 1200 to 1600 V/cm. Mir et al. [15] also demonstrated that a colony formation of four types of cell lines was reduced by the field intensity ranging over 1300 V/cm. Thus, it is evident that EP itself has the cytotoxic effect when the field intensity exceeds a certain level, although the sensitivity to EP may differ in cell lines. On the other hand, if the field intensity of EP is too low, the cells can not be electroporated and the expected augmentation of the drug's cytotoxic effect would not be obtained. In this respect, our in vitro studies showed that at the field intensity of 200 V/cm, the cytotoxic effect of BLM or CDDP began to be potentiated, and the potentiation reached a maximum at 400 V/cm. Since the further increase in field intensity up to 1000 V/cm did not alter this maximum potentiation, the field intensity between 400 and 1000 V/cm seems to be suitable for electrochemotherapy. We, therefore, used 600 V/cm as a standard intensity of EP for in vitro and in vivo electrochemotherapy, which can induce electroporation on MBT-2 cells without cytotoxicity.

In addition, in the SDI method that is used in evaluating cytocidal effects, the SD activity is determined as an index of cellular viability. The method is fast and yet its results can be evaluated easily. Therefore, it is used as an anticancer agent sensitivity test. It has been reported that results of this test are highly correlated with clinical outcome [12]. It has been said that the concentration of the anticancer agent that is used as a cutoff value in determining the sensitivity of the tissue to the agent is ten times that of the peak plasma concentration (PPC) of a man who has been receiving a normally accepted clinical dosage. These ten PPCs for BLM, CDDP and ADM correspond to 30.0, 24.9 and 6.0 μg/ml, respectively [24]. In the present study, the cytocidal effects of BLM and CDDP given at these concentrations were potentiated by electrochemotherapy. Although the results of an in vitro test using an experimental tumor in mice cannot simply be applied to clinical cases, we believe that electrochemotherapy shows promise in producing a favorable result when combined with anticancer agents at clinical dosages.

These results indicate that, under the suitable conditions of EP mentioned above, electrochemotherapy may be a promising modality for the treatment of bladder cancer. As demonstrated by the in vivo study, EP potentiates the action of anticancer agents only at the lesion where the pulses are applied. Moreover, this modality holds promise of ameliorating the adverse effect of anticancer agents by reducing their dosages or applying it to refractory cases that resist standard chemotherapy.

Recently, human studies for malignant melanoma, basal cell carcinoma and skin metastasis have showed

that electrochemotherapy could be applied clinically for the treatment of cutaneous and subcutaneous tumors [7, 8, 17, 25]. On the other hand, since most tumors occur inside the body, a major problem in the clinical setting of this therapy is that there is no adequate means to place the pulse-delivering electrodes at the tumor site. However, in this respect, bladder tumor may have an advantage over other tumors because this tumor is endoscopically manipulable and the electrodes can be placed with the aid of a cystoscope. Furthermore, Jaroszeski et al. [9] showed that the thick tumor tissue could be well treated by electrochemotherapy by using a needle array electrode in the tissue in a rat hepatoma model. Thus, bladder tumor seems to be a good candidate for electrochemotherapy in the future.

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