Anti-Cancer Drugs, 3, pp. 253–260

Anti-tumor effect of electrotherapy alone or in combination with interleukin-2 in mice with sarcoma and melanoma tumors

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Electrotherapy with direct current (DC) was performed on two murine tumor models, fibrosarcoma SA-1 and melanoma B16. Three Pt/Ir cathodes were inserted directly into the subcutaneous tumors and two anodes subcutaneously in the vicinity of the tumor. Significant tumor growth delay was achieved after electrotherapy and was dependent on DC intensity (0.6, 1.0, 1.4 and 1.8 mA). Melanoma B16 tumors were more sensitive to electrotherapy than SA-1 tumors. In order to enhance the antitumor effect of electrotherapy, combined treatment with interleukin-2 (IL-2) was performed. When both therapies were combined significant tumor growth delay and also higher curability rate was achieved. The results imply that electrotherapy can be an effective antitumor therapy and that the effects can be enhanced with additional IL-2 therapy.

Key words: Cell cultures, electrotherapy, experimental tumors, immunomodulators.

Introduction

Control of cell proliferation is currently under intensive investigation. Apart from other means, electrical direct current (DC) has also been recognized as one method of control, either enhancing or suppressing cell proliferation.[1–3] Electrical currents are in medical use in the treatment of chronic skin ulcers, nerve regeneration and bone healing.[4,5] The idea of controlling cell proliferation has also been employed in studies dealing with electrical currents as antitumor treatment (electrotherapy).[6–8] Many different forms of electrical currents, in terms of frequencies, pulse-shapes, amplitude, have been applied in different ways *in vitro*, on tumor models *in vivo* as well as in patients.[6–14] Reports on low level DC as an antitumor agent date back to 1959 when the first study was performed by Humphrey and Seal on experimental tumor bearing animals.[15] Subsequent studies suggest that the antitumor effect is dependent on current intensity and treatment time.[10,11,15] Since DC employed in electrotherapy studies was recognized as a local treatment, adjuvant cytotoxic treatment, radiotherapy or biological therapy have also been included.[13,16,17]

Besides these experimental studies there are also reports by Nordenström *et al.* on the use of electrotherapy in the treatment of pulmonary metastasis with extrathoracic primary cancer, either as a single treatment or as an electrochemical treatment in combination with chemotherapeutic agents.[7,12,13] Their studies have incited other authors to treat lung carcinomas and other malignant tumors.[18,19]

In all these studies different polarities of electric DC were employed, resulting in variable tumor responses. From the results reported it seems that electrotherapy effectiveness is dependent of the current polarity used; however, the effect could also be dependent on the biology of tumors treated.[6,10–13,17,20] Some of these biological parameters were studied on fibrosarcoma SA-1. A pre-

The work was supported by The Ministry of Science and Technology, Slovenia.

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liminary study has also been performed employing interferon (IFN)-α as adjuvant treatment to electrotherapy in order to enhance host antitumor mechanisms and to eradicate the remaining tumor cells after electrotherapy.[17] In the present study we combined single shot electrotherapy with interleukin-2 (IL-2) treatment. Immunotherapy with IL-2 was combined with electrotherapy with different current levels. These effects were compared on fibrosarcoma and melanoma tumor models.

Materials and methods

Animals

Female and male A/J and C57B1/6 mice were purchased from Rudjer Bošković Institute, Zagreb, Croatia. Animals were maintained at constant room temperature (24°C) at a natural day/night cycle in a conventional mouse colony. Mice in good condition, without signs of fungal or other infections, 8–10 weeks old, were used in the experiments.

Tumors

Melanoma B16 was maintained in C57B1/6 mice by serial transplantation. Tumor cells from the fourth isotransplantat generation were prepared for the described experiments by gentle mechanical dissaggregation. Fibrosarcoma SA-1 cells, syngeneic to A/J mice, were obtained from the ascitic form of the tumor. Solid subcutaneous tumors, dorsolaterally in animals, were initiated by injection of 5×10^5 viable SA-1 cells and 10^6 B16 melanoma cells. After the tumors reached 40–50 mm^3 in volume animals were randomly divided into experimental groups on day D0. On each consecutive day the tumor volume was calculated as $V = \pi \cdot e_1 \cdot e_2 \cdot e_3/6$, where e_j were three main mutually orthogonal tumor diameters measured by a vernier calliper gage. Experimental tumors were measured until the first tumor in the group reached 400 mm^3. Tumor doubling time (DT) was determined for individual tumors and tumor growth delay (GD) from the mean DT of experimental groups was calculated by:

$$GD_x = \overline{DT_x} - \overline{DT_c}$$

$$S^2 = \frac{(n_x - 1) \cdot S_x^2 + (n_c - 1) \cdot S_c^2}{n_x + n_c - 2} \cdot \left(\frac{1}{n_x} + \frac{1}{n_c}\right)$$

$$v = n_x + n_c - 2$$

where $\overline{DT}$ is the mean tumor doubling time, GD is the growth delay, S is the experimental standard deviation, n is the number of animals within the group; v is the degree of freedom; subscripts: x, experimental groups; c, control group.

Electrotherapy *in vivo*

Current and voltage were continuously monitored during electrotherapy with 0.6–1.8 mA DC, of 1 h duration. The DC source was designed and manufactured at the Faculty of Electrical and Computer Engineering, Ljubljana, Slovenia. Current was delivered through Pt/Ir (90/10%) alloy needle electrodes (0.7 mm diameter) with rounded tips. Three electrodes, inserted directly into the tumor site (active in further text), were insulated except for a 3 mm tip and were 20 mm long. Medical grade silicon tubes were used as insulators. Another two electrodes, inserted subcutaneously in the tumor vicinity (passive in further text), were bare, lightly radially bent and 22 mm long. Depending on the polarity of active electrodes during electrotherapy, electrodes were connected with a single lead to the appropriate current source terminal. The control groups were treated in the same way as the experimental groups with electrodes introduced into the tumors, except that no current was delivered.

IL-2 therapy

IL-2 with specific activity 3×10^6 U/mg was generously provided by Cetus Corporation (Emeryville, CA). Intraperitoneal treatment with 10^4 or 4×10^4 U IL-2 was started 1 h after electrotherapy. Animals were treated twice daily; in the morning and late in the evening for five consecutive days (D0 to D4). Animals in the control groups received physiological saline instead of IL-2 in the same schedule.

Determination of tumor necrosis

Tumors were excised, fixed in 10% phosphate buffered formalin, embedded in parafin for histological sectioning and then stained with hematoxylin & eosin. The extent of necrosis was assessed by light microscopy using an adaptation of the Chalkey Point method.[21]

Electrotherapy *in vitro*

Diploid lung Chinese hamster fibroblasts (V-79-379A) and SA-1 cells were grown in Eagle's MEM supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 ug/ml). Melanoma B16 cells were grown in RPMI 1640 medium supplemented with 10% FCS and antibiotics. For electrotherapy the cells were suspended in adequate culture medium (27 ml) without FCS at a concentration of 6-8 $\times$ 10^4 viable cells/ml (Trypan dye exclusion test) and placed in glass dishes (radius 21 mm, height 40 mm) with a teflon cover in which electrodes were fixed (the distance between electrodes was 30 mm). DC was delivered (0.6 or 1.0 mA) through Pt/Ir (90/10%) alloy electrodes immersed (depth of immersion 18 mm) into the cell suspension. During the treatment with DC (1 h) the dishes containing the cell suspension were placed on a shaker to prevent cell adhesion. Immediately after treatment with DC the cells were counted in a hemocytometer and the cell number/control (%) ratio calculated. Thereafter the cells were transferred to the tissue culture dishes in equal numbers for the determination of cell growth. For this purpose cells were counted in a hemocytometer after 48 h and the cell number/control (%) ratio was again calculated.

Statistical analysis

The non-parametric Mann–Whitney rank-sum test (single tailed) for testing medians was employed for statistical evaluation of the results *in vivo*. The test was performed for each day comparing tumor volumes between the experimental groups of interest. After determination of individual tumor doubling times, the Mann–Whitney rank-sum test was performed again. In all cases the exact level of significance was determined. Student's *t*-test was employed for statistical evaluation of the *in vitro* results, after equality of variances was tested by the *F*-test.

Results

Antitumor effect of electrotherapy

Electrotherapy was tested for its antitumor effect on SA-1 tumors. Depending on the polarity of the DC employed, anodes or cathodes were active electrodes. Antitumor effect was noticeable 24 h after electrotherapy with 0.6 mA DC for 60 min (Figure 1). Tumors in mice subjected to electrotherapy, regardless of the current polarity used, were significantly smaller than in the controls in both DC treated groups from D1 to D7 ($p < 0.001$, except in anodic treatment on D_7 where $p = 0.012$). Cathodic treatment was moderately more effective than anodic treatment, producing a substantial reduction of tumors on the first 2 days after treatment, although tumor growth delay was not statistically significantly different ($p = 0.173$; $GD = 2.5 \pm 0.7$ days cathodic electrotherapy and $GD = 2.2 \pm 0.6$ days anodic electrotherapy).

Tumors treated with cathodic electrotherapy were also significantly ($p = 0.005$) more necrotic 24 h after therapy (68.3 $\pm$ 12.5%) than the tumors treated with anodic DC (40.6 $\pm$ 15.9%), and both significantly more necrotic than tumors in the control group (20.0 $\pm$ 7.9% necrosis; $p < 0.001$, $p = 0.008$, respectively).

Electrotherapy combined with IL-2 treatment

In the following experiments single shot electrotherapy with 1.0 mA cathodic DC was tested on two tumor models, fibrosarcoma SA-1 and B16 melanoma, and was followed by treatment with IL-2. Electrotherapy alone had statistically signifi-

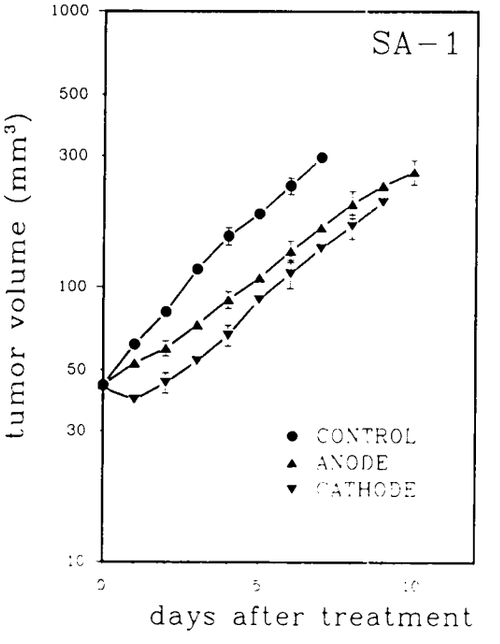

Figure 1. Antitumor effect of anodic and cathodic DC (0.6 mA, 1 h) on subcutaneous SA-1 tumors.

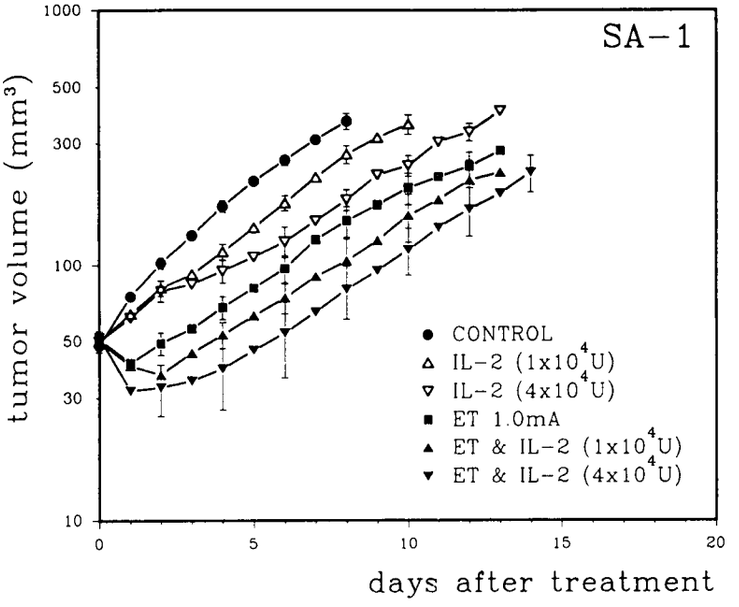

Figure 2. Antitumor effect of electrotherapy (cathodic DC, 1 mA, 1 h) on SA-1 tumors combined with intraperitoneal treatment with two different doses of IL-2 twice daily for five consecutive days.

cant antitumor effect on the first day after the treatment and throughout the observation period on both tumor models ($p < 0.001$) (Figures 2 and 3; Table 1). The antitumor effect of electrotherapy was better on B16 melanoma tumors than on SA-1 tumors ($p_{GD} = 0.01$).

IL-2 therapy with 10^4 or 4×10^4 U had an antitumor effect on both SA-1 and B16 melanoma tumors (Figures 2 and 3). Significant tumor growth delay was achieved with lower and higher IL-2 doses on SA-1 tumors from D3 onwards ($p = 0.001$), whereas the B16 melanoma with lower dose from D5 ($p = 0.001$) and with higher dose from D3 ($p = 0.0002$).

Table 1. Tumor growth delay[a] after electrotherapy (ET) alone or in combination with IL-2

	SA-1	B16
ET 1.0 mA 1 h	5.5 ± 1.6 (16)	8.7 ± 0.8 (14)
ET 1.4 mA 1 h	7.1 ± 0.9 (13)	11.2 ± 1.0 (13)
ET 1.8 mA 1 h	12.6 ± 1.6 (14)	17.2 ± 0.8 (11)
IL-2 (1×10^4 U)	2.3 ± 0.5 (15)	2.1 ± 0.6 (14)
ET 1.0 mA + IL-2	8.0 ± 1.7 (11)	13.0 ± 1.2 (13)
ET 1.4 mA + IL-2	11.1 ± 2.0 (14)	15.4 ± 1.6 (14)
ET 1.8 mA + IL-2	14.9 ± 2.9 (12)	—

[a] Tumor growth delay: mean ± SD (*n*).

In order to enhance host's antitumor mechanisms and to eradicate the remaining tumor cells after electrotherapy adjuvant IL-2 treatment was performed. IL-2 therapy following single shot electrotherapy contributed to the effectiveness of electrotherapy on both SA-1 and B16 melanoma tumor models (Figures 2 and 3). Growth of SA-1 tumors after combined treatment was delayed compared with electrotherapy alone but was not statistically significant with either of the IL-2 concentrations. At the same time combined treatment of melanoma B16 tumors caused a more rapid tumor regression than did electrotherapy alone and also greatly delayed tumor regrowth, especially with the higher IL-2 treatment dose (4×10^4 U) ($p = 0.02$).

Different doses of electrotherapy combined with IL-2

Since experiments in this study indicated that electrotherapy with higher doses had better antitumor effect we combined escalating DC doses (1.0, 1.4 and 1.8 mA cathodic DC) with lower dose of IL-2 on both SA-1 and melanoma B16 tumors (Table 1). Growth delay of SA-1 tumors was dependent on the DC level, but even with the

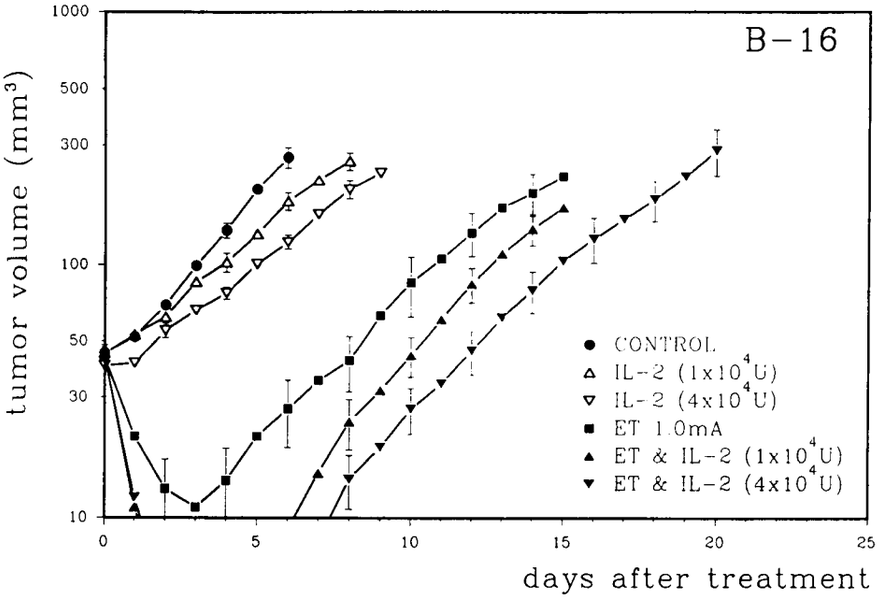

Figure 3. Antitumor effect of electrotherapy (cathodic DC, 1 mA 1 h) on B16 melanoma tumors combined with intraperitoneal treatment with two different doses of IL-2 twice daily for five consecutive days.

highest DC no tumor cures were achieved. A better antitumor effect was demonstrated on B16 melanoma tumors (Figure 4). Electrotherapy with 1.4 mA DC resulted in tumor regression for the first 7 days and 20% tumor cures, while with 1.8 mA DC 40% tumor cures were achieved (Figure 5).

In combined treatment of SA-1 tumors, IL-2 therapy (10^4 U) did not statistically significantly contribute to the effectiveness of electrotherapy. However, in melanoma B16 tumors IL-2 therapy significantly potentiated electrotherapy treatment (1.0 and 1.4 mA DC) ($p = 0.02$). Adjuvant IL-2

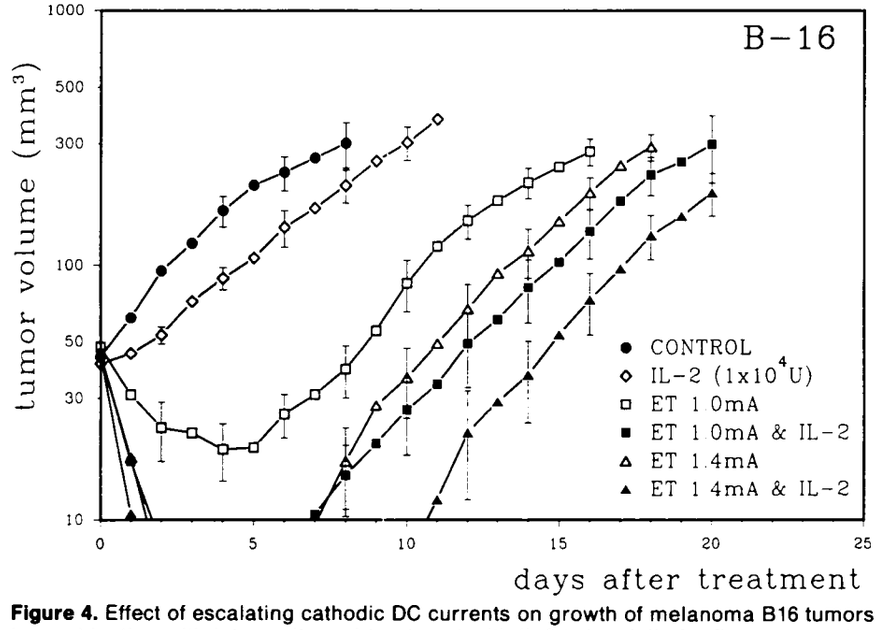

Figure 4. Effect of escalating cathodic DC currents on growth of melanoma B16 tumors in combination with intraperitoneal treatment with IL-2 twice daily for five consecutive days.

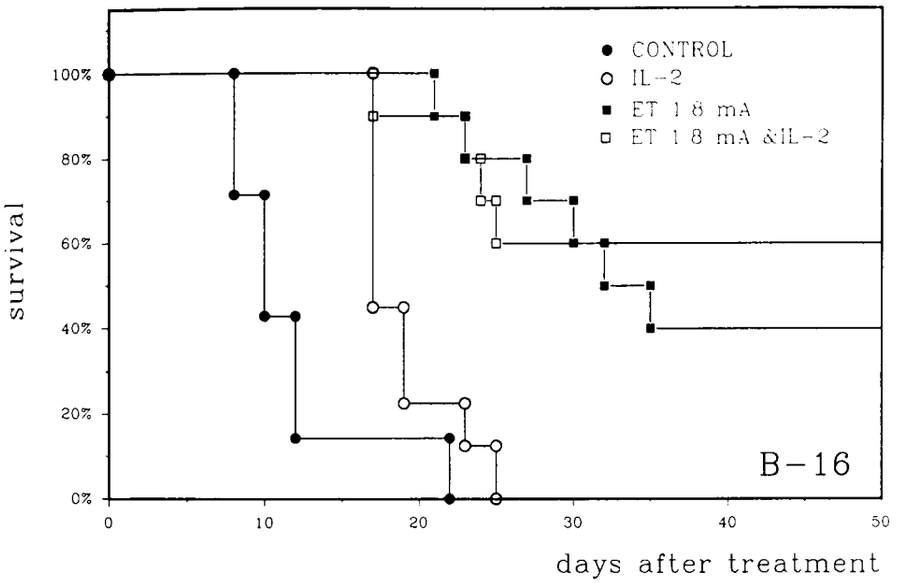

Figure 5. Survival of melanoma B16 bearing animals after treatment with cathodic DC and in combination with IL-2 (10^4 U, twice daily for five consecutive days).

therapy even increased the curability rate of electrotherapy with 1.8 mA DC from 40 to 60% (Figure 5).

Sensitivity of cells to electrotherapy *in vitro*

An *in vitro* model was established to investigate the influence of DC current on the cell growth rate. Cell number was determined immediately after 60 min of electrotherapy treatment. Regardless of the cells treated, V-79-379-A, SA-1 or B16 melanoma, the cell number decreased for 25% of control at 0.6 mA and 40% at 1.0 mA DC. The growth rate of the surviving cells in cell culture was further decreased compared with controls in a dose-dependent manner (Figure 6). Electrotherapy had less of an effect on the proliferation of normal V-79-379-A cells than on malignant SA-1 and B16 melanoma cells. The difference in electrosensitivity of SA-1 and B16 melanoma cells was prominent at 1.0 mA DC treatment. SA-1 cells were significantly more sensitive (25.6 $\pm$ 1.4% of control) compared with B16 melanoma (59.6 $\pm$ 4.3%, $p = 0.001$) and both B16 melanoma and SA-1 cells were significantly more sensitive than V-79-379-A cells (74.2 $\pm$ 3.7%, $p = 0.02$, $p = 0.001$, respectively).

Discussion

Electrotherapy delivered through Pt/Ir electrodes markedly retarded growth of fibrosarcoma SA-1 and melanoma B16 tumors. The antitumor effect was DC level dependent, 60 min treatment with 0.6 mA DC moderately delayed tumor growth, while treatment with 1.8 mA DC cured approximately 40% of B16 melanoma bearing animals. These results are in accordance with the reports of other investigators on different animal tumor models where the antitumor effect of DC varied from moderate to good and also seem to be tumor type dependent.[6,8,10,11,15,17] The two tumors tested in our study have not been used before, but proved to be interesting because their response to electrotherapy was different, SA-1 tumors being less sensitive than B16 melanoma. Electrotherapy did not induce SA-1 tumors to regress, but only delayed tumor growth depending on the current intensity. On B16 melanoma the antitumor effect was also dose dependent but with 1.0 mA DC electrotherapy tumors regressed for 7 days and eventually outgrew again. With 1.8 mA even 17.2 $\pm$ 0.8 days tumor growth delay was achieved. For better effectiveness of electrotherapy a multiple electrode array was used because uniform electrical current distribution over the entire tumor mass seemed to be essential.[10]

Since after electrotherapy some viable neoplastic tissue remains in the periphery of the tumor mass leading to eventual tumor regrowth, electrotherapy was recognized as a regional cancer treatment which should be accompanied by other treatments such as chemotherapy, radiotherapy or immunotherapy in order to eradicate the remaining clonogenic tumor

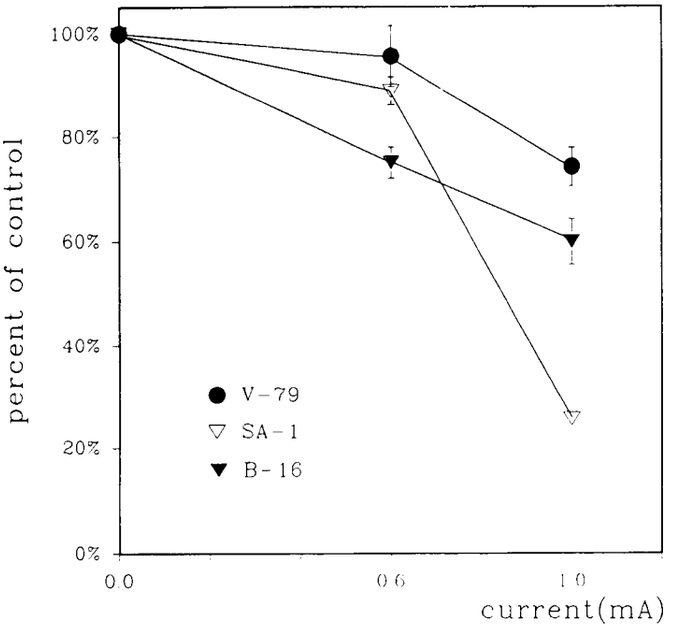

Figure 6. Growth of the cells *in vitro* after treatment with different DC currents.

cells.[10,12,13,16,17] In most of the reports electrotherapy potentiated chemotherapeutic treatment of the tumors,[13] radiotherapeutic effectiveness[16] or even enhanced specific and non-specific antitumor mechanisms of the host.[17,22,23]

Our preliminary study in combining electrotherapy with IFN-α showed that the combined treatments can interact in the treatment of SA-1 tumors.[17] The same idea was applied when combining systemic IL-2 immunotherapy with electrotherapy in the treatment of sarcoma and melanoma tumors. IL-2 treatment alone was effective on both tumors, to almost the same extent, and it delayed tumor growth for approximately 2 days. When both treatments were combined, IL-2 moderately contributed to the antitumor effect of electrotherapy on the SA-1 tumor model, whereas a significant tumor growth delay and higher curability was achieved in B16 melanoma. The difference in response of the two tumor models is difficult to explain. One of the reasons could be that since electrotherapy was more effective on B16 melanoma less viable tumor cells remained and therefore immunotherapy was more effective. Since normal and leukemic lymphocytes can show enhanced antileukemic cytotoxicity after DC treatment *in vitro*[23] this could also be one of the indirect antitumor effects of electrotherapy. Furthermore, lymphocyte and natural killer (NK) cell cytotoxicity was potentiated by IL-2 or IFN-α in combination with DC.[22] These observations were confirmed by enhanced expression of IL-2 receptors on the plasma membrane of lymphocytes.[24] Therefore adjuvant immunotherapy with IL-2 has a sound basis in the activation of host's antitumor mechanisms, contributing to the effectiveness of electrotherapy.

In addition, a temperature rise in the tumor mass, electrochemical reactions on electrodes or changes in pH surrounding the electrodes have been proposed as mechanisms of electrotherapy.[7,10,20,25] A temperature rise could not be the main reason for massive tumor destruction since theoretically and on the basis of measurements of David *et al.*[10] and ours (data not presented) the energy delivered with an electrical current does not raise the temperature by more than 1°C, which is not a cytotoxic hyperthermia, but might only induce increased blood flow through the tumor. Electrochemical reactions in the vicinity of the electrodes could not be ruled out, although stainless steel, Pt/Ir and other materials have similar antitumor effects.[10] Changes in the pH in the vicinity of the electrodes could be one of the major mechanisms in the antitumor effect of electrotherapy. Exact measurements must be performed to determine its role in the antitumor effect, because it is known that changes in extracellular pH modulate the cell cycle.[26]

Another unexplained mechanism is the effect of electrotherapy on cell proliferation. Therefore in our preliminary study *in vitro* the sensitivity of V-79 cells to electrotherapy[1] was compared with SA-1

and B16 malignant cell lines. The immediate cytotoxic effect of electrotherapy was the same on all three cell lines, but the late effects on the growth rate of cells were different. Both of the malignant cells, melanoma and sarcoma, had a slower growth rate than V-79 cells. This indicates that malignant cell proliferation can be suppressed with electrical currents and suggests the existence of a mechanism which affects cell division.[3]

Both tumors, sarcoma and melanoma, are immunogenic, their biological structure being different. Fibrosarcoma SA-1 has a solid structure, while melanoma B16 has a softer structure, which could be a consequence of better vascularization and extracellular components of the tumor.[27] Observations of Nordenström *et al.*[12,13] that small, less vascularized tumors are less responsive to electrotherapy than bigger, better vascularized tumors also points to the importance of the vascular component in the effectiveness of electrotherapy.

The data presented indicate that electrotherapy can be used in cancer treatment as a loco-regional treatment. Its antitumor mechanisms remain unexplained, but they are probably multiple and can therefore be potentiated by different adjuvant treatments, either local or systemic. Our results indicate the potential usefulness of combining electro and immunotherapy in cancer treatment.

Acknowledgements

We thank S Plesničar, S Reberšck, R Golouh, M Auersberg and V Kotnik for their helpful suggestions.

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(Received 3 March 1992; accepted 16 March 1992)