

## Enhanced Effectiveness of Radiochemotherapy with Tirapazamine by Local Application of Electric Pulses to Tumors

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Tumor hypoxia is associated with resistance to radiotherapy and anticancer chemotherapy. However, it can be exploited to therapeutic advantage by concomitantly using hypoxic cytotoxins, such as tirapazamine (TPZ). Tumor electroporation offers the means to further increase tumor hypoxia by temporarily reducing tumor blood flow and therefore increase the cytotoxicity of TPZ. The primary objective of this work was to determine whether electric pulses combined with TPZ and radiotherapy (electroradiochemotherapy) was more efficacious than radiochemotherapy (TPZ + radiation). In these studies using the SCCVII tumor model in C3H mice, electro-radiochemotherapy produced up to sixfold more tumor growth delay (TGD) than TPZ + radiation. In these studies, (1) large tumors ( $280 \pm 15 \text{ mm}^3$ ) responded better to electro-radiochemotherapy than small tumors ( $110 \pm 10 \text{ mm}^3$ ), (2) TGD correlated linearly with tumor volume at the time of electroradiochemotherapy, (3) electric pulses induced a rapid but reversible reduction in  $\text{O}_2$  saturation, and (4) the electric field was highest near the periphery of the tumor in a 3D computer model. The findings suggested that electroradiochemotherapy gained its therapeutic advantage over TPZ + radiation by enhancing the cytotoxic action of TPZ through reduced tumor oxygenation. The greater antitumor effect achieved in large tumors may be related to tumor morphology and the electric-field distribution. These results suggest that electro-pulsation of large solid tumors may be of benefit to patients treated with radiation in combination with agents that kill hypoxic cells. © 2004 by Radiation Research Society

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### INTRODUCTION

It is well known that oxygenated cells are more sensitive to radiation than hypoxic cells (1), because oxygen reacts

rapidly with free radicals produced by ionizing radiation to produce damaging reactive oxygen species. Furthermore, there is considerable evidence from studies with animal tumors that hypoxic cells are also resistant to most anticancer drugs. However, this is not because cells low in oxygen are intrinsically resistant to the drugs. It is rather because hypoxic cells, by definition, must be those furthest from functioning blood vessels. In addition, tumor blood vessels are very different from normal vasculature in that they are highly irregular and tortuous and therefore limit the accessibility of the drugs to the hypoxic regions of the tumor distant from intact vasculature. Impaired drug targeting combined with the fact that the majority of anticancer drugs are effective only against rapidly proliferating cells leads to resistance to anticancer drugs.

However, tumor hypoxia can be exploited to therapeutic advantage. One such strategy involves the use of drugs that are toxic under hypoxic conditions, such as tirapazamine (TPZ). Tirapazamine is a hypoxia-selective drug that was developed by Brown *et al.* in 1987 (2). Its higher differential toxicity to hypoxic cells may help overcome the radioresistance associated with hypoxia, and it is now in clinical trials in combination with chemotherapy and/or radiotherapy (3–8). At the molecular level, the activation of TPZ occurs through a one-electron reduction step that is modulated by low oxygenation in the presence of various intracellular reductase enzymes (9, 10). Reduction results in a TPZ radical that can initiate both single- and double-strand breaks in DNA, leading to chromosome aberrations and cell death (11).

Electrical pulses can also be used to overcome the problem of drug resistance. Under proper conditions, the application of short, intense electric pulses to cells or tissues can induce transient and reversible changes in the plasma membrane, which allows for the more efficient transmembrane passage of molecules (12, 13). This phenomenon is termed electroporation (14, 15). One of the clinical applications of electroporation is to facilitate cellular uptake of chemotherapeutic agents. This approach, known as electrochemotherapy, was first introduced by Okino *et al.* and Mir *et al.* (16, 17). Electrochemotherapy has been used to treat a va-

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riety of tumor types (18). Electroporation of cells in tumors was long thought to be the only mechanism leading to the antitumor effect of electrochemotherapy. However, Ramirez *et al.* (19), Sersa *et al.* (20, 21), and Gehl *et al.* (22) have shown that the application of electric pulses to tumors also resulted in temporary reduction in tumor blood flow. Therefore, it was hypothesized that the use of hypoxic cytotoxins in combination with the blood-modifying effects of electroporation would enhance the efficacy of the treatment compared to the use of the cytotoxin alone (20). Cemazar *et al.* showed increased cytotoxicity for TPZ after a single treatment with electric pulses in SA-1 fibrosarcoma tumors in mice, multiple treatments were more effective than the single treatment in terms of tumor growth delay (23). In these studies, the increased cytotoxicity of TPZ was attributed to increased tumor hypoxia, which resulted from reduced blood flow in the tumor.

Brown *et al.* have showed that TPZ is highly effective at potentiating tumor cell killing by radiation in the SCCVII mouse tumor (24). We hypothesized that the application of electric pulses in combination with TPZ and radiation (electroradiochemotherapy) would enhance the efficacy of TPZ in combination with radiotherapy. In a similar approach using cisplatin, electric pulses and radiation, electroporation increased the radiosensitizing effect of cisplatin (25). In our studies, we investigated the possibility of using TPZ instead of cisplatin. We also investigated the relationship between treatment outcome and the initial tumor volume at the time of the treatment. We further examined the effect of the electric pulses on tumor oxygenation using visible-light tissue oximetry. Finally, we compared the experimental results to a 3D computer-based model of the electric-field distribution.

## MATERIALS AND METHODS

### Bioreductive Agent

Tirapazamine was obtained from Dr. Martin Brown in the Department of Radiation Oncology, Stanford University. It was dissolved in physiological saline prior to use at a concentration of 1 mg/ml. Tirapazamine-treated mice received an intraperitoneal (i.p.) injection at a dose of 48 mg/kg.

### Mouse Tumor Model

Male C3H mice 7–8 weeks old were bred and housed under defined flora conditions in the Stanford University Department of Comparative Medicine. They were maintained under specific-pathogen-free conditions in the Stanford Research Animal Facility of the Department of Comparative Medicine. All animal experiments were approved by and complied with the regulations of the Stanford University Care Panel. Tumor cells were implanted subcutaneously by injecting 50  $\mu$ l NaCl (0.9%) containing  $5 \times 10^5$  viable SCCVII cells in the hind flank. This tumor is a tissue culture-adapted cell line of a squamous cell carcinoma that arose spontaneously in the abdominal wall of a C3H mouse in the laboratory of Dr. H. D. Suit, Massachusetts General Hospital, Boston, MA. The derivation of the cell line and the details of its handling have been published elsewhere (26). Approximately 2 weeks after implantation, mice with similar tumor volumes were selected and assigned randomly into groups of five

to eight mice. The animals were anesthetized by an i.p. injection of 70 mg/kg pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL) prior to treatment.

### In Vivo Treatment Protocol

Electric pulses were administered using two stainless steel parallel-plate electrodes (1 cm<sup>2</sup>) mounted on a caliper connected to a pulse generator (model PA-4000, Cyto Pulse Sciences, Inc., Columbia, MD). During treatment the electrodes were placed on opposite sides of the tumor. The distance between the electrodes was adjusted to optimize tumor coverage and to achieve a voltage to distance ratio of 1200 V/cm, which was used in all studies unless otherwise noted. Four rectangular pulses were applied in one direction followed by four rectangular pulses in the perpendicular direction. Sersa *et al.* showed that changing the electrode orientation during the delivery of the eight transcutaneous electric pulses improved the efficacy of electrochemotherapy (27). The pulse duration was 100  $\mu$ s and the repetition frequency was 1 Hz. A conductive gel (Parker Laboratories, New York) was used to ensure good contact between the electrodes and the skin. For irradiation of tumors, the animals were placed in individual lead boxes. The tumor protruded through a cut-out portion at the rear of the box. Irradiation was delivered using a 250 kV X-ray machine at a dose rate of 83 cGy/min. The tumors received a single dose of 7 Gy 30 min after drug injection as described elsewhere (28). An initial set of experiments was performed to determine whether the time between TPZ administration and electroporation had any effect on treatment outcome.

Tumor growth was monitored by tumor volume measurements every second day from the time of treatment by measuring the length (*l*), width (*w*) and height (*h*) of each tumor with Vernier calipers. Tumor volume was calculated using the formula  $l \times w \times h \times \pi/6$ . Tumor volume quadrupling time was observed for each mouse as the time taken by the tumor to grow to a volume four times the volume on the treatment day. Tumor growth delay (TGD) was calculated by subtracting the mean tumor volume quadrupling time of the control group from the tumor volume quadrupling times of individual mice in each treated group. The average TGD was then calculated for each treated group using the individual measurements. Differences between group means were tested for statistical significance by a Student's *t* test after a one-way analysis of variance (ANOVA, SigmaStat, SPSS).

### Measurement of Tumor Tissue Oxygenation

A visible-light tissue oximeter (T-Stat<sup>TM</sup>, Spectros Corp., Portola Valley, CA) was used for assessment of tumor oxygenation. The device employed a non-contact optical method to measure the oxygen saturation of hemoglobin in the capillaries, venules and arterioles. Briefly, this system emits low-power (2–100 mW), continuous, broadband light (UV to infrared) and spectroscopically analyzes the light that passes through tissue and returns to the monitor. The device solves for both scattering and absorbance in the blue to orange (475–600 nm) portion of the spectrum, which localizes measurements to a region several millimeters in depth beneath the monitoring site. The update speed is 20–250 ms, depending upon the probe used and degree of averaging. A more detailed description of the functionality of this device is reported elsewhere (29). The non-invasive nature of the device permitted us to make real-time *in vivo* measurements of oxygenation and hemoglobin in tumors during the electric-pulse treatment. The measurements were performed using a surface probe situated 10 mm above the subcutaneous tumor. The effective sampling volume in the tumor was  $\sim 5$  mm<sup>3</sup>. The parallel-plate electrodes were kept in place with a minimum of pressure to reduce the possibility of blood flow changes due to tissue compression. All animals were anesthetized prior the measurements and restrained to avoid motion artifacts. The electric pulses were delivered as described above, except that all eight electric pulses were administered in the same direction to avoid pressure artifacts from repositioning of the electrodes. Some animals served as controls where pulses were applied to the skin on the back and

the limbs to determine if tumor oxygen levels were affected by electric pulses delivered to sites other than the tumor.

#### Computer Simulation of the Electric-Field Distribution in a Solid Tumor Model

A 3D finite element model of a tumor was used to calculate the electric-field distribution due to the voltage on the parallel-plate electrodes. The computation was done with the Maxwell 3D Solver (Ansoft Corp., Pittsburgh, PA). The tumor was modeled as a half sphere 10 mm in diameter surrounded by a 0.5-mm-thick layer of dermis. The parallel-plate electrodes were modeled as  $10 \times 10 \times 0.1$ -mm slabs placed on either side of the tumor with a separation distance of 11 mm. One plate was grounded, while the other had a potential of 1320 V to achieve a voltage to distance ratio of 1200 V/cm. The electrical material properties were treated as isotropic homogenous parameters, with a conductivity of 0.125 S/m for tumor tissue, 0.046 S/m for dermis, and  $9.3 \times 10^6$  S/m for the electrodes (30). The ratio of the relative permittivities between the tumor tissue and dermis was 10:1. The relative permittivity of the dermis was set to 1.

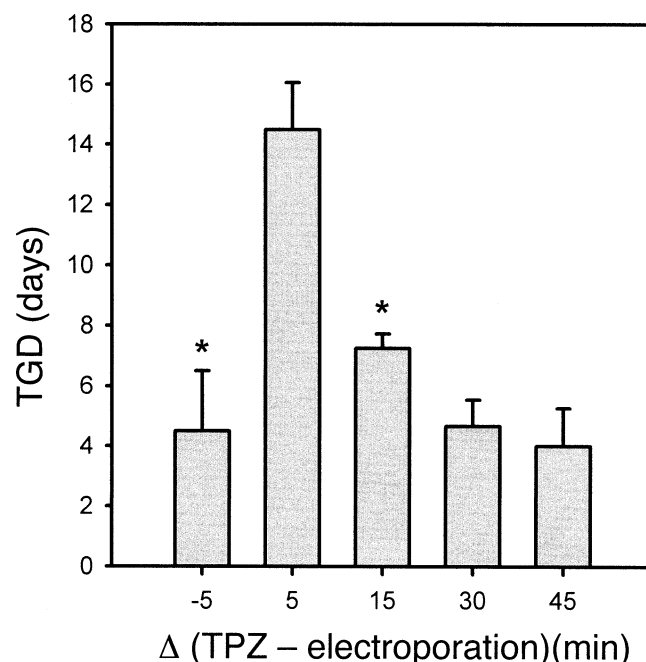
## RESULTS

### Sequence of Treatment Modalities

To maximize the efficacy of electroradiochemotherapy, we first determined the optimal sequence of administration of the different components of the therapy. Brown *et al.* showed that irradiation of tumors 30 min after i.p. administration of TPZ resulted in the best antitumor effect in SCCVII tumors (28). With this interval held constant, we found that the most critical variable affecting the outcome of electroradiochemotherapy was the timing between drug injection and electric-pulse application. We studied intervals from 5 min before drug administration to 45 min after drug injection. The results of these studies are summarized in Fig. 1, where TGD is displayed as function of time between drug and electric-pulse delivery. Of the conditions studied, delivering electric pulses 5 min after drug administration was most effective and led to a significantly higher TGD than the use of electric pulses 5 min prior to drug injection ( $P < 0.01$ ) or 15 min after drug injection ( $P < 0.02$ ). There was not a significant difference in TGD between tumors pulsed 30 and 45 min after drug administration.

### Effect of Electroradiochemotherapy on Large Tumors

Figure 2A shows the effects of electroradiochemotherapy, TPZ + radiation and TPZ + electroporation on the growth of large ( $230 \pm 10$  mm<sup>3</sup>) SCCVII tumors in mice, where the relative tumor volume [tumor volume divided by the tumor volume on day 0 (treatment day)] is shown as a function of time after treatment. The effect of a single dose of TPZ and that of the combined treatment with electric pulses and radiation (electroporation + radiation) are shown as well. A single dose of TPZ did not produce a significant change in tumor growth compared to the untreated tumors (TGD =  $1.0 \pm 0.2$ ,  $P > 0.1$ ). However, when TPZ was combined with electric pulses or radiation,

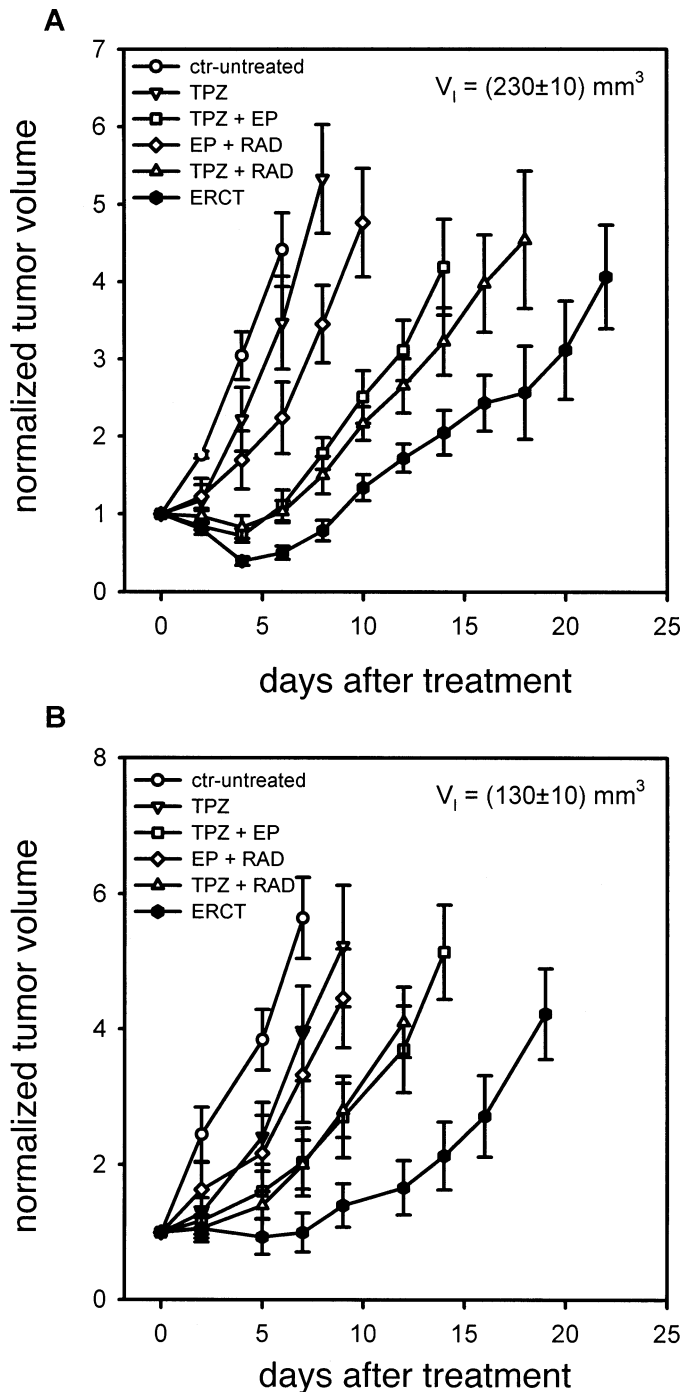


**FIG. 1.** Effect of treatment sequence on tumor growth. The TGD is plotted as a function of time between TPZ administration and electric-pulse delivery. Each experimental group consisted of six mice. For all groups, irradiation occurred 30 min after drug injection. Application of the electric pulses 5 min after drug delivery led to the longest TGDs. Data are expressed as means  $\pm$  SEM ( $n = 6$ ). \*Indicates significant difference from the 5-min group at  $P < 0.05$ .

the TGD was significantly increased ( $P < 0.01$ ) compared to the treatment with TPZ alone. The TGDs of the TPZ + electroporation and TPZ + radiation groups were  $7.5 \pm 1.5$  days and  $10.5 \pm 2.1$  days, respectively. There was no significant difference in TGD between the TPZ + electroporation and TPZ + radiation groups ( $P > 0.06$ ). The combined treatment with electric pulses and radiation resulted in a TGD of  $3.5 \pm 0.7$  days. The addition of electric pulses to TPZ + radiation resulted in a tumor growth delay of  $17.5 \pm 2.5$  days that was significantly higher than the TGD of the tumors treated with TPZ + radiation ( $P < 0.04$ ). Similarly, the addition of radiation to TPZ + electroporation resulted in significantly higher TGD compared to the tumors treated with TPZ + electroporation ( $P < 0.03$ ).

### Effect of Electroradiochemotherapy on Small Tumors

Figure 2B shows the tumor growth curves of SCCVII tumors with a volume of  $130 \pm 10$  mm<sup>3</sup> at the time of treatment. In this case too, a single dose of TPZ did not significantly affect tumor growth ( $P > 0.1$ ) compared to untreated control tumors, with a TGD of  $2.0 \pm 0.8$  days. The TGDs of the tumors treated with TPZ + electroporation and TPZ + radiation were  $7.5 \pm 1.4$  and  $7.0 \pm 1.0$ , respectively, which were significantly different from the TGD of the TPZ-treated tumors ( $P < 0.02$ ). However, there was no significant difference in TGD between these groups ( $P \geq 0.2$ ). The TGD of the tumors treated with electro-



**FIG. 2.** Effect of tumor volume at the time of the treatment on tumor growth after treatment. Panel A: Effect of the different treatment modalities on the growth of SCCVII tumors in C3H mice. The average tumor volume at the time of the treatment was  $230 \pm 10 \text{ mm}^3$ . The ordinate displays the tumor volume relative to the tumor volume at the time of the treatment as a function of time (days). Panel B: Effect of the different treatment modalities on the growth of SCCVII tumors that had an average tumor volume at the time of the treatment of  $130 \pm 10 \text{ mm}^3$ . Tumors in the electroradiochemotherapy group were treated as follows: Electric pulses [eight, 1200 V/cm (four in one direction followed by four pulses perpendicular to the first) with a pulse duration of 100  $\mu\text{s}$  and a repetition frequency of 1 Hz] were applied 5 min after TPZ injection (48 mg/kg, i.p.). Subsequent irradiation with 7 Gy followed 30 min after drug injection. Data are expressed as means  $\pm$  SEM ( $n = 8$ ). EP, electroporation; ERCT, electroradiochemotherapy; RAD, radiation.

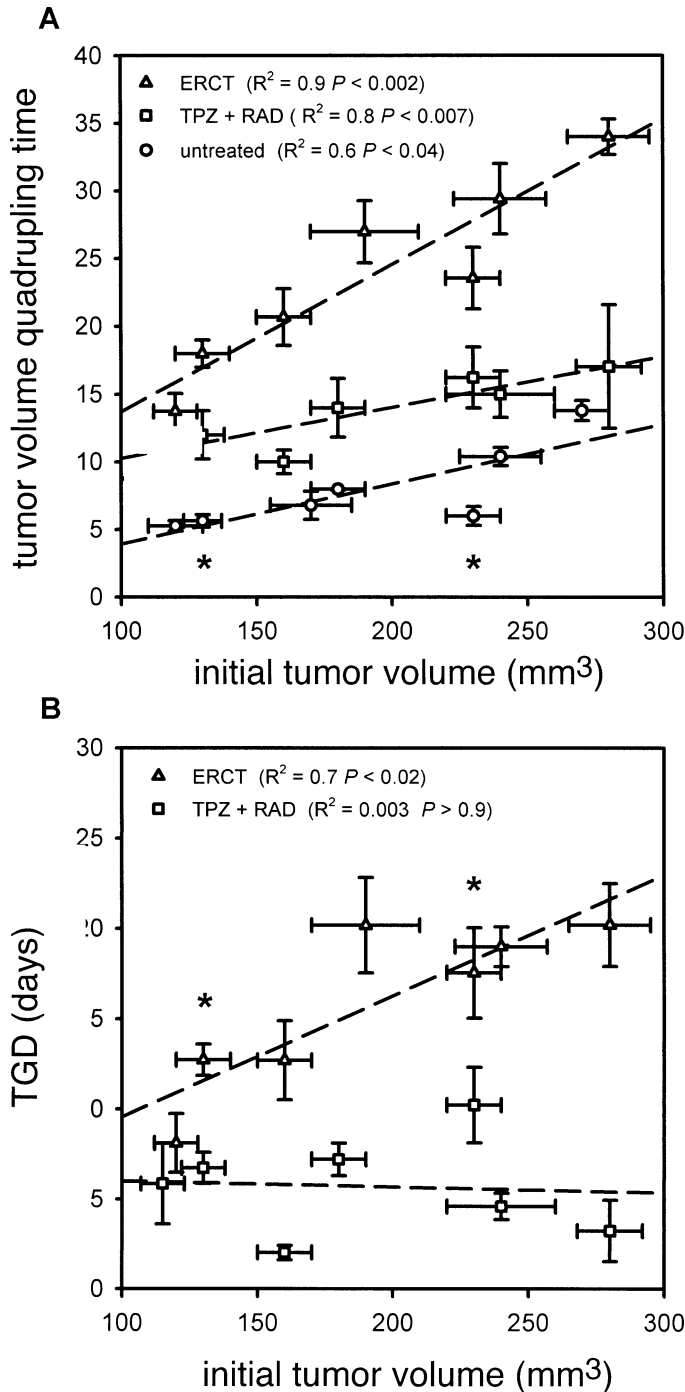
poration + radiation was  $3.0 \pm 0.8$  days. The TGD for the electroradiochemotherapy group was  $13.0 \pm 1.0$ , which was significantly different ( $P < 0.002$ ) from the TGDs of the TPZ + electroporation and TPZ + radiation groups. It should be noted that the TGDs of the tumors treated with electroporation + radiation, TPZ + electroporation and TPZ were similar for both small and large tumors.

#### *Relationship between Initial Tumor Volume and Therapeutic Response*

The effects of treatments with electroradiochemotherapy and TPZ + radiation were compared over a wide range of initial tumor volumes with proper control for all other biological and physical parameters. The results of these studies are summarized in Fig. 3A. The tumor volume quadrupling time of the untreated tumors and those treated with electroradiochemotherapy and TPZ + radiation is shown as a function of the tumor volume at the time of the treatment. The tumor volume quadrupling time–tumor volume relationship was linear for all tumors over the tumor volume range studied (data points were fitted by linear regression). However, the magnitude of this effect differed among the groups. While the lines of the untreated controls and the tumors treated with TPZ + radiation had similar slopes (slope =  $0.041 \pm 0.014$ ,  $R^2 = 0.6$ ,  $P < 0.04$  and slope =  $0.039 \pm 0.009$ ,  $R^2 = 0.8$ ,  $P < 0.007$ , respectively), the slope of the line for the tumors treated with electroradiochemotherapy was steeper (slope =  $0.108 \pm 0.019$ ,  $R^2 = 0.9$ ,  $P < 0.002$ ) [the slopes of the curves for electroradiochemotherapy and TPZ + radiation are significantly different at  $P < 0.05$  (two-tailed test), while those for TPZ + radiation and untreated controls are not ( $P > 0.5$ , two-tailed)]. This is reflected in the tumor growth delays of tumors treated with electroradiochemotherapy, which correlated linearly with the tumor volume at the time of treatment, in contrast to the tumor growth delay of the tumors treated with TPZ + radiation, which was not dependent upon the tumor volume at treatment (Fig. 3B). Figure 3 also reveals that for large tumors ( $280 \text{ mm}^3$ ), electroradiochemotherapy was sixfold more effective than TPZ + radiation in terms of TGD. The enhancement of electroradiochemotherapy over TPZ + radiation decreased with decreasing initial tumor volume. We did not observe any correlation between tumor volume quadrupling time and initial tumor volumes for the tumors treated with TPZ, TPZ + electroporation, radiation and electroporation + radiation (Fig. 2).

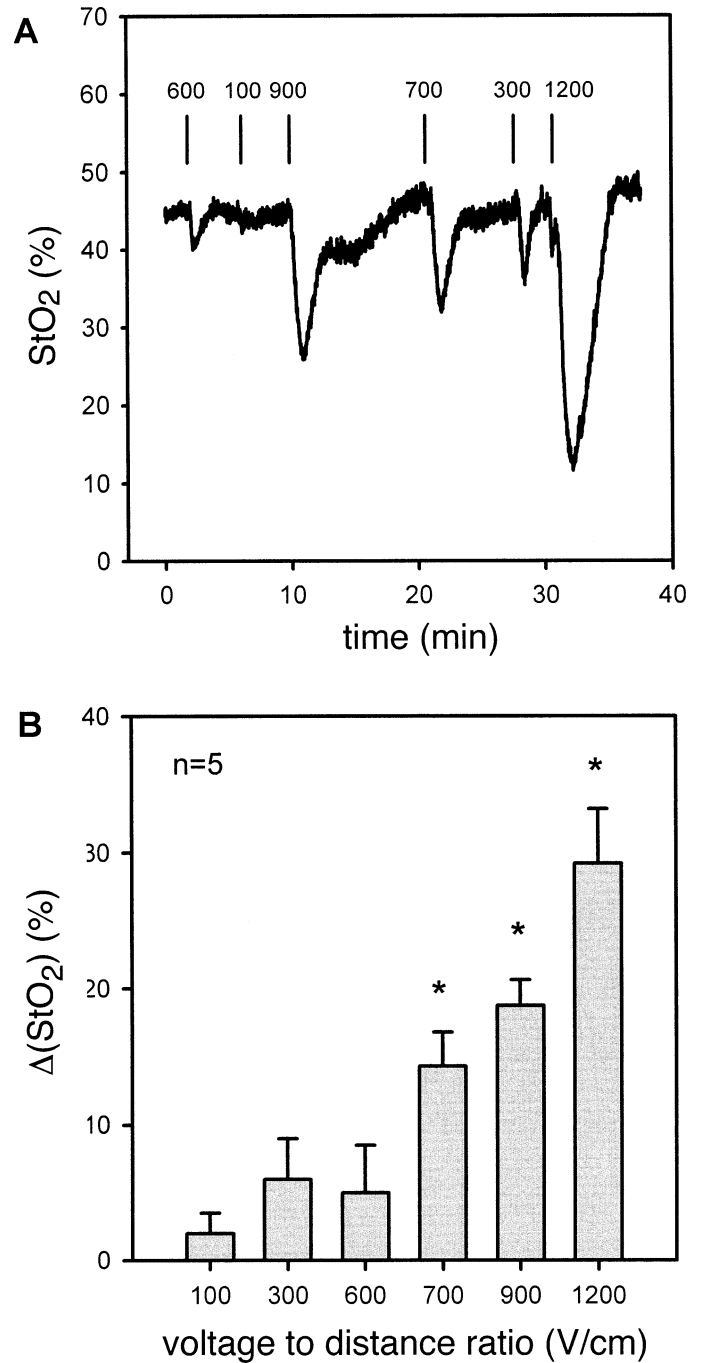
#### *Tumor Tissue Oxygenation*

Figure 4A shows a typical oximeter reading obtained before, during and after electric-pulse treatment. Pulses of different field strengths ranging from 100 V/cm to 1200 V/cm were applied to individual mouse tumors. The pulse sequence was chosen randomly to avoid possible artifacts that may be caused by sequential pulse delivery. The mean  $\text{O}_2$



**FIG. 3.** Effect of tumor volume at the time of the treatment on tumor growth. Panel A: Effect of the tumor volume at the time of the treatment on the tumor volume quadrupling time for the untreated, tumors and those treated with TPZ + radiation and electroradiochemotherapy. Linear regressions are shown as dotted lines. Panel B: TGD of the electroradiochemotherapy-treated tumors as a function of initial tumor volume. Data are expressed as means  $\pm$  SEM ( $n = 8$ ). The asterisks indicate the data sets shown in Fig. 2. RAD, radiation; ERCT, electroradiochemotherapy.

saturation of all tumors examined ( $n = 5$ ) was  $45 \pm 8\%$  (Fig. 4A). Application of 100 V/cm to the tumors had a negligible effect on oxygenation. In contrast, pulses of 300 V/cm induced a decrease in oxygen saturation to 35%. The



**FIG. 4.** Panel A: Real-time tumor oximeter reading before, during and after eight electric pulses of different magnitudes. Tick marks indicate the times of electric-pulse delivery. The magnitude of the voltage to distance ratio is given in V/cm. The pulse duration was 100  $\mu$ s with a repetition frequency of 1 Hz. In contrast to the other studies, the eight pulses were applied in the same direction for methodological reasons (see the Materials and Methods). Panel B: Summary of the oximetry studies. Each experimental group consisted of five mice. Data are expressed as means  $\pm$  SEM. \*Indicates significant difference between the groups at  $P < 0.05$ .

drop in oxygenation increased with increased field strength. Oxygen saturation reached a minimum of 12% at 1200 V/cm. Tumor oxygen saturation was restored after approximately 3 min for tumors treated with 300 V/cm and 5–7

min for tumors treated with 700 V/cm. Tumors treated with 1200 V/cm recovered to pretreatment oxygen saturation levels after approximately 10 min. Figure 4B summarizes the results of our oximetry studies and shows the mean change in tumor oxygenation (five mice per field strength) as a function of the applied field strength. Although there was not a significant change in oxygenation for the 300 V/cm and 600 V/cm pulses ( $P > 0.1$ ), the application of pulses of higher strength led to significant differences in  $O_2$  saturation. The observed drop in tumor oxygenation after electric-pulse delivery was specific to the local application of the pulses to the tumor. In control animals in which the pulses were applied to sites other than tumor (e.g. skin on the back and on the limbs), tumor oxygenation was unaffected (data not shown).

#### Computer Simulation of the Electric-Field Distribution

The electric-field distribution generated by the plate electrodes in the tumor model is shown in Fig. 5A as a wire mesh and volume rendering. The highest electric field was achieved at the interface of the electrodes and the skin (not shown for clarity; see below). The electric-field strength decreased with increased distance from the electrodes and reached a minimum at the center of the tumor. Figure 5B shows the profile of the electric-field strength along the y axis of the model. Since the ratio of the relative permittivities between the tumor tissue and dermis was 10:1, a 10-fold higher electric field was observed in the dermis compared to the periphery of the tumor. The electric field decreased toward the center of the tumor and reached a minimum of approximately 350 V/cm near the center of the tumor.

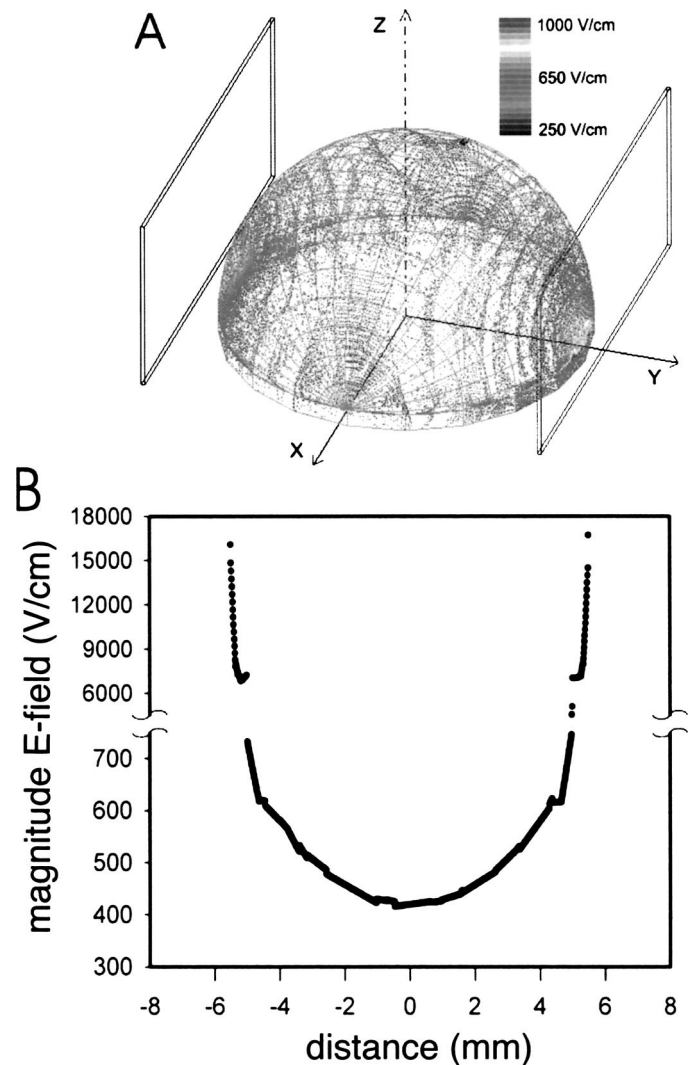
## DISCUSSION

#### Effect of the Treatment Schedule

To maximize the efficacy of electroradiochemotherapy, the sequence and timing of administration of the different components of the therapy must be optimized. We observed that the best tumor response was achieved when the electric pulses were administered 5 min after drug injection and 30 min prior to irradiation. Cemazar *et al.* reported an optimal interval of 20 min between TPZ administration and electric-pulse delivery (23). Pulses delivered 3 min after drug injection led to only a minimal prolongation in TGD that did not differ significantly from results for treatment with TPZ alone. The difference between our results and those of Cemazar *et al.* may be related to the use of different animal and tumor models as well as by the use of radiation in our studies.

#### Electroradiochemotherapy in Comparison to the Combined Treatment with TPZ + Radiation

Our results demonstrated that electroradiochemotherapy enhanced the efficacy of combined treatment with TPZ and

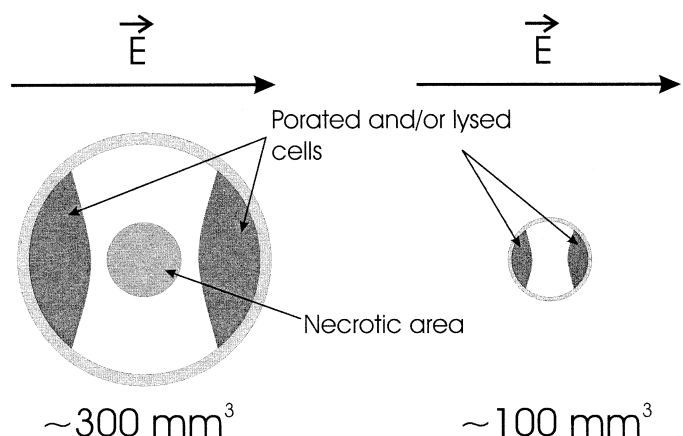


**FIG. 5.** Calculations of the electric-field distribution in a tumor model using the finite element method. Panel A: Electric-field distribution in the 3D model of a tumor. The values of the magnitude of the electric field are given in V/cm. Panel B: Magnitude of the electric field along a line situated in the bottom plane of the model through center of the tumor.

radiotherapy. It is unlikely that the electric pulses enhanced the effects of radiation directly, since the acute effects of electroporation on tumor oxygenation were short-lived and probably were no longer present at the time of irradiation. Furthermore, our oximetry studies showed that a recovery to pretreatment oxygenation levels occurred within 10 min (Fig. 4). In contrast, irradiation of the tumors shortly after electroporation resulted in less TGD. It is therefore more likely that electroporation increased the cytotoxicity of TPZ by inducing hypoxia secondary to a short-lived reduction in tumor oxygenation. Subsequent irradiation of the reoxygenated tumor led to further enhancement of the treatment as reported previously (24).

#### Dependence of Treatment Outcome on Tumor Volume

Electroradiochemotherapy was more effective in large tumors than small tumors (Fig. 1), and there was a linear



**FIG. 6.** Schematics of a large ( $300 \text{ mm}^3$ ) and small ( $100 \text{ mm}^3$ ) tumor exposed to an electric field. In contrast to small tumors, large tumors can have extensive areas of necrosis. The proportion of cells affected by the electric field is similar in small and large tumors (dark gray-shaded areas). However, taking necrosis into account, cell killing is proportionally higher in large tumors compared to small ones. This leads to longer tumor volume quadrupling times for large tumors.

correlation between the TGD and the tumor volume at the time of treatment (Fig. 3B), suggesting that the dependence of cell killing on tumor volume was specific to electroradiochemotherapy.

Given that the action of TPZ was dependent on tumor hypoxia, one might expect the volume dependence of the effect of electroradiochemotherapy to be explainable by differences in tumor hypoxia between tumors of different sizes. However, this is unlikely since tumor hypoxia is known not to depend on the tumor size and grade and the extent of necrosis (31).

It is possible that the electric pulses induce a higher level of hypoxia in large tumors compared to small ones. This could result in increased cytotoxicity of TPZ in large tumors compared to small tumors. While our oximetry studies indicated that the electric pulses could transiently reduce the tumor oxygen status, the volume dependence of this effect was not investigated.

It is also possible that this volume effect may be related to morphological differences between large and small tumors. While small tumors are composed largely of viable cells, large tumors can have extensive areas of necrosis, often located near the center. Calculation of the electric-field distribution in tumors (using a finite element method) demonstrated that the electric field was not uniform throughout the tumor but was highest around the electrodes and decreased toward the center (Fig. 5). It is therefore likely that viable tumor cells near the periphery of both large and small tumors were affected by electric pulses to a greater degree than those near the center. If the necrotic subpopulation in large tumors is added to cell killing as a consequence of the electric pulses, a greater proportion of viable cells would be affected in large tumors than in small tumors (Fig. 6). As a consequence, the TGD should be longer for larger tumors than for small ones.

A similar volume dependence has been reported for hyperthermia, for treatment with flavone acetic acid (32, 33), and more recently for the vascular targeting agent combrestatin A-4 phosphate (34). The tumor volume dependence of electroradiochemotherapy may have important implications for conventional therapies such as chemotherapy and radiotherapy, which are generally less effective on large solid tumors than small ones (35, 36). For example, if the effects seen with the mouse model can be successfully translated to humans, then electroradiochemotherapy may be useful as a method to sensitize tumors to radiation and chemotherapy in patients with larger tumors in which chemotherapy and radiotherapy tend to work less well. In this case, it would be necessary to identify an optimum interval between pulse application and irradiation to ensure that the transient changes in blood flow due to electroporation have passed at the time of irradiation. This is especially important, since in other studies the transient reduction in blood flow was reported to last significantly longer than in our studies (see below).

#### *Effects of Electric Pulses on Tumor Oxygenation*

Our real-time oxygenation measurements showed an immediate drop in tumor oxygenation after the electric pulses and a recovery to pretreatment levels within 10–12 min. Since the measurements were performed non-invasively using a surface probe situated 10 mm above the tumor,  $\text{O}_2$  saturation was also measured in the skin that surrounded the tumor. However, the thin layer of the dermis did not prohibit the collection of data from the underlying tumor since the measurements were localized to a region several millimeters in depth beneath the monitoring site. Therefore, the collected oximetry data are composed of contributions from the dermis (surrounding the tumor) and from the tumor tissue.

The electric-pulse protocol used for the oximetry studies was modified from the protocols used for the other studies. Here, the eight electric pulses were delivered in the same direction compared to all other experiments, in which the two sets of pulses were administered with a perpendicular offset. Since we observed that the application of four pulses in one direction caused an identical change in oxygenation as another set of four pulses administered in the perpendicular direction, we decided to apply all pulses in one direction. This had the advantage of eliminating pressure artifacts caused by repositioning of the electrodes.

The observed change in tumor oxygenation was specific to the application of the electric pulses to the tumor. No change in tumor oxygenation was observed when the pulses were applied on other sites.

Although Sersa *et al.* (20) reported effects of electric pulses on tumor blood flow that lasted several hours, our data suggested that in the SCCVII tumor model the effect of electric pulses on perfusion was short-lived. This discrepancy between the two studies may be due to differences

in the tumor model, measurement technique, or electric pulse strength. Based on reports from the literature, vasoconstriction after electric pulses probably resulted from either myogenic spasm of the arteriole vessel walls (37) or reflexory vasoconstriction of the afferent arterioles (22).

#### Computer Simulations of the Electric-Field Distribution

Our computer simulations showed that the electric-field strength was not homogeneous across the tumor, and that it decreased with increased distance from the electrodes. A minimum was reached at the center of the tumor. The simulations also showed a tenfold higher electric field in the surrounding dermis at the interface to the tumor. This is a consequence of the highly resistive stratum corneum. This aspect is rarely discussed and must be taken into account in studies of the use of plate electrodes.

In a static electric field, when two dielectrics such as tumor and the dermis are in contact with no free charges at the interface, one obtains

$$E_{Tumor} = \frac{\epsilon_{Dermis}}{\epsilon_{Tumor}} E_{Dermis},$$

where  $\epsilon_{Dermis}$  and  $\epsilon_{Tumor}$  are the relative permittivities of the dermis and of the tumor, respectively. A ratio of 1:10 between the relative permittivities of the dermis and the tumor resulted in a tenfold higher electric field in the dermis, as calculated in our computer simulations. In reality, however, the influence of the skin resistance is not that strong. The skin will become electroporated during the treatment and the permittivity will increase markedly during the electric-pulse treatment. Engstrom *et al.* showed that the increase in permittivity depended on the duration of pulses (unpublished results).

A more realistic model of electroporation *in vivo* must take temporal effects into account, especially when pulses of long durations are applied. The delivery of multiple pulses of long durations (e.g. 100 ms–10 s) to the tissue will lead to an increase in temperature, which would be expected to change the resistance of the tissue. A change in tissue resistance will affect the currents generated by the following pulses. This temporal change in the electrical properties was not taken into account in the simulations. The calculations were done assuming a static electric field across the tumor. To obtain more precise results, electrical parameters like conductivity, resistance and relative permittivity must be treated as time-dependent variables. Furthermore, the calculations assume a homogeneous model that does not take into account the complex morphology of real tumors.

Despite the simplicity of our model, the calculations nevertheless showed a first-order approximation of the electric-field distribution in tissue.

In summary, electric-pulse treatment combined with radiochemotherapy proved to be an effective treatment for superficial tumors in a mouse model. The enhanced effec-

tiveness of electroradiochemotherapy appeared to result from an acute reduction in tumor blood flow with an associated reduction in tumor oxygenation and enhancement of the cytotoxicity of TPZ in combination with radiation. To verify more fully the mechanism of action of electroradiochemotherapy, future studies will be designed to examine the effect of electric pulses on (1) the intratumor concentration of TPZ before, during and after treatment and (2) the oxygen saturation between small and large tumors during treatment.

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