Alterations in the mortality and growth cycle of cervical cancer cells treated with electroporation at different electric strengths

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Summary

Objective: To explore the biological effects of electric fields of various strengths on Hela cells. *Methods:* Electroporation experiments were performed using Hela cells. Changes in cell mortality, cell vitality, cell cycle, and apoptosis status were examined. In addition, temperature changes in the surrounding tissue were measured. *Results:* Cell proliferation was markedly inhibited after treatment with field strengths of 2-2.5 kV/cm. The expression of caspase-3 increased significantly in cells treated with field strengths of 1.5-2.5 kV/cm. Field strengths of 1.75-2.5 kV/cm produced complete cancer cell ablation. G2 phase frequency increased significantly after treatment with field strengths of 2-2.5 kV/cm. During this process, the maximum temperature increase in the pulsed electric field was 4.9 ± 1.17°C under free air convection. *Conclusions:* IRE can be used alone for the treatment of cancer, and its thermal effect is negligible. Cell death was caused by the effects of IRE and apoptosis. The tumor cells must be destroyed completely, or the altered cell cycle may lead to tumor recurrence and accelerated growth.

Key words: Pulsed electric field; Cervical cancer cells; Cell cycle; Apoptosis.

Introduction

The development of techniques based on pulsed electric fields, such as electrochemotherapy, electrogenetherapy, and the newly developed irreversible electroporation (IRE) has been rapid. These techniques are all based on the phenomenon of membrane electroporation. The term electroporation describes an electromagnetic biological phenomenon of increased cell membrane permeability after a pulsed electric field is applied to the cells. The increased membrane permeability is caused by altered transmembrane potentials and the formation of nanochannels in the bilayer lipid membranes [1-3]. Depending on whether the permeability of the cell membranes can return to the normal physiological state, electroporation is divided into reversible electroporation (RE) and irreversible electroporation (IRE), which is also known as irreversible electrical breakdown (IREB) [4-6].

RE allows chemotherapeutic drugs (such as bleomycin and cisplatinum) and macromolecules, which do not normally cross the lipid bilayer membrane, to enter the cells freely. Using RE, chemotherapeutic drugs are injected into tumor cells in a pulsed electric field; this injection is typically performed with the following classical parameters: field strength, 1000 V/cm; pulse width, 100 s; and number of pulses (p), 8. These drugs then enter the tumor cells and exert a strong local anti-cancer effect. The use of RE can reduce the systemic drug dose required, selectively increase local tumor cell toxicity, and avoid the adverse effects caused by systemic chemotherapy [7-11]. Gene therapy is an alternative method for cancer treatment, and a wide variety of vectors, such as adenovirus and liposomes, are used in gene transfer. However, these vectors are not satisfactory because of their biologically unsafe nature, high immunogenicity, and lack of stability. In contrast, when used as a vector for gene transfer, the pulsed electric field is free of these disadvantages.

IRE has been studied extensively *in vitro*. IRE can effectively kill microorganisms and has been used for water sterilization [12]. Recently, IRE was applied to rat liver and was found to completely ablate the target regions without causing thermal damage to the surrounding normal tissue. The ablation area had a clear border and the large blood vessels and bile ducts of the target area were intact [13]. Rubinsky used IRE as a new and minimally invasive surgical technique to ablate a volume of tissue in large animals and then observed the long-term effects. Because of the presence of intact and functional large blood vessels in the ablation area, the recovery of the target region was extremely rapid. The integrity of the blood vessels and the connective tissue indicated that IRE affected only the cell membranes. The area for ablation can be predetermined by the Laplace equation [14]. These results suggest that the use of IRE as a minimally invasive technique in cancer treatment is promising, especially for tumors that are located near large blood vessels, where surgery is difficult.

The karyoplasmic ratio is large in tumor cells, and the specific inductive capacity of cancer cells is larger than that of normal cells [15]. These characteristics make it possible for pulsed electric fields to selectively kill cancer cells rather than normal cells. This selectivity provides an advantage over other thermal ablation methods for the treatment of cancer. However, the cell death pathway that is activated by pulsed electric fields is still unclear. Both necrosis and apoptosis may occur in the cells in the target

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Fig. 1

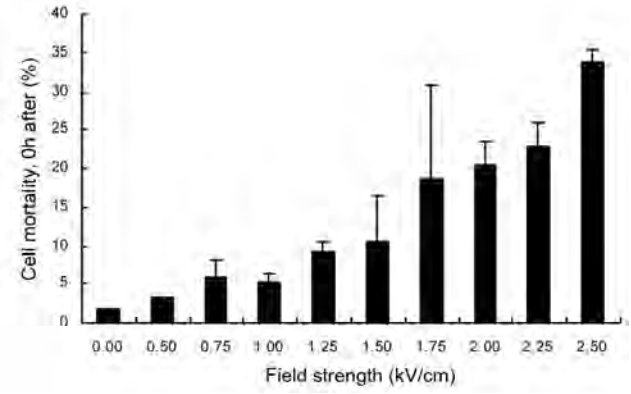

Fig. 2

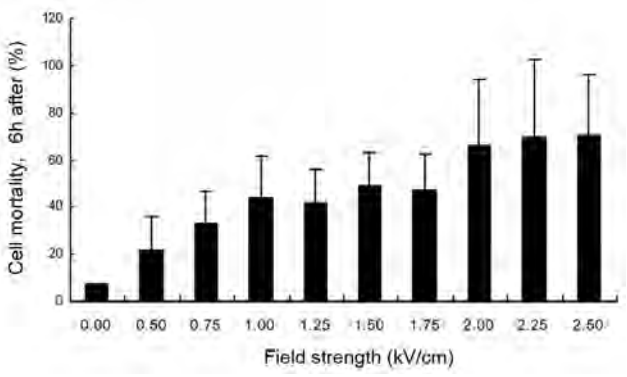

Fig. 3

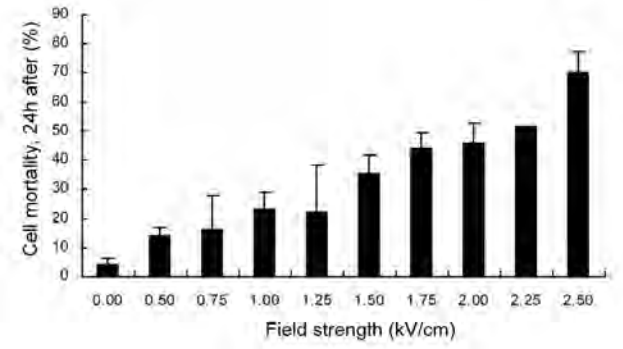

Figure 1. — Cell mortality as determined by trypan blue staining 0 h after the application of field strengths ranging from 0–2.5 kV/cm.

Figure 2. — Cell mortality as determined by trypan blue staining 6 h after the application of field strengths ranging from 0–2.5 kV/cm.

Figure 3. — Cell mortality as determined by trypan blue staining 24 h after the application of field strengths ranging from 0–2.5 kV/cm.

area, and a combination of the morphological features of the two forms of cell death has been observed. The strong electric field around the electrode may lead to coagulative necrosis, while apoptosis may dominate where the electric field is weaker.

There are many unanswered questions regarding pulsed electric fields. Can it activate apoptosis or will it cause a mild injury that will be repairable and will allow cell survival? If the cells do survive, do they display changes in their cell cycle and growth state? What is the field intensity that will cause ablation of the tumor cells with a minimal thermal effect? To answer these questions, we used pulses with a fixed pulse length and frequency, made gradual changes to the electric field strength, and simulated the attenuation of the electric field within the tissue. We also recorded the responses of Hela cells to different field strengths.

Materials and Methods

Cell culture

Hela cell lines were provided by the Ultrasonic Research Institute of Chongqing Medical University. The cells were cultured in RPMI 1640 medium (HyClone, Logan, UT, USA) with 10% neonatal calf serum (Sijiqing, Hangzhou, China) at 37°C under 5% CO_2. Cells in the logarithmic phase of growth were digested with 0.25% trypsin (HyClone), and the digestion was terminated by addition of serum. The cells were then placed into 10-ml centrifuge tubes and centrifuged for 5 min at 1000 rpm, and the supernatant was discarded. The cells were washed three times with phosphate-buffered saline (PBS, HyClone) and resuspended at a concentration of 1×10^6 cells/ml.

Electroporation

The pulse width was 100 s and the frequency was 1 Hz, with eight pulses in a set. On the basis of the field-strength gradient, the cell suspension was divided into a control group that did not receive any electrical field treatment and 0.5-, 0.75-, 1-, 1.25-, 1.5-, 1.75-, 2-, 2.25-, and 2.5-kV/cm groups. Each group was treated with one set of pulses (8 pulses in total). The 1.5-2.25-kV/cm groups were treated with three more sets of pulses, and the gap between each pulse was maintained at 1 min in order to eliminate thermal effects. A total of 760 µl of cell suspension from each group was added to the electrode cuvette (Bio-Rad Inc., Hercules, CA, USA; gap distance of the electrode cuvette, 4 mm), and the pulse generator (Department of Education of High Voltage and Electrical New Technology, Chongqing University, China) was connected to the experimental apparatus. Real-time wave formation was recorded by an oscilloscope (Tektronix TDS3032B; Beaverton, OR, USA). The differences between the set voltage, the pulse width, and the recordings on the oscilloscope were less than 10%. The temperature was measured immediately after the pulse by using a thermocouple device (Model: JK-8; Changzhou Jin Ai Lian Electronic Technology, China; test accuracy: 0.1°C). The theoretical temperature rise of the differential electric field was calculated using the formula $\Delta T = \frac{E^2 \times t}{r \times \rho \times {}^{c}p}$,

where r indicates the impedance of the solution, ρ indicates the density of the PBS solution, and ^{c}p indicates the specific heat of the PBS solution (Table 1).

Table 1. — *Theoretical temperature increase and actual temperature increase under different electrical parameters*.*

Field strength (kV/cm)	Theoretical temperature increase (°C)	Actual temperature increase (°C)
0.5	0.68	0.16 (0.15)
0.75	1.53	0.30 (0.26)
1	2.72	0.56 (0.37)
1.25	4.25	0.90 (0.43)
1.5	6.12	1.50 (0.87)
1.75	8.33	2.13 (0.68)
2	10.88	3.10 (1.65)
2.25	13.77	4.43 (2.0)
2.5	17	4.90 (1.17)

* Values are presented as means (SD).

Trypan blue staining

At 0 h after treatment with the pulsed electric field, 100 µl of the cell suspension was added to 0.4% trypan blue solution and allowed to stain for 5 min. The blue cells and the total number of cells were counted with a hemacytometer (Shanghai Precision Instrument Co., Ltd., Shanghai, China). The mortality rate was calculated as follows: (the total number of cells in the control group – the number of viable cells in the experimental group)/the total number of cells in the control group × 100%. Next, 100 µl of the cell suspension was cultured in a 24-well plate, and the supernatant was then separately collected after six and 24 hours. Adherent cells were digested with 0.25% trypsin, and digestion was terminated by addition of serum. After centrifugation at 1000 rpm for 5 min, the cell pellet was resuspended with 100 µl of PBS. The same amount of 0.4% trypan blue solution was added, and the cells were counted with the method specified above.

Giemsa staining

Cells treated with different electric field parameters were suspended in 10 µl of the medium, transferred to a 6-well plate (10 × 10 mm), and then cultured with coverslips at 37°C under 5% CO_2. The plate was gently rotated to uniformly disperse the cells. After the surviving cells adhered to the coverslips, the coverslips were washed three times with PBS, and the cells were fixed with 4% paraformaldehyde for 10 min. The coverslips were allowed to air dry. Staining with 100 µl of Giemsa (1:50) was performed for 20 min. The coverslips were rinsed three times with water and allowed to air dry.

MTT assay

A total of 100 µl of cells from each group were treated with pulsed electric fields, and then, 300 µl of the culture medium was added to 96-well plates for 24 h for culture. Next, 20 µl of MTT solution was added, and the cells were allowed to grow for an additional 4 h. The supernatant was then removed, 150 µl of dimethyl sulfoxide was added to each well, and the plates were oscillated for 10 min. An enzyme-linked immunoassay (ELISA) was used to measure the absorption value at a wavelength of 490 nm.

Immunohistochemistry

For immunohistochemical analysis, 10 µl of the treated cell suspension from each group was cultured on a coverslip (10 × 10 mm) in 30 µl of medium in 6-well plates, which were then rotated gently to uniformly disperse the cells. The cells were cultured at 37°C under 5% CO_2 for 6 h. The presence of caspase-3 was tested by SP immunohistochemistry, and the working dilution of both the primary (rabbit anti-human polyclonal antibody; Bio-Rad Inc.) and secondary antibodies (species anti-rabbit; Bio-Rad, Inc.) was 1:200.

Flow cytometry

After treatment with pulsed electric fields for 6 h, 500 µl (1×10^5 cells) of cells from each group were stained with annexin V-FITC and propidium iodide. After complete mixing, the solution was allowed to react for 15 min at room temperature in the dark. The cells were tested by flow cytometry, and FACScan CellQuest was used for analysis.

Statistical analysis

The SPSS 10.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to perform single-factor analysis of variance. The results were expressed as mean (SD). If there were differences, the LSD *t* test for homogeneous variances or the Games-Howell test for non-homogeneous variances was used to compare differences between the groups. The level of significance was $p < 0.05$.

Results

Trypan blue cell count 0 h after treatment with pulsed electric field

After 0 h, the trypan blue cell count showed no significant differences in the cell mortality between the control group and the 0.5-1.5-kV/cm groups, while there were significant differences between the cell mortality in the control group and the 1.75-2.5-kV/cm groups ($p < 0.01$). The result for the 1.75-kV/cm group was different from those for the 0-1-kV/cm groups, but not from those for the 1.25-2.25-kV/cm groups. The result for the 1.75-kV/cm group was also different from that for the 2.5-kV/cm group. The result for the 2.5-kV/cm group showed differences when compared with those for the 0-1.75-kV/cm groups ($p < 0.01$) and the 2-2.25-kV/cm groups ($p < 0.05$; Figure 1).

Trypan blue cell count 6 h after treatment with pulsed electric field

Six hours after treatment with the previously mentioned electrical parameters, the trypan blue cell count showed significant differences between the cell mortality in the 2-kV/cm group and the 0-1.75-kV/cm groups ($p < 0.05$). In addition, significant differences were found among the 2.25-kV/cm group, the 2.5-kV/cm group, and the 0-1.75-kV/cm groups ($p < 0.01$). No differences were found among the 0-1.75-kV/cm groups.

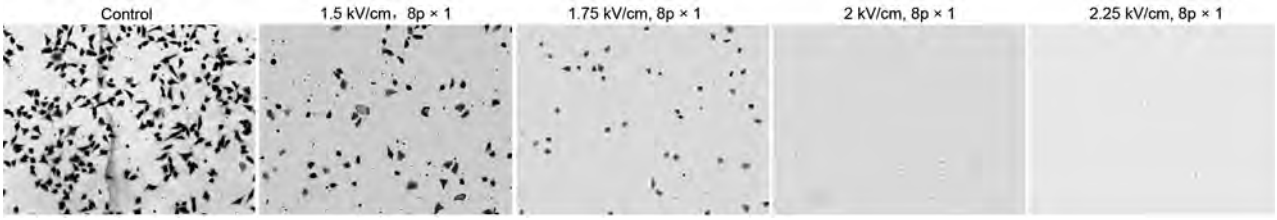

Figure 4. — Giemsa staining of 6-h creep plates that showing that the number of surviving cells decreased significantly as the field strength increased.

Trypan blue cell count 24 h after treatment with pulsed electric field

Twenty-four hours after treatment with the previously mentioned electrical parameters, the trypan blue cell count showed no significant differences among the control group and the 0.5-0.75-kV/cm groups ($p > 0.05$). Significant differences were found among the control group and the 1-1.25-kV/cm groups ($p < 0.05$) and among the control group and the 1.5-2.5-kV/cm groups ($p < 0.01$). These results suggest that as the exposure time increased, the cells treated with a field strength greater than 1 kV/cm and less than 1.75 kV/cm showed a relatively slow process of death. Cells treated with a field strength of 2-2.5 kV/cm showed a rapid process of death.

Giemsa staining

After 6 h of treatment with an electric field, the surviving cells (Hela cells attached at 6 h) were fixed *in situ* by Giemsa staining. At this time, pyknosis, which is characteristic of apoptotic cells, could be observed, and completely dead and non-adherent cells were lost in the process of staining.

Cell viability testing with MTT

MTT value under different field strengths (8 p × 1 set)

There were significant differences among the control group and the other groups (Figure 10, $p < 0.05$ and $p < 0.01$). Significant differences were also found among the values for the 0.5-1.75-kV/cm groups. A significant difference was found between the values for the 2-kV/cm group and the 2.25-kV/cm group ($p < 0.05$).

MTT value under the action of field strength (1.5-2.25 kV/cm, 8 p × 3 sets)

Significant differences were found among the MTT values for the 1.5-kV/cm 8 $p \times 2$ group, the 1.75-, 2-, and 2.25-kV/cm 8 $p \times 2$ groups, and the 1.5-kV/cm 8 $p \times 3$ group ($p < 0.01$). Cells were completely ablated under the 1.75-2.25-kV/cm 8 $p \times 3$ conditions, and there were no viable cells. Cells were not completely ablated under the 1.5-kV/cm 8 $p \times 3$ conditions, and no differences were found compared to the 1.75-2.25-kV/cm 8 $p \times 2$ conditions ($p > 0.05$).

Detection of apoptosis and changes in the cell cycle by flow cytometry after electric field treatment

Detection of apoptosis

Significant differences in apoptosis were found among the control group and the 2- and 2.25-kV/cm groups and between the 1.25-kV/cm group and the 2.25-kV/cm group ($p < 0.05$). Significant differences in apoptosis were found among the control group, the 0.5-1.75-kV/cm groups, and the 2.5-kV/cm group. No significant differences in apoptosis were found among the control group and the 0.5-1.75-kV/cm groups. When the field strength was greater than 2 kV/cm, apoptosis increased significantly (Figure 7).

Changes in the cell cycle

Flow cytometry analysis showed that the number of cells (6 h under electric field) in the G2 phase increased significantly when the field strength was 2-2.5 kV/cm (compared with the number of cells in the 0-2-kV/cm group, $p < 0.01$; compared with the number of cells in the 2.25-kV/cm group, $p < 0.05$). No significant differences were found between the 2.25-kV/cm group and the 1.25-kV/cm, 1.5-kV/cm, and 2-kV/cm groups with $p > 0.05$. However, significant differences were found between the values for the 2.25-kV/cm group and the 0.75-kV/cm, 1-kV/cm, and 1.75-kV/cm groups with $p < 0.05$. When the values for the 2.25-kV/cm group were compared with those for the control group and the 0.5-kV/cm group, significant differences were found with $p < 0.01$ while no significant differences were found among the values for the 0-2-kV/cm groups. No differences in the number of G1 phase and S phase cells were found among the groups (Figures 8 and 9).

Detection of caspase-3 expression by immunohistochemical analysis of Hela cells after electric field treatment

SP immunohistochemistry showed significantly increased caspase-3 protein expression with the 1.5-2.25-kV/cm 8 $p \times 1$ pulsed electric fields when compared with the expression in the control group. Brown-yellow immunohistochemical particles were located in the cytoplasm and the nucleus (Figure 8).

Fig. 5

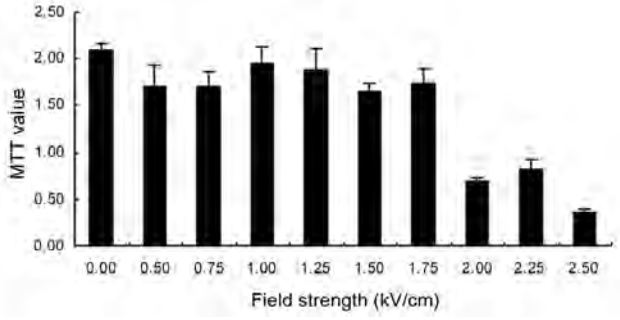

Fig. 6

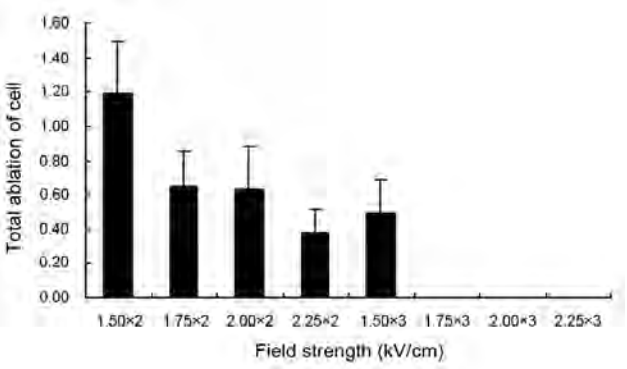

Fig. 7

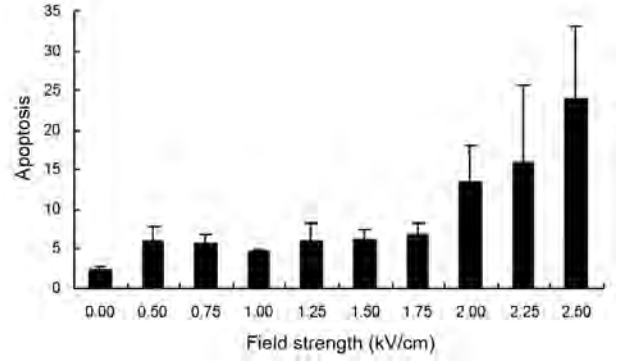

Fig. 8

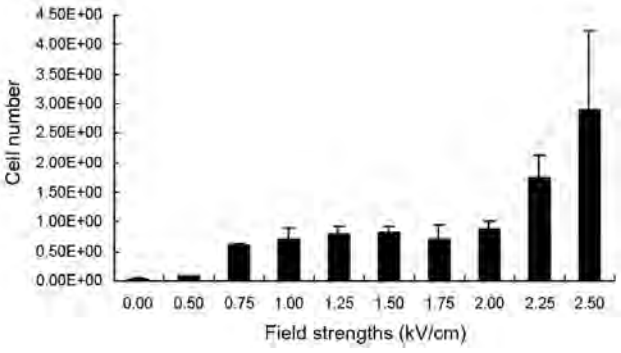

Figure 5. — MTT assay results showing a significant inhibition of Hela cell activity 24 h after treatment with different field strengths (2-2.5 kV/cm 8 $p \times 1$).
Figure 6. — Total ablation of cells under the electric field strengths of 1.75-2.25 kV/cm 8 $p \times 3$.
Figure 7. — Flow cytometry results that showed increasing apoptosis as the electric field intensity increases.
Figure 8. — The change in number of cells in the G2 phase after electric field treatment as detected by flow cytometry.

Real-time temperature increase in cells under different experimental parameters

The theoretical temperature increase of the differential field strength was calculated according to the previously mentioned formula. The real-time temperature increase was 5°C when the field strength was 2.5 kV/cm. The cell temperature increase was negligible after treatment with 0.5-2.5 kV/cm because the temperature was not high enough to damage the cells.

Discussion

With increasing electric field strength, RE shifted to IRE, and the increasing energy was able to ablate the tumor cells completely. However, the ultimate goal of this study was to determine the field strength that was able to ablate the diseased tissues with the minimum amount of energy so that the patients were able to tolerate the adverse effects. Our research shows that the 1.5-2.5-kV/cm levels of field strength were close to the median lethal dosage, and that an increased number of pulses was able to ablate the tumor cells completely. Thus, field strength plays a relatively important role, but the role of the pulse number is also important. It has been demonstrated that an increased number of pulses with optimal intervals can kill tumor cells without heat superposition. Under the conditions of air convection and proper temperature used in this study, the heat generated by energy dissipation had a negligible impact on the cells, and there was no thermal effect. This is consistent with the findings of a previous study [16].

In this study, we stimulated the cells with gradually increasing electric field strengths in order to observe the changes in the mortality rates of the tumor cells at 0, 6, and 24 h after treatment with the various electric fields. The results showed that 0-6 h after treatment with electric fields of 0.5-1.75 kV/cm, no significant differences in cell mortality were found. However, after the cells were treated for 24 h with an electric field strength of 1 kV/cm, the mortality rate increased significantly. When the field strength reached 2 kV/cm for 24 h, immediate cell death occurred in more than 60% of the population and cell debris was seen, which indicated that the cells showed IRE and disintegrated when the field strength reached a certain level. Thus, there was an obvious dose-effect relationship between cell death and the electric field strength. The process of cell death after treatment with a field strength of 1-1.75 kV/cm was slow and may have involved apoptosis. Although the flow cytometry test showed significantly increased apoptosis at 6 h after treatment with field strengths above 2 kV/cm, slow death continued to occur at 24 h after treatment in some cells. At the same time, the immunohistochemical assay showed

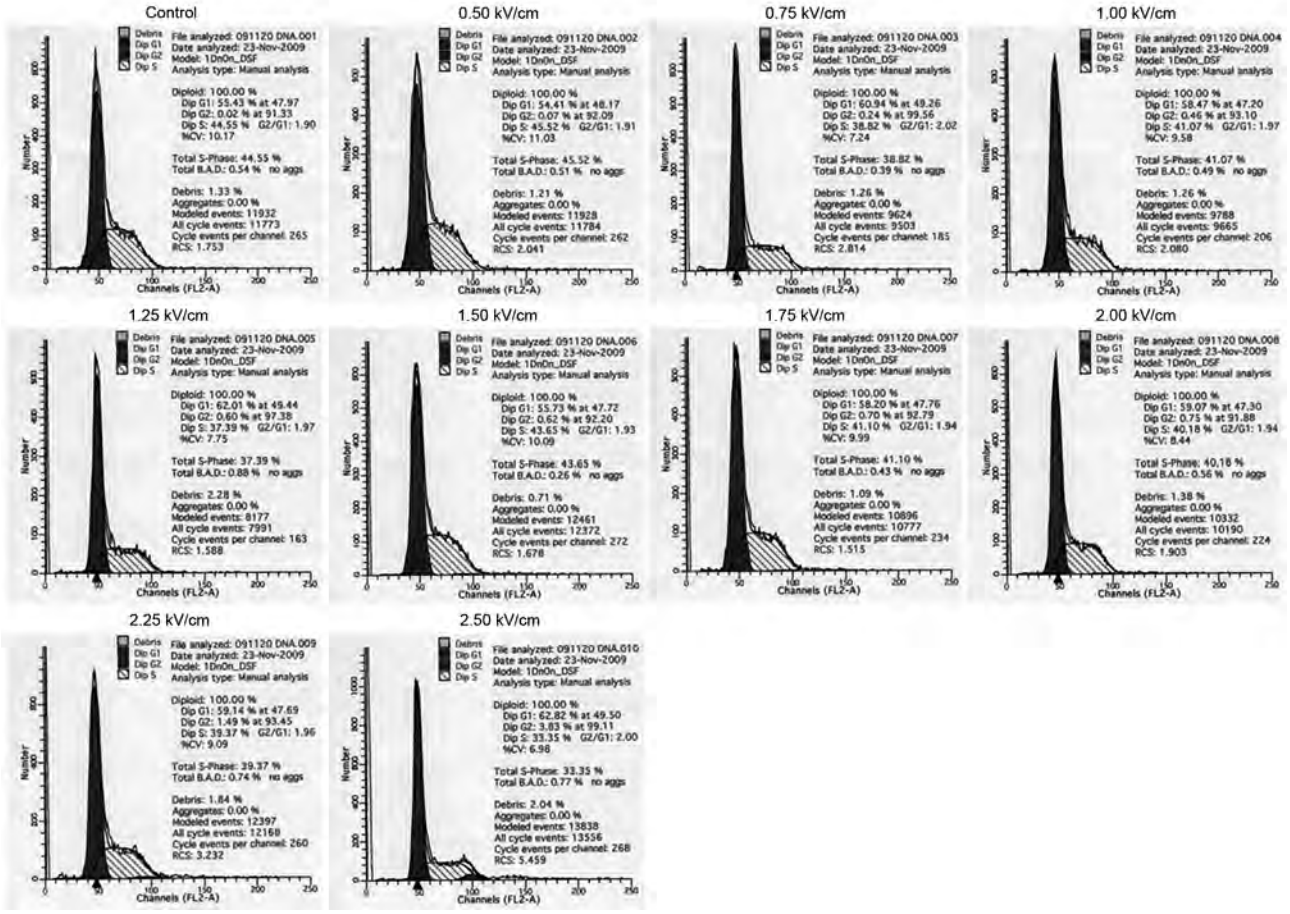

Figure 9. — Cell cycle (G2) changes after electric field treatment as detected by flow cytometry.

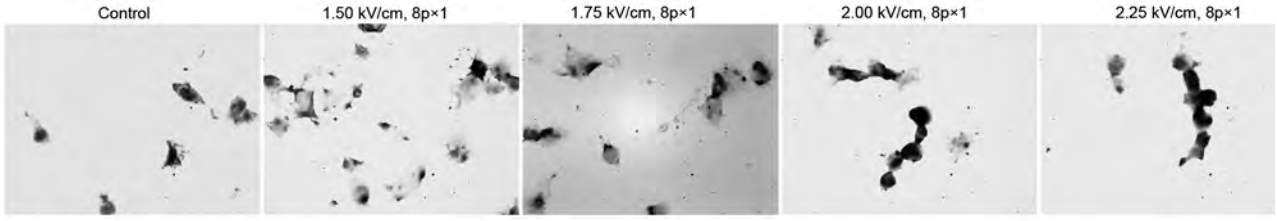

Figure 10. — Caspase-3 immunoreactivity resulting from the SP immunohistochemical method in Hela cells of the control group and other groups treated with different electrical parameters (4 × 100).

that the expression of the apoptotic protein caspase-3 in cells treated with a field strength greater than 1.5 kV/cm was significantly higher than that in the control cells, which was consistent with the results of the flow cytometry test. Therefore, the treatment of cells with a pulsed electric field of a certain intensity can cause cell death and cell apoptosis.

Notably, the 24-h MTT cell viability test showed that at 6 h after treatment with the 1-1.75-kV/cm electric fields, the cell viability detected was still close to that of the untreated control group, although the trypan blue cell count showed a 40% mortality rate. These data show that the complete ablation of tumor cells by the pulsed electric field was particularly important, and these results are in agreement with the results of Miller *et al*. [17]. In order to explore the mechanisms underlying these results, we studied the changes in the cell cycle by flow cytometry after treatment with an electric field and found that the numbers of cells in the G2 phase in the treatment groups were higher than that in the control group. This difference was especially prominent between the control group and the 2.25-kV/cm and the 2.5-kV/cm groups. The unablated cells had a changed cell cycle phase, and the proliferation of these cells may have been accelerated. This observation suggests that in the clinical application of pulsed electric fields for the treatment of cancer, the complete killing of the tumor cells is very important to ensure that any recurrence or accelerated growth of the tumor can be avoided.

How can we optimize the combination of electrical parameters for ablating the tumor? On the basis of the original parameters, we chose field strengths above 1.5 kV/cm, which is close to the median lethal dosage, and the number of pulses was increased to three sets. We found that cervical cancer Hela cells were completely ablated without thermal effects by a field strength of 1.75-2.5 kV/cm, 1 Hz, 8 $p \times 3$. We did not try pulses with the lower field strengths. We anticipate that the results of these experiments can also be used for *in vivo* tumor tissue as long as the geometric properties of the tumor cells do not change. In addition, because the electric field affects the membrane, successful tumor tissue ablation is also closely related to the electrode shape [18, 19].

Conclusion

In summary, IRE may be used alone as a novel and minimally invasive technique for the complete ablation of cancer tissues without a thermal effect. Both the electric field intensity and the number of pulses are important. Coagulation necrosis and apoptosis are involved in the cell death induced by IRE. It is worth noting that the complete destruction of tumor cells is critical when a pulsed electric field is used for tumor ablation, or the change in the cell cycle from the electric field treatment may lead to tumor recurrence or accelerated growth of the tumor cells.

Acknowledgment

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References

[1] Weaver J.C., Chizmadzhev Y.A.: "Theory of Electroporation: A Review". *Bioelectrochem. Bioenerg,* 1996, 41135.
[2] Chang D.C.: "Guide to Electroporation and Electrofusion". San Diego: CA. Academic Press, Inc., 1992.
[3] Huang Y., Rubinsky B.: "Micro-Electroporation: Improving the EfŽciency and. Understanding of Electrical Permeabilization of Cells". *Biomedical Microdevices,* 2000, 2145.
[4] James C.W.: "Electroporation of cells and tissues". *IEEE Transaction on Plasma Sci.,* 2000, *28*, 24.
[5] Ulrich Z., Uwe F., Heiko M., Petra G., Katja H., Vladimir S.: "Electromanipulation of mammalian cells: fundamentals and application". *IEEE Transaction on Plasma Sci.,* 2000, *28,* 72.
[6] Dev S.B., Rabussay D.P., Widera G., Hofmann G.A.: "Medical applications of electroporation". *IEEE Transactions on Plasma Sci.,* 2000, *28,* 206.
[7] Heller R., Gilbert R., Jaroszeski M.J.: "Clinical applications of electrochemotherapy". *Adv. Drug. Deliv. Rev.,* 1999, *35,* 119.
[8] Mir L.M., Moller P.H., Andre F., Gehl J.: "Electric pulse-mediated gene delivery to various animal tissues". *Adv. Genet.*, 2005, *54*, 83.
[9] Mir L.M., Glass L.F., Sersa G., Teissie J., Domenge C., Miklavcic D. *et al.*: "Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy". *Br. J. Cancer,* 1998, *77,* 2336.
[10] Kranjc S., Cemazar M., Grosel A., Sentjurc M., Sersa G.: "Radiosensitising effect of electrochemotherapy with bleomycin in LPB sarcoma cells and tumors in mice". *BMC Cancer*, 2005, 5115.
[11] Soden D.M., Larkin J.O., Collins C.G., Tangney M., Aarons S., Piggott J. *et al.*: "Successful application of targeted electrochemotherapy using novel flexible electrodes and low dose bleomycin to solid tumours". *Cancer Lett.*, 2006, *232,* 300.
[12] Rowan N.J., MacGregor S.J., Anderson J.G., Fouracre R.A., Farish O.: "Pulsed electric field inactivation of diarrhoeagenic Bacillus cereus through irreversible electroporation". *Lett. Appl. Microbiol.,* 2000, *31*, 110.
[13] Edd J.F., Horowitz L., Davalos R.V., Mir L.M., Rubinsky B.: "In vivo results of a new focal tissue ablation technique: irreversible electroporation". *IEEE Trans. Biomed. Eng.,* 2006, *53,* 1409.
[14] Rubinsky B., Onik G., Mikus P.: "Irreversible electroporation: a new ablation modality–clinical implications". *Technol. Cancer Res. Treat.,* 2007, *6*, 37.
[15] Yao C.G., Sun C.X., Xiong L., Mi Y., Wang S.B.: "Irreversible electrical breakdown of malignant tumor cell under steep pulsed electric fields". *J. Chongqing Univ. (Nat. Sci. Edit),* 2004, *6,* 27.
[16] Coster H.G., Zimmermann U.: "Dielectric breakdown in the membranes of Valonia utricularis. The role of energy dissipation". *Biochim. Biophys Acta,* 1975, *382,* 410.
[17] Miller L., Leor J., Rubinsky B.: "Cancer cells ablation with irreversible electroporation". *Technol. Cancer Res. Treat.,* 2005, *4,* 699.
[18] Miklavcic D., Corovic S., Pucihar G., Pavselj N.: "Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy". *EJC Supplements,* 2006, *4,* 445.
[19] Miklavcic D., Beravs K., Semrov D., Cemazar M., Demsar F., Sersa G.: "The importance of electric field distribution for effective in vivo electroporation of tissues". *Biophys J.,* 1998, *74,* 2152.

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