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Optimal Electric Conditions in Electrical Impulse Chemotherapy

Motonori Okino,[1,3] Hiroshi Tomie,[1] Hirohiko Kanesada,[1] Masaru Marumoto,[1] Kensuke Esato[1] and Hiroshi Suzuki[2]

[1]*The First Department of Surgery, Yamaguchi University School of Medicine, 1144 Kogushi, Ube 755 and* [2]*Institute of Medical Electronics, Nihon Medicus Co., Ltd., 315-1 Mukohjima, Minamihanashima, Matsudo 271*

The relationship was examined between the tumoricidal effect and the electrical variables of *in vivo* electrical impulse chemotherapy. Donryu rats subcutaneously inoculated with AH-109AY hepatocellular carcinomas were given a single high-voltage electrical impulse of varying voltage and duration, 30 min after an intramuscular injection of 4 mg/kg bleomycin. As the voltage (V) was increased from 0 to 5 kV, the tumoricidal effect (E) increased in proportion to the square of the voltage. As the pulse duration (D) was increased from 2.5 to 5.8 ms, the tumoricidal effect increased in direct proportion to it. Combining these results yielded the formula, $E=\gamma V^2 D$, which indicates that tumoricidal efficacy was proportional to the applied electrical energy. When the electrical energy was kept constant while varying the voltage and duration of pulse, the anticancer effect was the same, confirming this relationship for lower energy levels.

Key words: Electrical impulse chemotherapy — Bleomycin — AH-109A hepatocellular carcinoma — Optimal electric conditions

We have previously reported the strong tumoricidal effect of applying a single high-voltage electrical impulse to a tumor during treatment with an antineoplastic drug, i.e., electrical impulse chemotherapy.[1,2] Administration of a high-voltage electrical impulse (5 kV/cm for 2 ms) during bleomycin administration (20 mg/kg i.m.) resulted in a significant decrease in the size of AH-109A hepatocellular carcinomas subcutaneously inoculated into Donryu rats. Tumors decreased to an average of 17% of their initial size 4 days after treatment, and the average survival time was about 220% of untreated controls. Neither the high-voltage electrical impulse nor bleomycin alone demonstrated any tumoricidal effect.

We postulated that the mechanism underlying this strong tumoricidal effect was the transport of a large quantity of anticancer drug through the pores created in the tumor cell membrane by the high-voltage electrical impulse. Previous *in vitro* studies have demonstrated that high-voltage electrical impulses (1–20 kV/cm for 1–1000 μs) can induce structural changes in the biological membranes of many animal and plant cells, resulting in reversible pore formation in cell membranes (electroporation), or irreversible membrane fusion (electrofusion).[3–5] However, the existence of this, or a similar phenomenon, has not been confirmed in *in vivo* studies. In our previous study, field strength and impulse duration were kept constant. Recently, we have developed a new high-voltage impulse generator which can create impulses with a range of potentials and durations.

In the present study, we assessed the dependency of the antitumor effect of electrical impulse chemotherapy on field strength and impulse duration in order to maximize the tumoricidal effect, gain some insight into its underlying mechanism, and minimize damage to surrounding tissues.

MATERIALS AND METHODS

Animal and tumor systems Six-week-old male Donryu rats were purchased from Seiwa Experimental Animals, Co., Ltd. The AH-109A hepatocellular carcinoma cell line, supplied by Dr. H. Satoh of the Sasaki Institute, was maintained intraperitoneally in our laboratory. The AY-109AY hepatocellular carcinoma cell line was provided by the Institute of Yamanouchi Co., Ltd.

Apparatus An improved high-voltage electrical impulse generator (ECTES-16) was constructed by Nihon Medicus Co., Ltd. (Fig. 1). The ECTES-16 produces a single electrical impulse of variable voltage (0–10 kV) and duration (0–7.5 ms). The impulse is synchronized to the R wave of the eletrocardiogram in order to avoid ventricular fibrillation and cardiac arrest. The ECTES-16 generates a rectangular wave, unlike our previous generator which generated a spike wave by capacitor discharge (Fig. 2). The applied electrical impulses were recorded on a digital memory scope (Yokogawa DL 1200) through a 1/5000 attenuator (Nihon Medicus AT 3).

Experiment 1 AH-109A hepatocellular carcinoma cells (3×10^6) were injected subcutaneously into the back of 100 male Donryu rats weighing 150 to 200 g. Tumors

[3] To whom request for reprints should be addressed.

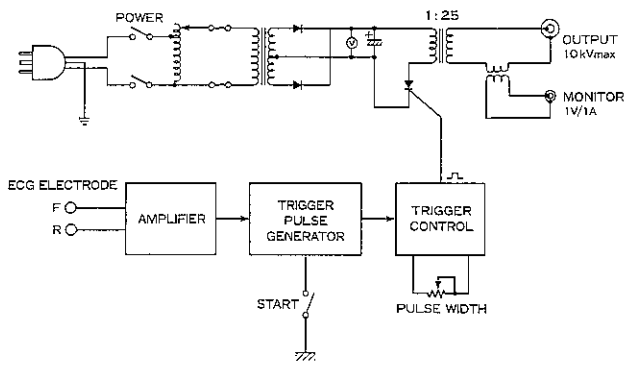

Fig. 1. Block diagram of the new high-voltage electrical impulse generator (ECTES-16).

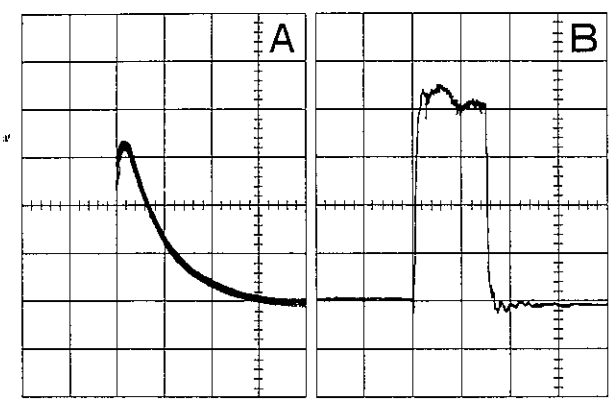

Fig. 2. Comparison of wave forms generated by old and new models. A: spike wave generated by the old model, B: rectangular wave generated by the new model.

1–2 cm in diameter had developed in 72 rats by the 7th postinoculation day. These 72 rats were divided randomly into four groups of 18 rats each.

One group served as a control (group C) and received no treatment. The second group received bleomycin 4 mg/kg ingected intramuscularly in the right thigh (group B). The third group received a single electrical impulse across the tumor (group I). The last group received a single electrical impulse across the tumor 30 min after an intramuscular injection of 4 mg/kg bleomycin (group BI). The electrical impulse (5 kV for 3 ms) was given using bare iron electrodes, 2 cm long and 0.5 mm in diameter, inserted through the skin and positioned on opposite sides of the tumor at the tumor margin. The distance between the two electrodes was 2 cm. All treatment was performed under ether anesthesia.

Experiment 2 Sixty male Donryu rats with an AY-109AY solid tumor 1.5–2.5 cm in diameter were divided randomly into six equal groups 12 days after tumor inoculation. All rats received a single electrical impulse of 3 ms duration, 30 min after an intramuscular injection of 4 mg/kg bleomycin in the right thigh. Rats in the different groups received either a sham shock of 0 kV, or a real shock of 1, 2, 3, 4 or 5 kV, applied through parallel subcutaneously placed electrodes 2.5 cm long and 2.5 cm apart.

Experiment 3 Forty-five male Donryu rats with AY-109AY solid tumors 1.5–2.5 cm in diameter were divided randomly into five equal groups. Thirty min after an intramuscular injection of 4 mg/kg bleomycin, a single electrical impulse of 3 ms duration was applied to each tumor, as in experiment 1. Rats in the different groups received either a sham shock of 0 kV, or a real shock of 2.5, 5, 7.5 or 10 kV.

Experiment 4 Twenty-four male Donryu rats with AY-109AY tumors 1.5–2.5 cm in diameter were divided randomly into four equal groups. All rats in these groups received a single electrical impulse of 5 kV, 30 min after an intramuscular injection of 4 mg/kg bleomycin. Rats in the different groups received either a sham shock of 0 ms, or a real shock of 0.25, 1.3 or 2.3 ms.

Experiment 5 Thirty-five male Donryu rats with AY-109AY tumors 1.5–2.5 cm in diameter were divided randomly into five equal groups. These rats were subjected to the same protocol as those in experiment 4 except that impulse duration was 0 ms for controls, and 0.8, 1.8, 3.3 or 5.8 ms for the experimental groups.

Experiment 6 Twenty-eight male Donryu rats with AY-109AY solid tumors of 1.5–2.5 cm diameter were divided randomly into four equal groups. All rats except the controls (group 0) received a single electrical impulse, 30 min after an intramuscular injection of 4 mg/kg bleomycin. The applied voltages and durations were 7 kV and 0.7 ms in the first group (group 1), 5 kV and 1.9 ms in the second group (group 2), and 4 kV and 7.25 ms in the third group (group 3). The rats in group 0 received an intramuscular injection of 4 mg/kg bleomycin only.

Measurement The length, width and height of the tumors were measured by using calipers on the day of treatment and every day thereafter. *Sn*, the tumor size at day *n* after treatment is the tumor size in mm^3 estimated as $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. The relative tumor size (RTS) was calculated using the formula

$$\text{RTS} = Sn/So.$$

The final relative tumor size (f-RTS) was formulated using the following equation

$$\text{f-RTS} = (\text{RTS}_{5th} + \text{RTS}_{6th} + \text{RTS}_{7th})/3,$$

where RTSnth is the RTS at day *n*.

The tumoricidal effect (*E*) was expressed by the following formula

$$E=(C/T)-1,$$

where C/T is the quotient of the mean f-RTS of the control group divided by the mean f-RTS of any group.

Results are reported as the mean ± SE, and the significance of differences between groups was determined by using Student's t test and analysis of variance.

RESULTS

In our first experiment, tumors continued to grow in the untreated controls, and in the groups treated with bleomycin or electrical impulse alone (Fig. 3). Seven days post-treatment, the relative tumor size was 11.3 ± 1.6 in the untreated group (C), 10.6 ± 1.0 in the electrical impulse only group (I), and 7.9 ± 0.59 in the bleomycin only group (B). No significant difference in relative tumor size existed between groups I and C. However, a significant difference did exist between groups B and C ($P<0.01$).

The concomitant use of electrical impulse and bleomycin (IB) caused a significant regression in tumor size. The relative tumor size of group IB was 0.5 ± 0.05 at 3 days after treatment and decreased further to 0.32 ± 0.05 at 7 days after treatment. The effect is highly significant compared to the other three groups ($P<0.0001$).

In experiment 2, we tested the effect of increasing voltages on tumor growth. As the voltage of the applied electrical impulse was increased from 0 kV to 5 kV, the relative tumor size on post-treatment day 7 decreased from 3.0 ± 0.33 to 0.52 ± 0.07 (Fig. 4). Significant differences in relative tumor size on post-treatment day 7 existed between group 0 kV and groups 2 kV, 3 kV, 4 kV

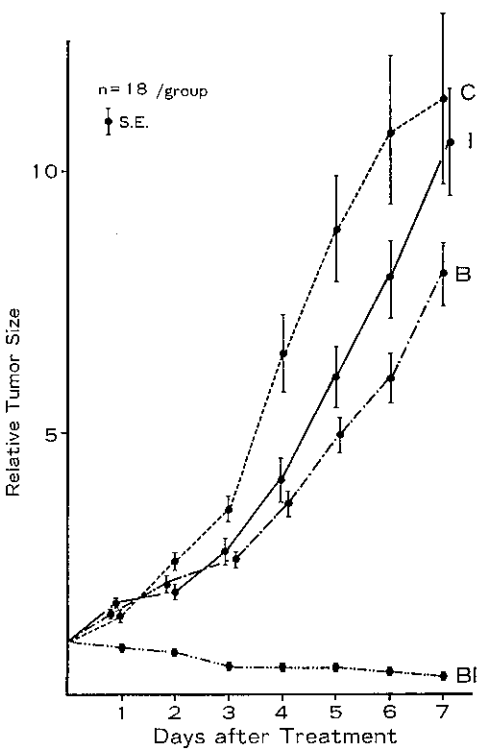

Fig. 3. Relative tumor size after treatment. C, control; I, electrical impulse alone; B, bleomycin alone (4 mg/kg, i.m.); BI, electrical impulse (5 kV for 3 ms) administered 30 min after bleomycin injection (4 mg/kg i.m.).

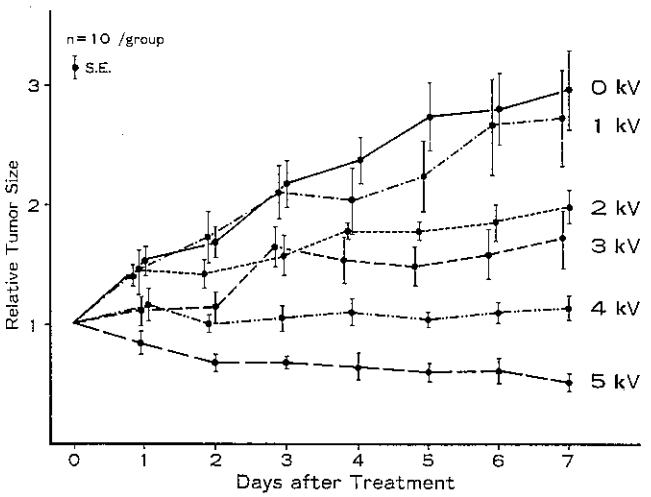

Fig. 4. Sequential changes in relative tumor size after electrical impulse chemotherapy with voltages up to 5 kV.

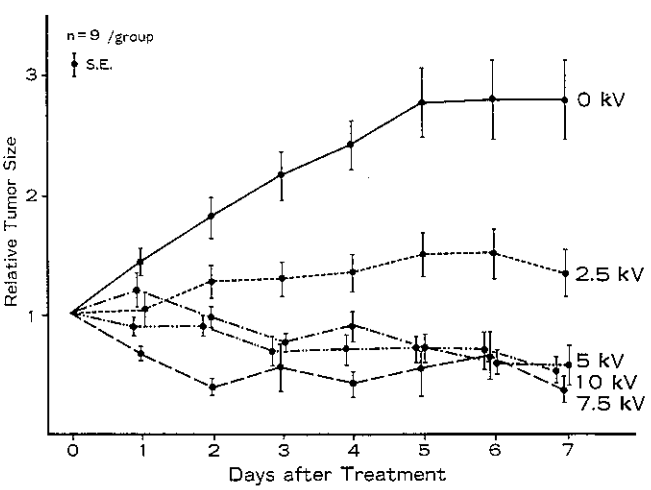

Fig. 5. Sequential changes in relative tumor size after electrical impulse chemotherapy with larger increments of voltage.

and 5 kV. However, no significant difference in tumor growth was found between groups 1 kV and 0 kV. Tumors regressed only in group 5 kV, decreasing significantly from the initial size.

We then repeated these experiments with larger increments of voltage in experiment 3. Tumors had grown to 2.8 ± 0.32 times their initial size by post-treatment day 7 in sham-treated controls (group 0 kV) (Fig. 5). In group 2.5 kV, tumor growth was inhibited by electrical impulse chemotherapy; mean tumor size was only 1.35 ± 0.2 times the initial size, but the difference in tumor size was not significant between post-treatment day 7 and day 0. In groups 5 kV, 7.5 kV and 10 kV, tumors regressed, with a significant difference from the initial size on post-treatment day 7 ($P < 0.01$). The relative tumor size was 0.58 ± 0.17 in group 5 kV, 0.38 ± 0.11 in group 7.5 kV and 0.52 ± 0.14 in group 10 kV on post-treatment day 7. No significant difference in tumor growth existed between any of these three groups.

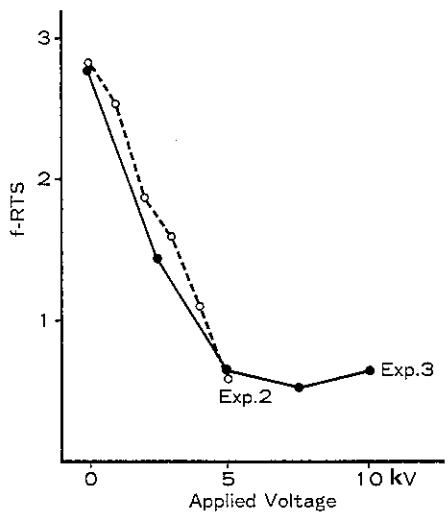

Fig. 6. Correlation between applied voltage and final relative tumor size (f-RTS).

The correlation between the final relative tumor size (f-RTS) and the applied voltage was investigated in experiments 2 and 3 (Fig. 6). F-RTS decreased linearly from about 3 to 0.6 as the applied voltage was increased from 0 kV to 5 kV in both experiments 2 and 3. The f-RTS remained relatively constant at applied voltage greater than 5 kV.

The correlation between the tumoricidal effect (E) expressed by $\{(C/T)-1\}$ and the applied voltage (V) was analyzed in experiments 2 and 3, using a logarithmic scale (Fig. 7). The linear relationship by the least-squares approximation was:

$$\log E = 2.04 \log V - 0.93,$$

with a correlation coefficient (r) of 0.98.

The final relative tumor size (f-RTS) decreased exponentially as the duration of the electric impulse was increased in experiments 4 and 5 (Fig. 8). The f-RTS was almost constant at intervals longer than about 4 ms. The correlation between the tumoricidal effect (E) and the duration of electrical impulse (D) was analyzed quantitatively for impulses shorter than 4 ms in experiments 4 and 5 using a logarithmic scale (Fig. 9). The linear

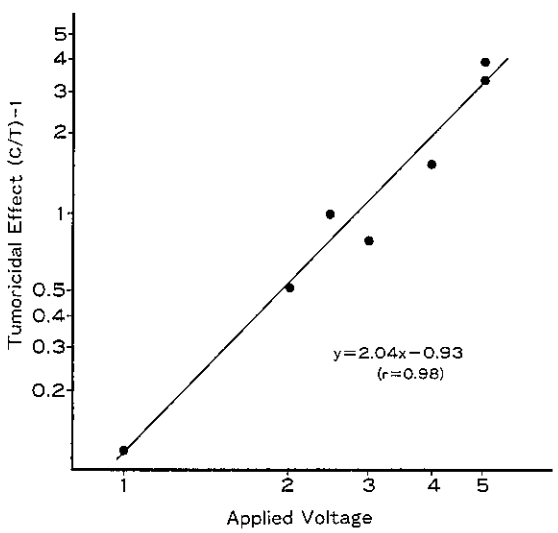

Fig. 7. Correlation between applied voltage and tumoricidal effect on a log-log graph.

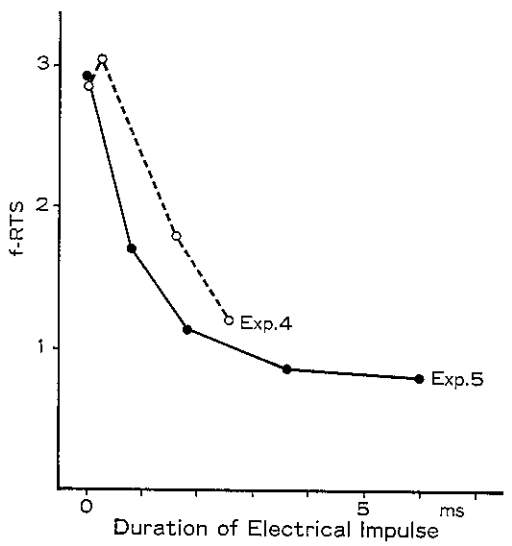

Fig. 8. Correlation between pulse duration and final relative tumor size (f-RTS).

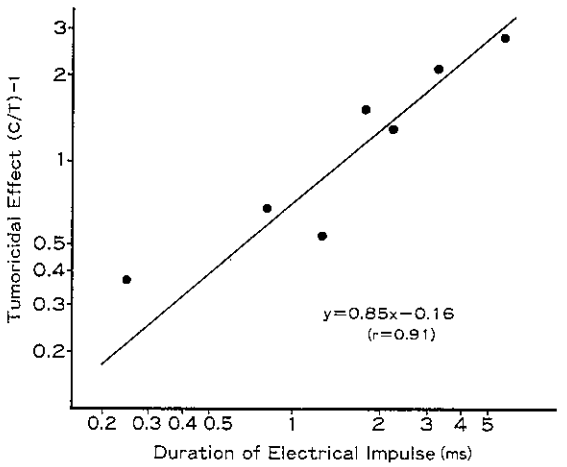

Fig. 9. Correlation between pulse duration and tumoricidal effect on a log-log graph.

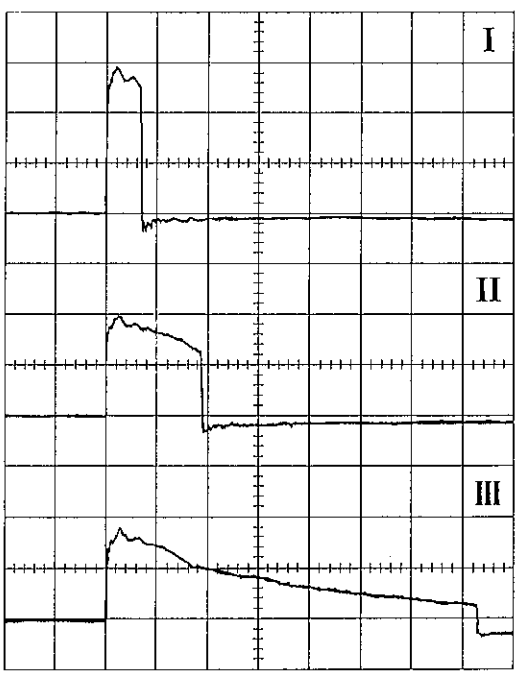

Fig. 10. Waveforms of applied electrical impulses with nearly constant electric energy. Group I received 7 kV for 0.7 ms. Group II received 5 kV for 1.9 ms. Group III received 4 kV for 7.25 ms.

relationship by the least-squares spproximation was:

$$\log E = 0.85 \log D - 0.16,$$

with a correlation coefficient (r) of 0.91.

Table I. Final Relative Tumor Size (f-RTS) under Different Electrical Conditions with a Constant Electric Energy

Group	0	I	II	III
Nominal voltage (kV)	0	7	5	4
Pulse duration (ms)	0	0.7	1.9	7.25
Electric energy/ε (J)	0	1.32	1.31	1.45
f-RTS	3.0	1.09	0.89	1.06
(±SE)	(±0.34)	(±0.14)	(±0.17)	(±0.23)

To test whether the tumoricidal effect depended on the electric energy applied, we administered impulses of different voltages and duration calculated to deliver equivalent energy. Actual energies in row 3 vary slightly because waveforms of longer pulses were not exactly square (Fig. 10). Tumoricidal effect did not differ significantly between experimental groups. N=7 in each group.

We next investigated the relation of tumoricidal effect to electrical energy applied in experiment 6 (Fig. 10). As pulse duration was increased, the end voltage decreased, resulting in non-rectangular wave forms. Thus, we did not obtain the value of electric energy by using the formula $E=\varepsilon V^2 D$, but by integrating as follows

$$\text{Electric energy} = \varepsilon \int_0^D V(t)^2 dt,$$

where $V(t)$ is voltage, D is pulse duration, and ε is a constant.

Electric energy/ε obtained by approximate calculation was 1.32 in group 1, 1.31 in group 2, and 1.45 in group 3. The f-RTS was 1.09±0.14 in group 1, 0.89±0.17 in group 2, 1.06±0.23 in group 3, and 3.0±0.34 in group 0 (Table I). No significant difference in f-RTS existed between the three experimental groups.

DISCUSSION

Electrical impulse chemotherapy combines two interventions: chemotherapy and high-voltage electrical impulses. The dependency of tumoricidal effect on the dose of the antineoplastic drug bleomycin has been reported previously.[6] Originally, we administered a constant dose of 20 mg/kg bleomycin to Donryu rats with AH-109A subcutaneous solid tumors. The dose of bleomycin needed to cause tumor regression with application of a high-voltage electrical impulse (5 kV for 3 ms with an interpolar distance of 2.5 cm) was more than 1.25 mg/kg. In the present study, we used a higher dosage of bleomycin, 4 mg/kg.

Many aspects of electrical impulse chemotherapy may be varied. Among them are the wave form of electrical impulse, the shape and material of the electrodes through which the electrical impulses are applied, and the number of applied electrical impulses. The waveform of the high-

voltage electrical impulse used in all of these experiments was rectangular. In this study, all electrical conditions other than voltage and duration of the electrical impulse were kept constant; the distance between the two electrodes was 2.5 cm, the electrodes were needle-shaped, and only one electrical impulse was applied.

In experiment 1, we reproduced our previous results despite the change in the waveform of the applied electrical impulse from spike to rectangular.[2] Tumors regressed only in group BI, while tumor growth in group B was inhibited only slightly. Having confirmed that a strong tumoricidal effect can be induced by a high-voltage electrical impulse with rectangular form, we proceeded to examine the effects of varying impulse voltage and duration. Histological changes of tumor tissues between the treatment with bleomycin alone and the combined use were analogous to those described in our previous report.[2]

In experiment 1, we used the AH-109A hepatocellular carcinoma employed in the previous experiment.[2] However, AH-109A hepatocellular carcinoma grows rapidly and metastasizes soon after inoculation, resulting in a wide distribution of RTSes. AH-109AY, a substrain of AH-109A, has a low malignant potential and thus a narrow distribution of RTSes. We therefore used the AH-109AY cell line for this series of experiments, since the purpose was to quantify local control of solid tumors by electrical impulse chemotherapy. To confirm the reproducibility of each study, paired experiments for each parameter were performed; exp. 2 and 3 for various voltages, exp. 4 and 5 for various durations.

In experiments 2 and 3, only the voltage was varied, with a constant 3 ms duration of the electrical impulse. To quantify the dependency on voltage, we expressed the tumoricidal effect (E) by means of the following formula: $E=(C/T)-1$. We used E to evaluate the tumoricidal effect, because it varies inversely with T, reaching zero when T is equal to the control. A graph of E versus voltage (V) suggested an exponential relationship, and we postulated the following formula: $E=\alpha V^{\beta}$. Taking the logarithm of both sides of the equation, we have $\log E=\log\alpha+\beta\log V$ and we obtained $\beta=2.04$ by least-squares approximation. The quality of fit to the data can be seen in the logarithmic graph of Fig. 7. We therefore concluded that the tumoricidal effect was in direct proportion to the square of the electric potential.

$$E=\alpha V^2$$

We analyzed experiments 4 and 5 in the same manner as experiment 2 and obtained the value $\delta=0.85$ for the formula $E=\gamma D^{\delta}$. We conclude that the anticancer effect was directly proportional to pulse duration:

$$E=\gamma D.$$

Combining the results of the present study we suggest the following formula to quantify the tumoricidal effect of electrical impulse chemotherapy

$$E=\varepsilon V^2 D,$$

where ε is a constant determined by electric conditions other than voltage and pulse duration. The right side of the equation, $\varepsilon V^2 D$ is equivalent to the electric energy of a direct current. We therefore propose that the tumoricidal effect of electrical impulse chemotherapy is directly proportional to the electrical energy of the pulse.

To confirm this hypothesis, we performed experiment 6. f-RTS values remained almost constant when the energy of the applied electrical impulse was kept constant while varying the impulse voltage and duration. This result strongly supports our hypothesis that the tumoricidal effect of electrical impulse chemotherapy is directly proportional to the applied electrical energy. This relationship held only in the lower range of electrical energy. The tumoricidal effect reaches a plateau as in experiments 2 and 5, and does not then increase any further with further increase of electric energy.

We have found no other report on *in vivo* electroporation, though many studies have appeared on the dependence of *in vitro* electroporation on electrical variables. Fromm *et al.* have studied the expression of genes transferred into plant cells by electroporation.[7] Gene expression increased geometrically as the electric field strength increased from 200 V/cm to 400 V/cm with a pulse duration of 54 ms. However, when the electric field strength exceeded 400 V/cm, gene expression decreased steeply, due to destruction of the cell membrane. Similarly, when pulse duration increased from 0 to 100 ms at an electric field strength of 200 V/cm, gene expression increased in direct proportion to pulse duration.

Hashimoto *et al.* have studied the transformation of intact yeast cells *in vitro* by electroinjection of plasmid DNA, and found a geometric increase in transformants as the electric field strength was increased from 0 kV/cm to 6 kV/cm.[8] Their results were thus similar to ours, although their cell survival rate decreased at high voltages.

Neumann *et al.* have studied gene transfer into mouse lyoma cells by electroporation in a high electric field.[9] Again, gene expression appeared to increase geometrically as the electric field strength increased from 0 kV/cm to 8 kV/cm. This curve reached a plateau at 6–7 kV/cm. However, they performed no quantitative analysis.

Other reports of *in vitro* electroinjection of genes into animal cells also show that gene expression increases as electric field strength increases, with a dependence suggestive of a high-order relationship with a plateau at 4–7 kV/cm.[10-12]

Our conclusion that the tumoricidal effect of electrical impulse chemotherapy is directly proportional to the electrical energy applied to the tumor is consistent with previous studies on gene transfer *in vitro* by electroinjection. We therefore hypothesize that electrical impulse chemotherapy exerts its effect by *in vivo* electroinjection of antineoplastic drugs into tumor cells, since its dependence on electrical parameters is completely consistent with what is known of the dependence of *in vitro* electroinjection of genes into animal cells.

Our maximal tumoricidal effect was achieved at a field strength of 5 kV/2.5 cm (2 kV/cm) or greater for 3 ms. Since we expect that any adverse effects on surrounding normal tissues will also be in proportion to electric energy, it may be possible to minimize adverse effects while maximizing the tumoricidal effect by using an electric pulse of 2 kV/cm for 3 ms.

Before electrical impulse chemotherapy becomes practical for clinical use, it will be essential to clarify the difference in sensitivity to electrical impulse chemotherapy between tumor and normal tissue. The specificity of electrical impulse chemotherapy will depend on two factors: the efficiency of electroporation of tumor tissue relative to normal tissue and the distribution of anticancer drugs *in vivo*.

There is reason to believe both may be favorable for electric impulse chemotherapy. Although the difference in electroporation efficiency between malignant cells and nonmalignant cells is unknown, spontaneous cell fusion occurs 100 times more frequently in malignant than in nonmalignant cells, probably because of a structural fragility of cancer cell membranes.[13] It therefore seems likely that the efficiency of electroporation in malignant cells may be significantly higher than that in normal cells.

Malignant tumor tissue also generally concentrates antineoplastic drugs more than normal tissue. By determining the period after drug administration when the difference in concentration between malignant tissues and surrounding nonmalignant tissues is maximal, it may be possible to attain tumor regression with minimal damage to surrounding normal tissue.

Finally, electrical impulse chemotherapy may be relatively safe. There were no deaths after electrical impulse chemotherapy among the 350 rats used in these experiments. The combination of relative safety with considerable antitumor efficacy suggests that electric impulse chemotherapy may make a significant contribution to the medical therapy of cancer.

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