Host's immune response in electrotherapy of murine tumors by direct current

Damijan Miklavčič [1], Dongjian An [2], Jean Belehradek Jr [3] and Lluis M. Mir [3]

1. University of Ljubljana, Faculty of Electrical Engineering, Trzaska 25, 1000 Ljubljana, Slovenia
2. Department of Thoracic Surgery, Xiang Dong Hospital, Liling 412200, Hunan, People's Republic of China
3. LPPMB, URA 147 CNRS, Institut Gustave-Roussy, 39, rue Camille-Desmoulins, 94805 Villejuif, France

Correspondence: Dr. L.M. Mir, LPPMB, URA 147 CNRS, Institut Gustave-Roussy, 39, rue C.-Desmoulins, 94805 Villejuif Cedex, France. Fax: +33 (0)1 42 11 52 76, e-mail: luismir@igr.fr

Abstract. Electrotherapy by low level direct current has been demonstrated to have antitumor effects in different murine tumor models and in clinics. Electrotherapy in "field" configuration, where electrodes are placed subcutaneously outside of the tumor in a way that tumor lies in between the electrodes, was performed in immunodeficient nude and immunocompetent mice. Electrotherapy was much more effective in immunocompetent mice based on the observed tumor growth retardation, thus demonstrating that antitumor effectiveness of electrotherapy greatly depends on host's immune response. Further experiments were conducted by combining electrotherapy with concomitant immunotherapy in order to potentiate the antitumor effect of electrotherapy. Immunotherapy consisted of local delivery of genetically engineered cells selected for IL-2 secretion. This combined treatment was much more effective than any of the treatments alone.

Keywords: electrotherapy, direct current, interleukin-2, immunotherapy.

INTRODUCTION

Electrotherapy by low level direct current has been previously shown to have an antitumor effect in different murine tumor models [1-6] as well as in clinical trials [7, 8]. Depending on the electrode positioning with respect to the tumor, we account on different underlying mechanisms in the observed tumor growth retardation. When one or both of the electrodes are inserted in the tumor, the major part of the response is ascribed to the cell killing due to extreme increase/decrease of the pH in the vicinity of the electrodes [4, 6]. It is possible to eradicate most of the tumor mass with appropriate spacing of multiple electrodes in the tumor and with direct current of long duration [9]. However, if the electrodes are not placed in the tumor, but in its surroundings, in a way that current passes through the tumor, similar tumor growth retardation was obtained at the currents used. Furthermore, in this "field" configuration neither temperature rise nor changes in pH in the tumor were found [10]. Tumor growth retardation in electrotherapy by low level direct current was also not correlated to the current density in the tumor [11] nor to the metal deposited from the electrodes [12]. Furthermore, electrotherapy in "field" configuration was combined with local delivery of IFN-α and TNF-α [13, 14]. In the combination of electrotherapy with TNF-α, the treatment resulted in 40% long term complete responses *i.e.* cures, whereas none of the treatments alone produced any complete response. In order to understand further the mechanisms and to determine possible involvement of host's immune response in the observed antitumor effect of electrotherapy we performed a single shot electrotherapy by 0.6 or 1.0 mA of one hour duration in field configuration on LPB tumors inoculated subcutaneously in syngeneic C57Bl/6 mice and in immunodeficient Swiss nude mice. As a concomitant treatment to electrotherapy we injected intratumorally or peritumorally histoincompatible CHO cells genetically engineered and selected for the secretion of high levels of interleukin-2 (IL-2).

MATERIALS AND METHODS

Mice and tumors. C57Bl/6 and nu/nu Swiss nude female mice, 6 to 8 weeks old, were inoculated subcutaneously in the left flank with, respectively, 0.8×10^6 and 1.6×10^6 cells of the LPB sarcoma cell line. The LPB cell line is a clonal derivative of TBL.Cl2, a methylcholanthrene-induced C57Bl/6 mouse sarcoma cell line [15]. Tumors of 5 to 7 mm in diameter were obtained 8 to 10 days later. Tumors were randomly distributed in experimental groups consisted of 7 to 9 mice. Tumor growth was followed by measuring the two largest perpendicular tumor diameters (a and b, where $a \geq b$). Tumor volume (V) was estimated by equation $V = \pi ab^2/6$, and for each tumor doubling time in days was determined as the time needed by the tumor to double its initial volume. For each experimental group mean tumor volume and mean doubling time (DT, in days) were calculated with corresponding standard deviations for presentation of results. Tumor non palpable at day 60 after the treatment were designated as cures. Statistical evaluation

was performed by means of Student t-test comparing the tumor doubling times in different experimental groups of interest. Values of p less than 0.05 were considered as indicating statistical significance.

Electrotherapy. When the tumor reached approximately 80 mm^3 in C57Bl/6 mice and 40 mm^3 in nu/nu mice a single shot electrotherapy was performed as described previously [14]. Briefly, anaesthesia was induced using a mixture of ketamine and xilazine with addition of atropin. Then, Pt-Ir needle electrodes of 1.0 mm diameter and 22 mm long were inserted subcutaneously parallel to each other on each side of the tumor. Each electrode was 5-8 mm apart from the edge of the tumor. Then a current of 0.6 or 1.0 mA was delivered for one hour.

Immunotherapy. Genetically manipulated Chinese hamster ovary (CHO) cells, transfected with the IL-2 gene and selected for high secretion of IL-2 [16, 17] were injected in different time schedules in order to achieve the best response of combined IL-2 and electrotherapy treatment. It has been reported previously that, when combined to electrochemotherapy, three consecutive injections of IL-2 secreting cells give better results in comparison to single injections [18, 19]. Therefore a series of three injections were used. In the preliminary experiments 10^6 IL-2 secreting cells were injected intratumorally or peritumorally on the day of electrotherapy (day 0), 1 to 2 hours after the end of electrotherapy, and on two additional days, *i.e.* days 2 and 5 or days 1 and 2. Experimental groups which did not receive IL-2 secreting cells received a volume of culture medium (MEM) identical to that in which CHO cells were otherwise injected. Additional control experiments were performed, where instead of the IL-2 secreting cells, untransfected wild type CHO cells were injected at the tumor site with or without previous electrotherapy. Neither given alone or in combination with electrotherapy this CHO cells did affect tumor growth with respect to control or electrotherapy alone, respectively.

RESULTS AND DISCUSSION

In immunodeficient nu/nu mice, electrotherapy with 0.6 mA resulted in noticeable but not statistically significant tumor growth retardation. Higher current of 1.0 mA was then employed and significant tumor growth delay was observed (Table 1). In addition, electrotherapy by 0.6 and 1.0 mA produced significant tumor growth delay in immunocompetent mice on the same tumors ($p = 0.005$ for 0.6 mA, Table 2, and $p \leq 0.001$ for 0.6 and 1.0 mA, Table 3). Based on this results, which clearly demonstrate that electrotherapy is less efficient in immunodeficient mice, and previous experiences, where electrochemotherapy was significantly potentiated using genetically engineered CHO cells selected for secretion of IL-2 [18, 19], electrotherapy was combined with immunotherapy using IL-2 secreting cells in order to potentiate its antitumoral effectiveness. Two different therapeutic schedules were employed, namely single shot electrotherapy followed by intratumoral or peritumoral subcutaneous injection of IL-2 secreting cells either on day 0, 1 and 2 or on day 0, 2 and 5, as described in Materials and Methods. The former experimental schedule gave better results (Table 2). In further experiments a single shot electrotherapy of one hour duration by 0.6 and

Table 1
Effect of electrotherapy on tumor growth in nude mice

	Experiment 1		Experiment 2		Experiment 3	
	DT (days ± s.d.)	n	DT (days ± s.d.)	n	DT (days ± s.d.)	n
Control	2.8 ± 1.5	8	1.8 ± 0.8	7	1.9 ± 0.8	7
DC 0.6mA 1 h	3.4 ± 1.0	8	2.5 ± 1.0	7	2.3 ± 0.8	6
DC 1.0mA 1 h	4.5 ± 1.2*	8	2.7 ± 0.4*	7	3.2 ± 1.2*	7

*: $p < 0.05$ with respect to the control group.
Tumor doubling time following the one hour electrotherapy on day 0 by direct current (DC) of 0.6 and 1.0 mA in nude mice in three different experiments. Mean tumor doubling time (DT, in days) from each experimental group is given together with standard deviation (s.d.) and number of animals in group (n).

Table 2
Effects in immunocompetent mice of the electrotherapy alone or in combination with immunotherapy using different schemes

Experimental groups	Experimental scheme 1 Days 0, 1, 2			Experimental scheme 2 Days 0, 2, 5		
	DT (days ± s.d.)	n	cures	DT (days ± s.d.)	n	cures
Control	2.3 ± 1.1	9	0	4.9 ± 0.8	7	0
CHO IL-2	7.9 ± 4.0#	9	1	5.7 ± 2.8	7	0
DC 0.6mA 1 h	9.3 ± 3.6	9	0	8.8 ± 2.5	7	0
DC 0.6 + CHO IL 2	13.9 ± 6.6#	9	2	10.0 ± 3.7	7	0

#: In groups where cures were obtained, the mean of the doubling time is calculated only with the doubling times from tumors which regrew after treatment (*i.e.* cured animals were excluded).
Mean tumor growth doubling time (DT, in days) with standard deviation (s.d.), number of animals in group (n) and number of cures in immunocompetent mice following either electrotherapy or treatments with the IL-2 secreting cells alone or in combination, using different experimental schemes for the IL-2 secreting cells injections.

Table 3
Effect of electrotherapy in combination with immunotherapy on tumor growth in immunocompetent mice

	Experiment 1			Experiment 2		
	DT (days ± s.d.)	n	cures	DT (days ± s.d.)	n	cures
Control	4.0 ± 2.2	8	0	2.8 ± 0.7	8	0
DC 0.6mA 1 h	6.3 ± 2.9	8	0	7.5 ± 2.0	8	0
DC 0.6 + CHO IL-2	7.5 ± 0.4#	8	3	22.6 ± 14.0#	8	2
DC 1.0 mA 1h	11.7 ± 5.4	8	0	10.4 ± 3.1	8	0
DC 1.0 + CHO IL-2	20.6 ± 27.9#	8	2	18.3 ± 8.8#	8	3

: In groups where cures were obtained, the mean of the doubling time is calculated only with the doubling times from tumors which regrew after treatment (*i.e.* cured animals were excluded).
Mean tumor growth doubling time (DT in days) with standard deviation (s.d.), number of animals in each experimental group (n) and number of cures in immunocompetent mice following either a one hour electrotherapy on day 0 by direct current (DC) of 0.6 or 1.0 mA, or immunotherapy alone, or in combination, using the experimental scheme where the IL-2 secreting cells were given on days 0, 1 and 2.

Table 4
Cure rates obtained by electrotherapy in combination with immunotherapy

	cures	n	cures (%)
Control	0	25	0
CHO IL-2	3	25	12
DC 0.6mA 1 h	0	25	0
DC 0.6 + CHO IL-2	7	25	28
DC 1.0mA 1 h	0	16	0
DC 1.0 + CHO IL-2	5	16	31

Cumulative number of cures, number of animals included (n) and percentage of cures obtained from three repetitions of experiments in which mice were treated either by a one hour electrotherapy on day 0 using direct currents of 0.6 or 1.0 mA, or by immunotherapy alone where IL-2 secreting cells were given on days 0, 1 and 2, or by the combination of these two treatments.

1.0 mA was followed by three injections of CHO cells secreting IL-2 on days 0, 1 and 2. Tumor growth delay due to electrotherapy was potentiated by inoculations of IL-2 secreting cells at both currents used (Figure 1, Tables 3 and 4). Treatment by IL-2 secreting cells alone in schedule day 0, 1 and 2 resulted also in significant tumor growth delay and in the obtention of 12% cures. However, combination of electrotherapy with immunotherapy resulted in 28% and 31% cures at 0.6 and 1.0 mA electrotherapy, respectively (Table 4). Exact determination of additivity or synergism of both therapies, electrotherapy and immunotherapy, is difficult to determine due to the different endpoints of experiments, *i.e.* tumor growth delay or cures. In spite of that, our results show that the combined treatment of electrotherapy and immunotherapy in our experimental conditions is more efficient than any of the treatments given alone.

Our experiments demonstrate that antitumor effectiveness of electrotherapy by low level direct current when electrodes are placed outside the tumor depends greatly on the immune host response. However it has been previously demonstrated by other investigators that, by placing electrodes directly into the tumor of immunodeficient animals and using higher currents, electrotherapy, used as a single treatment, is effective and produces significant tumor growth delay [4, 5]. This effect in immunodeficient animals is in accordance with the hypothesis that antitumor effectiveness of electrotherapy where electrodes are placed within

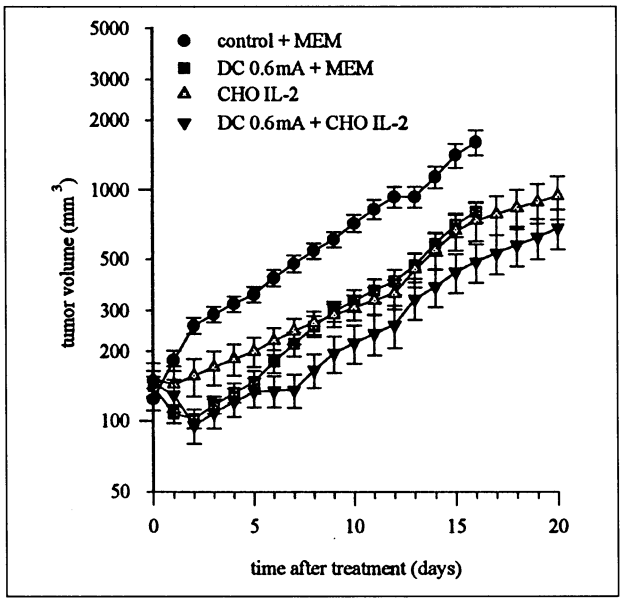

Figure 1.
Effect of electrotherapy and immunotherapy on tumor growth in immunocompetent mice.
Tumor growth after one hour of electrotherapy on day 0 by 0.6 mA and immunotherapy by injecting 10^6 genetically engineered CHO cells selected for IL-2 secretion given intratumorally or peritumorally on day 0 (1-2 hours after the end of electrotherapy) and on day 1 and 2 is presented as mean tumor volume in each of the experimental groups, with vertical bars corresponding to standard error of the mean.

the tumor is due mainly to induced extreme changes in pH around the electrodes, which causes tissue necrosis around electrodes [20].

When electrodes are placed outside the tumor, the effects of DC that result in the reported tumor growth delay have not yet been elucidated. In particular, it is not known if DC can directly provoke the cell death of some tumor cells. Such cell death might liberate some hidden antigens and induce an immune response (even in the immunocompetent mice that are not immunostimulated with IL-2) which could explain the better response of immunocompetent mice and that of the combined therapy. However these effects could also be explained by non cytotoxic consequences of DC application on tumor cells. Indeed, it is known that external electric fields induce changes of the transmembrane potential. These changes could affect

cell membrane surface and the direct or indirect consequences could be modifications in the number of MHC molecules present at the cell surface. Therefore, an increase of tumor cells recognition by NK cells or an increase of tumor antigen presentation could be consequences of DC application. This could result in an increased sensitivity of tumor cells to NK or to cytotoxic T cells as well as in increased antitumor effects when an IL-2-based immmunotherapy is combined to DC.

However, direct effects of DC on the immune cells can not be excluded. Indeed, it has been previously reported by other authors [21] that *in vitro* short-duration (5-10 s) exposure of human lymphocytes to direct current enhanced their cytotoxicity. This was demonstrated by the increase in the number of trypan blue stained target cells, of tumor-binding cells, and of lymphocytes with activated nucleoli. Lymphocytes cytotoxicity was further potentiated after direct current treatment using media containing IL-2. In the experiments reported, authors were using direct current for a very short time in comparison to our experimental conditions. The current densities used in these experiments were 1 to 50 mA/cm^2. As we previously reported [11], the current density in our experimental conditions *in vivo* reaches values of 0.2 to 0.5 mA/cm^2 in the tumor and over 2 mA/cm^2 closer to the electrodes outside of the tumor. These values are comparable to the ones used in the *in vitro* experiments reported [21]. It is important to point out that the reported increase in the cytotoxic effects of human lymphocytes treated by direct current treatment was detected while whole lymphocyte population was exposed to direct currents, without previous selection of specific subpopulations. Thus the antitumor effects here reported could result from similar effects on lymphocytes after *in vivo* exposure of tumor to direct currents.

In conclusion, the results of our study demonstrate that antitumor effectiveness of electrotherapy by low level direct current as here applied greatly depends on host's immune response. Furthermore electrotherapy in combination with immunotherapy using IL-2 secreting cells resulted in potentiation of antitumor effectiveness in immunocompetent mice. Possible mechanisms of action of electrotherapy on immune system remain to be further elaborated.

ACKNOWLEDGEMENTS. This study was performed at the Institute Gustave-Roussy, Villejuif, France within the Proteus programme of scientific, technological and cultural co-operation between France and Slovenia and was partially supported by postdoctoral grant from Ministère de l'Éducation Supérieure et de la Recherche de la République Française to D.M. and by a grant from the government of the People's Republic of China to D.A. Authors wish to thank to Mr. Patrice Ardouin, Mrs. Annie Rouchès and Miss Bernadette Leon for their assistance during the preparation of the experiments.

REFERENCES

1. Humphrey C E, Seal E H. 1959. Biophysical approach towards tumor regression in mice. *Science* 130: 388.

2. David S L, Absolom D R, Smith C R, Gams J, Herbert M A. 1985. Effect of low level direct current on *in vivo* tumor growth in hamsters. *Cancer Res.* 45: 5626.

3. Marino A A, Morris D, Arnold T. 1986. Electrical treatment of Lewis lung carcinoma in mice. *J. Surg. Res.* 41: 198.

4. Samuelsson L, Jonsson L, Lamm I L, Linden C J, Ewers S B. 1990. Electrolysis with different electrode materials and combined with irradiation for treatment of experimental rat tumors. *Acta Radiol.* 32: 178.

5. Heiberg E, Nalesnik W J, Janney C. 1991. Effects of varying potential and electrolytic dosage in direct current treatment of tumors. *Acta Radiol.* 32: 174.

6. Griffin D T, Dodd N J F, Moore J V, Pullan B R, Taylor T V. 1994. The effects of low-level direct current therapy on a preclinical mammary carcinoma: tumour regression and systemic biochemical sequelae. *Br. J. Cancer* 69: 875.

7. Xin Y L. 1994. Advances in the treatment of malignant tumours by electrochemical therapy (ECT). *Eur. J. Surg.* Suppl. 574: 31.

8. Plesnicar A, Serša G, Vodovnik L, Jancar J, Zaletel-Kragelj L, Plesnicar S. 1994. Electric treatment of human melanoma skin lesions with low level direct electric current: an assessment of clinical experience following a preliminary study in five patients. *Eur. J. Surg.* Suppl. 574: 45.

9. Serša G, Miklavčič D. 1993. The feasibility of low level direct current electrotherapy for regional cancer treatment. *Reg. Cancer Treat.* 1: 31.

10. Miklavčič D, Serša G, Kryzanowski M, Novakovic S, Bobanovic F, Golouh R, Vodovnik L. 1993. Tumor treatment by direct electric current – tumor temperature and pH, electrode material and configuration. *Bioelectrochem. Bioenerg.* 30: 209.

11. Miklavčič D, Šemrov D, Valencic V, Serša G, Vodovnik L. 1997. Tumor treatment by direct electric current: computation of electric current and power density distribution. *Electro- and Magnetobiology* 16: 119.

12. Miklavčič D, Fajgelj A, Serša G. 1994. Tumor treatment by direct electric current: electrode material deposition. *Bioelectrochem. Bioenerg.* 35: 93.

13. Serša G, Miklavčič D. 1993. Combined treatment of murine SA-1 tumors by human leukocyte interferon alpha and electrotherapy. *Radiol. Oncol.* 27: 280.

14. Serša G, Golouh R, Miklavčič D. 1994. Anti-tumor effect of tumor necrosis factor combined with electrotherapy on mouse sarcoma. *Anti Cancer Drugs* 5: 69.

15. Belehradek J Jr, Barski G, Thonier M. 1972. Evolution of cell-mediated antitumor immunity in mice bearing a syngeneic chemically induced tumor. Influence of tumor growth, surgical removal and treatment with irradiated tumor cells. *Int. J. Cancer* 9: 461.

16. Roth C, Mir L M, Cressent M, Quintin-Colonna F, Belehradek J Jr, Ley V, Fradelizi D, Kourilsky P. 1992. Inhibition de la croissance tumorale induite par l'injection de cellules histoincompatibles produisant de l'interleukine-2. *C. R. Acad. Sci. Paris III* 314: 499.

17. Roth C, Mir L M, Cressent M, Quintin-Colonna F, Ley V, Fradelizi D, Kourilsky P. 1992. Inhibition of tumor growth by

histoincompatible cells expressing interleukin-2. *Int. Immunol.* 4: 1429.

18. Mir L M, Roth C, Orlowski S, Belehradek J Jr, Fradelizi D, Paoletti C, Kourilsky P. 1992. Potentialisation de l'effet antitumoral de l'électrochimiothérapie par une immunothérapie à l'aide de cellules allogéniques productrices d'interleukine-2. *C. R. Acad. Sci. Paris III* 314: 539.

19. Mir L M, Roth C, Orlowski S, Quintin-Colonna F, Fradelizi D, Belehradek J Jr, Kourilsky P. 1995. Systemic antitumor effects of electrochemotherapy combined with histoincompatible cells secreting interleukin-2. *J. Immunother. Emphasis Tumor Immunol.* 17: 30.

20. Miklavčič D, Serša G, Novakovic S, Reberšek S. 1990. Tumor bio-electric potential and its possible exploitation for tumor growth retardation. *J. Bioelectr.* 9: 133.

21. Chudomel V, Soucek J, Hruba A, Jerabek J, Schwarz J, Smetana K. 1989. Positive effect of direct current on cytotoxicity of human lymphocytes. *Neoplasma* 35: 573.