Metastatic potential of melanoma cells is not affected by electrochemotherapy

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Electrochemotherapy is a local treatment combining chemotherapy and application of electric pulses to the tumour. Electrochemotherapy with bleomycin and cisplatin has shown its effectiveness in controlling local tumour growth in the treatment of malignant melanoma. However, the effect of electrochemotherapy on the metastatic potential of tumour cells is not known. Prevention of metastasis is an important aspect of successful treatment; however, it is known that metastasis can be induced by different treatment modalities. Therefore, the aim of this study was to evaluate the effect of electrochemotherapy with cisplatin on the metastatic potential of human malignant melanoma cells. Cells treated by electrochemotherapy with cisplatin were tested for their ability to migrate and invade through Matrigel-coated porous membrane. In addition, RNA was isolated from cells after treatment and differentially expressed genes were investigated by microarray analysis to evaluate the effect of electrochemotherapy with cisplatin on gene expression. There were no significant changes observed in cell migration and invasion of melanoma cells after electrochemotherapy. In addition, there were no changes observed in cell adhesion on Matrigel. Gene expression analysis showed that a very low number of genes were differentially expressed after electrochemotherapy with cisplatin. Two genes, *LAMB3* and *CD63* involved in cell migration, were both downregulated after electrochemotherapy with cisplatin and the expression of metastasis promoting genes was not increased after electrochemotherapy. Our data suggest that electrochemotherapy does not increase the metastatic behaviour of human melanoma cells. *Melanoma Res* 21:196–205 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Electrochemotherapy (ECT) is a local treatment combining chemotherapy and electroporation [1]. Electroporation is a highly effective method for transient modification of cell membrane permeability by means of series of controlled electric pulses (EPs) [2]. It is effective in facilitating the transport of different molecules through the cell membrane, including chemotherapeutic drugs [3]. So far, two chemotherapeutic drugs have proved to be effective in ECT, bleomycin and cisplatin (CDDP). Electroporation with CDDP or bleomycin significantly enhances the cytotoxicity of these drugs in different tumour cell lines, CDDP-resistant cell lines and solid tumours [4–7]. In clinical trials, ECT with CDDP or bleomycin have been effective in the treatment of skin tumour nodules of various types of tumours, including malignant melanoma [8–10]. The results on malignant melanoma nodules showed that ECT with CDDP or bleomycin is effective in controlling local tumour growth with approximately 80% objective response rate [10,11].

Besides good local tumour control, the prevention of metastatic spread is also an important aspect of successful treatment. It is generally accepted that alterations in the local microenvironment during wound healing after surgery provide a favourable climate for cancer cell growth [12]. In addition, radiation-induced metastasis can occur *in vivo*, after conventional radiotherapy. Irradiation of tumour cells can modulate their migratory behaviour. Depending on the cell line and radiation type used, a decrease or increase in cell migration was observed [13].

Metastasis is the spread of tumour cells from the original growth site to other sites in the body. The development of metastasis is a complex multi-step process, which requires the migratory potential of tumour cells to migrate through the primary tumour mass, intravasate into, survive in, and extravasate from the vascular system into a secondary organ and proliferate at that site. The metastatic potential of tumour cells is believed to be regulated by interactions between the tumour cells and their extracellular environment. These interactions can

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be modified by the accumulation of genetic changes and by the transient alterations in gene expression induced by the local tumour microenvironment [14].

Formation of metastases can be directly linked to migratory potential of the cells [13]. So far, no studies have dealt with the effect of ECT on migratory potential of human melanoma cells. Clinically, biological properties and metastatic potential of cells that have not been or were suboptimally affected by ECT, is a relevant problem. Namely, in the case of insufficient drug distribution in the tumour, and suboptimal electroporation of the tissue some cells may survive the treatment [15]. Furthermore, the treatment could affect the local tumour microenvironment by inducing transient alterations in gene expression that would promote the metastatic behaviour of tumour cells that survived the initial treatment. To date, it had been shown that ECT with bleomycin does not increase metastatic spread of liver tumours in rabbits [16]. In clinical trials, there were no reports of increased metastatic spread of tumours after ECT [17]. It was also shown that none of the lesions that were in complete response after ECT with bleomycin relapsed during the follow-up of 21 months [18]. Nevertheless, it is not known whether ECT with CDDP affects metastatic potential of melanoma cells. Therefore, the aim of this study was to evaluate the effect of ECT with CDDP on metastatic potential of human melanoma cells *in vitro* by migration, invasion and adhesion assays. The effect of ECT on gene expression was further investigated on the same cells.

Materials and methods

Cell line

Human malignant melanoma cells (SK-MEL28) were derived from a melanoma metastasis of a 51-year-old male patient (HTB-72; American Type Culture Collection, USA). These cells are tumourigenic *in vivo*, in nude and severe combined immune-deficient mice [19,20]. SK-MEL28 cells were grown as monolayer in minimum essential medium (MEM) with Glutamax (Gibco, Invitrogen, Paisley, UK), supplemented with 10% fetal calf serum (FCS) (Invitrogen) and gentamicin (30 μg/ml) (Gibco, Invitrogen). The doubling time of these cells was 48 h. Cells were routinely subcultured twice a week and incubated in an atmosphere with 5% CO_2 at 37°C.

Drug

CDDP was obtained from Aventis (Paris, France) as a crystalline powder. CDDP was dissolved in sterile H_2O at a concentration of 1 mg/ml. For each experiment, a fresh solution of CDDP was prepared. The final concentrations of CDDP were prepared in MEM. For ECT 2, 8, 20, 40 and 80 μg/ml solutions of CDDP were used.

Electrochemotherapy protocol

Confluent cell cultures were trypsinized, washed in MEM with FCS for trypsin inactivation and once in electroporation buffer (125 mmol/l saccharose, 10 mmol/l K_2HPO_4, 2.5 mmol/l KH_2PO_4, 2 mmol/l $MgCl_2{\cdot}6H_20$) at 4°C. Final cell suspension was prepared in electroporation buffer at 4°C at a concentration of 22×10^6 cells/ml. For clonogenic assay, 90 μl of final cell suspension was mixed with 10 μl of CDDP solution in a concentration range from 2 to 80 μg/ml. Fifty microlitres (μl) of the mixture (1×10^6 cells) was placed between two parallel electrodes with 2 mm gap in between and subjected to eight square wave EPs with electric field intensity of 1300 V/cm, pulse duration of 100 μs and frequency of 1 Hz. EPs were generated by inhouse build electroporator (University of Ljubljana, Faculty of Electrical Engineering, Ljubljana, Slovenia). Another 50 μl of mixture (1×10^6 cells) served as a control for CDDP treatment alone. For adhesion, migration and invasion assays, the volume of cell suspension subjected to EPs was 100 μl (2×10^6 cells). After electroporation, cells were incubated at room temperature for 5 min, diluted in 2 ml of growth media and then plated for clonogenic, adhesion, migration, invasion and microarray assays.

Clonogenic assay

For clonogenic assay, SK-MEL28 cells were plated at a concentration of 300 cells/dish for control and CDDP concentrations of 2, 8 and 20 μg/ml and 600 cells/dish for CDDP concentrations of 40 and 80 μg/ml, respectively, for chemotherapy and ECT. After 16 days, colonies were fixed, stained with crystal violet and counted. The survival curve for the ECT-treated cells was normalized for the cytotoxicity of EPs treatment alone. Surviving fraction of cells treated with EPs alone was 0.57 ± 0.04 (data not shown). The inhibitory concentration of each treatment that reduced cell survival to 50% (IC_{50}) was determined graphically in each experiment. The experiment was repeated four times in triplicates.

Proliferation assay

After the ECT protocol, cells were plated in 96-well plates (TPP, Trasadingen, Switzerland) for proliferation assay. For this experiment, 1.5×10^4 cells/well were seeded in two separate 96-well plates and left for 48 and 72 h. After 48 and 72 h, a solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium with phenazine methosulfate (ratio 20 : 1) (Promega, Madison, USA) was added to each well and, after 2 h, absorbance was measured at 492 nm using a microplate reader (Tecan, Salzburg, Austria). The experiment was repeated twice in sextuplicates.

Adhesion assay

Cell adhesion assay was determined 48 h posttreatment. For adhesion assay, 96-well plates were coated with Matrigel (BD Bioscience, USA) diluted in serum-free

MEM according to manufacturer's instructions. These plates were incubated for 1 h at room temperature to allow Matrigel polymerization. Unbound material was aspirated and the 96-well plates were gently washed with serum-free MEM. CDDP- or ECT-treated cells were trypsinized, washed in MEM with serum for trypsin inactivation, centrifuged, resuspended in serum-free MEM and counted. Cells (3×10^4 per well) were plated in preprepared 96-well plates in 200 µl serum-free MEM. After 2 h, medium and unbound cells were removed from the wells and attached cells were gently washed with $1 \times$ phosphate buffered saline and fresh serum-free MEM with 3-(4,5-dimethylthiazol-2yl)-2,5-diphenol tetrazolium bromide (0.5 mg/ml) (Calbiochem, Germany) was added. Cells were then further incubated for 2 h, media was then removed and formazan crystals were dissolved in dimethyl sulfoxide (Sigma Aldrich, Steinheim, Germany). Absorbance was measured at 595 nm using microplate reader (Tecan). The experiment was repeated four times in septuplicates.

Migration and invasion assay

For migration assay, uncoated inserts with polycarbonate membrane with 8 µm pores (TPP) in 24-well plates were used. Forty-eight hours after CDDP or ECT, cells were trypsinized, washed in MEM with FCS for trypsin inactivation, centrifuged, resuspended in serum-free MEM and counted. Cells (9×10^4 per well) were plated in inserts (TPP) with 8 µm pores in 24 well plates in 400 µl serum-free MEM and 400 µl MEM with 10% FCS as a chemo-attractant was added to these wells in the 24-well plates. After 22 h, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenol tetrazolium bromide was added to inserts and wells and was further incubated for 2 h. The migrated cells were washed off at the bottom of the insert and were collected in the original well, whereas the inserts were transferred to a clean well. Formazan crystals were dissolved in dimethyl sulfoxide. Absorbance was measured at 595 nm using microplate reader (Tecan). The experiment was repeated three times in quadruplicate. Migration (%) is the ratio between the absorbance of the cells collected in the original well over the sum of absorbance of the cells collected in the original well and the cells collected in the insert.

For invasion assay, the inside of the insert was coated with Matrigel diluted in serum-free MEM according to manufacturer's instructions. Inserts were incubated for 1 h at room temperature to allow Matrigel polymerization. The inserts were washed with serum-free MEM. Invasion was tested in the presence of the Matrigel but otherwise as described for the migration assay. The experiment was repeated three times in quadruplicates. Invasion (%) is the ratio between absorbance of the cells collected in the original well over the sum of absorbance of the cells collected in the original well and the cells collected in the insert.

Microarray assay

RNA from CDDP- and ECT-treated cells (with 40 µg/ml) was isolated using Tri Reagent (Sigma Aldrich) and a PureLink Micro-to-Midi Total RNA Purification System (Invitrogen), according to the manufacturer's instructions. In brief, 16 h after treatment, cells were trypsinized, washed in MEM with FCS for trypsin inactivation and resuspended in phosphate buffered saline. After centrifugation, all excess liquid was removed and 1 ml of Tri Reagent was added to each sample. Samples were mixed and centrifuged. The aqueous phase was transferred to a fresh microcentrifuge tube and an equal amount of 70% ethanol was added. Samples were transferred to a PureLink Micro-to-Midi Total RNA Purification System column (Invitrogen) and processed according to the manufacturer's protocol. All samples were washed from the column with 75 µl of RNAse free water.

The quality of isolated RNA was checked on a Bioanalyzer 2100 (Agilent, Santa Clara, California, USA) using RNA 6000 Nano Labchip (Agilent) and 6000 RNA ladder as reference (Ambion, Austin, Texas, USA). Concentration and quantity of RNA were determined with ND-1000 (Nanodrop, Wilmington, Delaware, USA).

Preparation of aaRNA was done using an Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion) according to the manufacturer's recommendations. For each hybridization, we labelled 5 µg of untreated (Cy3) and 5 µg of treated (Cy5) mRNAs. After removing the excess dye, the RNAs were dissolved in Nexterion Hybridization solution (Schott Nexterion, Jena, Germany).

Microarrays were prepared with Human Apoptosis Subset v2.0 and Human Cancer Subset v3.0 (Operon, Ebersberg, Germany) 70mer oligonucleotides and Nexterion 70mer Oligo Microarraying Kit (Schott Nexterion) slides. A single array contained 2698 different genes, each gene being replicated at least four times on each array. Oligonucleotides were spotted using an MG1000 spotter (MicroGrid, Boston, Massachusetts, USA), immobilized and stored according to the manufacturer's instructions (Schott Nexterion). All hybridizations were carried out on HS400 (Tecan) according to the manufacturer's instructions (Schott Nexterion). We used an LS200 scanner (Tecan) at 6 µm resolution for scanning the microarrays.

Data were analyzed using Array-Pro Analyzer 4.5 (Media Cybernetics, Bethesda, Maryland, USA) for feature extraction after imaging of microarrays. Acuity (Molecular devices, USA) was used for filtration of bad signals, LOWESS normalization and microarray data analysis. Features showing signal intensity greater than 65 000 were flagged as bad, whereas features with signal less than two times the intensity of background or coefficient of variation (ratio between standard deviation of the background and the median feature intensity) greater than 0.3 were considered not significantly expressed and were filtered out. Log_2 ratios were normalized using LOWESS

fit [21] and the median from four replicates was used to calculate the average gene expression for a single sample. Differentially expressed genes were selected based on direct comparison between treated and untreated cells, where difference in expression was used as a cut-off for detection. Only those genes that showed a differential expression of more than 1.5-fold in all replicates were considered as differentially expressed. The standard error was calculated for all differentially expressed genes.

Raw data from the experiment are available at the Gene Expression Omnibus database under the serial code GSE15138.

Statistical analysis

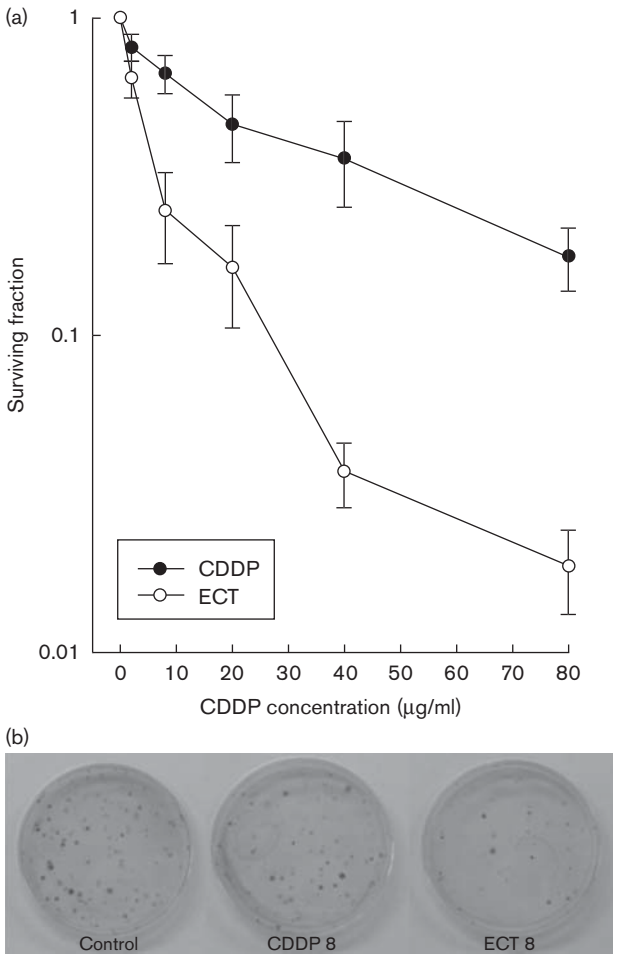
Data obtained from clonogenic, proliferation, adhesion, migration and invasion assays were tested for normality distribution using Kolmogorov–Smirnov test. All pair-wise multiple comparisons were tested using Holm–Sidak method after one-way analysis of variance. SigmaPlot 11.0 (Systat Software Inc., San Jose, California, USA) was used for statistical analysis. P values of less than 0.05 were considered significant. Data are expressed as mean value ± standard error of the mean.

Results

Clonogenic assay

The cytotoxicity of CDDP after electroporation of melanoma SK-MEL28 cells was determined by a colony-forming assay. Exposure of cells to EPs resulted in an increase in CDDP cytotoxicity (Fig. 1). Throughout the range of CDDP concentrations investigated, cells exposed to EPs were more sensitive to CDDP than those unexposed, the difference being statistically significant at a concentration of 8 μg/ml and above. The cells exposed to EPs were 4.8-fold more sensitive to CDDP as determined at IC_{50} value. The IC_{50} value for treatment with CDDP was 28.8 μg/ml, whereas for the ECT, the IC_{50} value was 6.0 μg/ml.

Fig. 1



Potentiation of cisplatin (CDDP) cytotoxicity *in vitro* by electroporation of human melanoma SK-MEL28 cells. (a) Surviving fraction of SK-MEL28 cells after CDDP treatment and after electrochemotherapy (ECT) with CDDP. Cells exposed to electric pulses and CDDP were 4.8-fold more sensitive than cells exposed to CDDP only. (b) Clonogenic assays of human melanoma SK-MEL28 cells after no treatment (left), treatment with 8 μg/ml CDDP (middle) and after ECT with 8 μg/ml CDDP (right).

Proliferation assay

Proliferation of SK-MEL28 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay to assess the viability of cells at the time of metastatic potential determination. CDDP did not affect cell proliferation at 48 and 72 h posttreatment compared with control ($P > 0.05$). Similarly, ECT did not affect cell proliferation, except for the highest concentration used, in which proliferation was significantly lower at 48 and 72 h after ECT (Fig. 2).

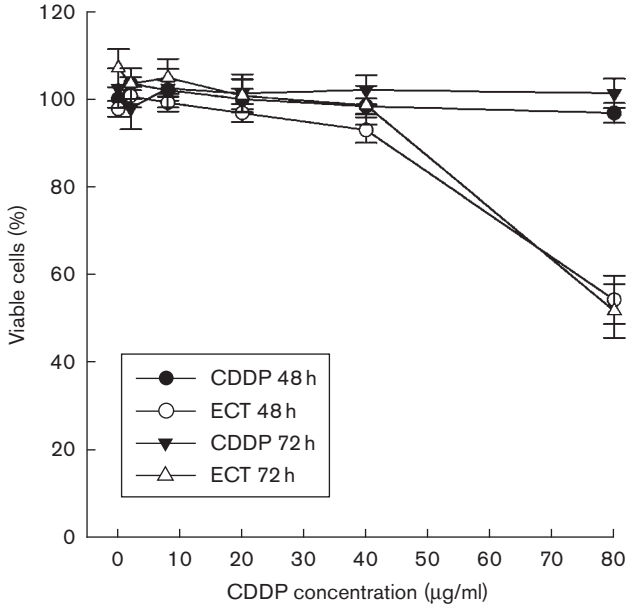
Adhesion assay

Adhesion of SK-MEL28 cells was determined 48 h after CDDP treatment or ECT. Throughout the tested concentrations, neither CDDP treatment nor ECT affected cell adhesion in comparison with control cells. Interestingly, although not significant, there was a trend that cell adhesion was lower after ECT in comparison with CDDP treatment (Fig. 3).

Migration and invasion assays

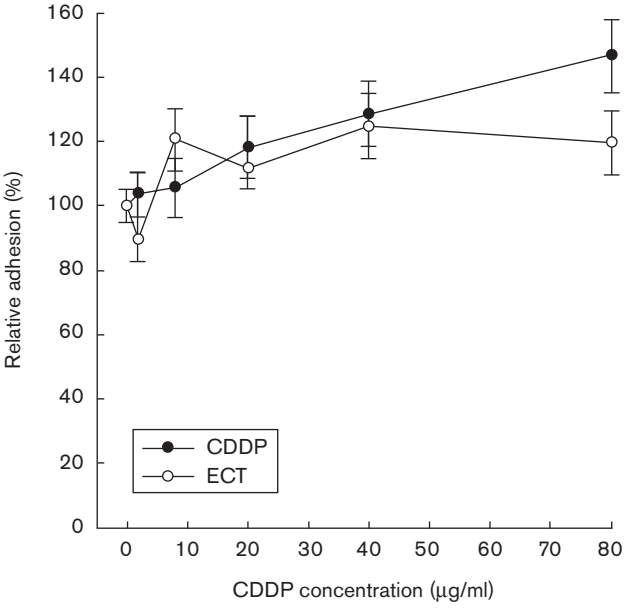
Migration and invasion of SK-MEL28 cells were determined 48 h after CDDP treatment or ECT. Approximately 21% of the control cells migrated through the insert's membrane and 15% of the control cells invaded through the Matrigel under our experimental conditions. Throughout the tested concentrations, neither CDDP treatment nor ECT affected cell migration and invasion in comparison with control cells (Fig. 4). Interestingly, although not significant, cell invasion was lower after ECT in comparison with control and CDDP treated cells.

Fig. 2



Proliferation of SK-MEL28 cells 48 and 72 h after cisplatin (CDDP) treatment or electrochemotherapy (ECT) with CDDP. Results were normalized to control cells after 48 h. Proliferation was significantly lower after ECT with CDDP concentration 80 μg/ml, both after 48 and 72 h. Symbols represent mean values ± standard error of pooled data from two independent experiments.

Fig. 3



Adhesion of SK-MEL28 cells 48 h after cisplatin (CDDP) treatment or electrochemotherapy (ECT) with CDDP. Results were normalized to control cells. There was no significant difference in cell adhesion after CDDP treatment and ECT with CDDP. Symbols represent mean values ± standard error of pooled data from four independent experiments.

Microarray assay

Differentially expressed genes were identified by microarray analysis to evaluate the effects of treatment with CDDP alone or ECT on gene expression. In total, 2698 genes were analyzed. We compared CDDP treatment (CDDP group), application of EP group and ECT group to control group. In the CDDP group, 13 genes (0.5%) were found to be differentially expressed; of which five genes were downregulated and eight genes were upregulated (Table 1). In the EP group, 34 genes (1.3%) were found to be differentially expressed; of which 20 genes were downregulated and 14 genes were upregulated (Table 2). In the ECT group, 13 genes (0.5%) were found to be differentially expressed; of which 11 genes were downregulated and two genes were upregulated (Table 3). These differentially expressed genes are involved in different cellular processes. The major functional categories for these genes include apoptosis, cell cycle, cell proliferation, cell–cell signalling, metabolism, response to stress, signal transduction, transcription and translation.

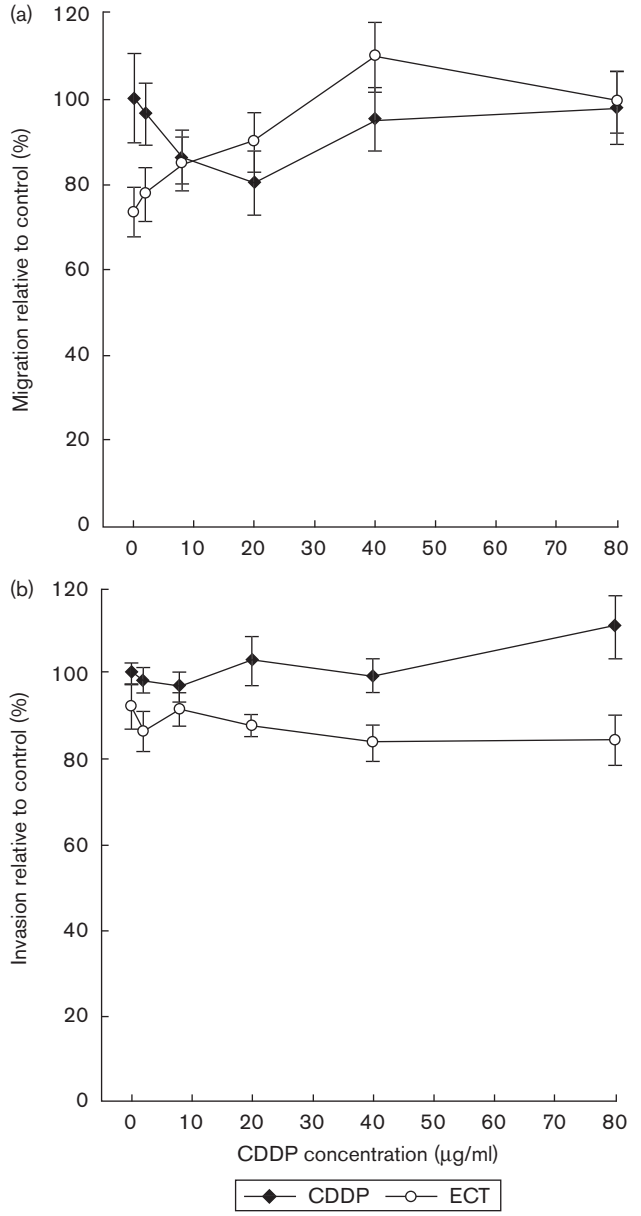
Specifically, in all groups two genes were downregulated. These are RPL31, coding for ribosomal protein L31 and EEF1A1, coding for elongation factor-1 α1. Furthermore, in EP and ECT groups, H4FN, coding for histone H4, TNFRSF14, coding for tumour necrosis factor receptor superfamily member 14 precursor and NQO1, coding for NAD(P)H dehydrogenase 1, were down-regulated, whereas HSPA1B, coding for heat-shock 70 kDa protein 1, was up-regulated. In CDDP and EP groups, CGB5, coding for choriogonadotropin β chain precursor, was downregulated and CCNF, coding for G2/mitotic specific cyclin F and AD7c-NTP, coding for neuronal thread protein, were up-regulated. Furthermore, in ECT group, LAMB3 and CD63 were downregulated. These genes code for β3 chain of laminin 5 and CD63 antigen, respectively, and are involved in cell adhesion and migration.

Discussion

In this study, we showed that metastatic potential of human melanoma SK-MEL28 cells is not affected by ECT with CDDP. In addition, gene expression analysis showed that a very low number of genes are differentially expressed after ECT with CDDP. Furthermore, expression of genes *LAMB3* and *CD63*, coding for proteins promoting metastasis of tumour cells, is downregulated after ECT with CDDP.

Several studies have already evaluated the effect of ECT with CDDP *in vitro*, although not on human melanoma cells. SK-MEL28 cells are sensitive to ECT with CDDP. Their IC_{50} value being 6.0 μg/ml, compared with other human and murine tumour cell lines, in which IC_{50} value was in the range of 0.6–20.5 μg/ml [4–6,22–26].

Fig. 4



Migration and invasion of human melanoma SK-MEL28 cells 72 h after cisplatin (CDDP) treatment or electrochemotherapy (ECT) with CDDP. Results were normalized to control cells. (a) There was no difference in cell migration observed after CDDP treatment and ECT with CDDP. (b) There was no difference in cell invasion observed after CDDP treatment and ECT with CDDP. Symbols represent mean values ± standard error of pooled data from three independent experiments.

Mechanisms involved in anti-tumour effectiveness of ECT are the electroporation of the cells, the tumour drug entrapment, vascular disrupting effect and involvement of immune response [27]. Clinical results have shown that ECT with CDDP, given either systemically or intratumourally, is an effective treatment with no side effects and can be repeated several times with equally good anti-tumour effectiveness in the treatment of malignant melanoma [8,9,28,29].

Of late, it has become evident that the microenvironment plays an important role in metastasis induction. Prevention of metastasis formation is an important aspect of successful treatment. It was revealed, and is now generally accepted, that alterations in the local microenvironment, such as rapid growth of tumour cells and lower number of clonogenic cells needed for tumour growth, provide a suitable environment for wound-induced tumour growth after surgery [12,30,31]. In addition, irradiation of tumour cells can modulate their migratory behaviour. Photon irradiation promotes cell migration and invasive capabilities, whereas in contrast, particle irradiation suppresses metastatic potential of cancer cells [13,32]. Increased metastatic dissemination in human melanoma xenografts [33] and increased migration and invasion of glioma cells was shown after sublethal radiation treatment [34]. It is obvious that different mechanisms are involved in induction of metastasis. It seems that different treatments affect the metastatic potential differentially, possibly through different mechanisms. Although ECT is mainly considered as a local treatment, it is also important to evaluate its effect on migratory potential of the cells exposed to EP and the drug.

Owing to the direct linkage between formation of metastasis and tumour cell migration [13], we were interested in the possible effects of ECT on the motility of SK-MEL28 cells. We compared CDDP treatment, ECT, or application of EP alone to elucidate any effects on metastatic potential of SK-MEL28. Our experiment defined metastatic potential as a characteristic of individual tumour cells that enables them to migrate, invade and adhere to extracellular matrix proteins.

We tested the ability of SK-MEL28 cells that were viable 48 h after ECT, to migrate through the cell culture insert's porous membrane, and to invade through the Matrigel layer and traverse the cell culture insert's membrane. As expected, migration activity of SK-MEL28 was not affected by ECT. There were no significant changes in cell migration observed between cells exposed to CDDP, ECT, or application of EP alone. Evaluation of migration and invasion activities of different epithelial tumour and melanoma cells *in vitro* showed that, among several melanoma cell lines, SK-MEL28 is a cell line with highly migrating cells [35].

Likewise the migration activity, the invasion activity of SK-MEL28 on Matrigel layer was not affected by ECT. There were no significant changes in cell invasion observed between cells exposed to CDDP, ECT, or application of EP alone. Interestingly, there was a trend that cell invasion was even lower after ECT that after CDDP treatment. Our results show that control cells were able to migrate through Matrigel layer, although to a

Table 1 Down-regulated and up-regulated genes after CDDP treatment

Gene symbol (RefSeq)	Fold change[a]	Protein product	Process
Down-regulated genes			
RPL31 (NM_000993)	1.8 ± 0.2	Ribosomal protein L31	Translation
EEF1A1 (NM_001402)	1.8 ± 0.3	Elongation factor-1 α1	Translation
CKS2 (NM_001827)	1.6 ± 0.2	Cyclin-dependent kinase regulatory subunit 2	Cell cycle, cell division, cell proliferation, signal transduction
CCNB1 (NM_031966)	1.5 ± 0.2	G2/mitotic specific cyclin B1	Cell cycle, protein metabolic process, cell division
CGB5 (NM_033142)	1.4 ± 0.1	Choriogonadotropin β chain precursor	Apoptosis, cell–cell signalling, female gamete generation, signal transduction
Up-regulated genes			
FUT1 (NM_000148)	1.8 ± 0.3	Fucosyltransferase 1	Carbohydrate metabolic process
AD7c-NTP (NM_014486)	1.7 ± 0.2	Neuronal thread protein	Cell death
LASS2 (NM_013384)	1.6 ± 0.04	Tumour metastasis suppressor	Lipid metabolic process, transcription
CCNF (NM_001761)	1.4 ± 0.1	G2/mitotic specific cyclin F	Cell cycle, cell division, anatomical structure development
TNFSF4 (NM_003326)	1.4 ± 0.2	Tumour necrosis factor ligand superfamily member 4	Response to stress, cytokine production, cell proliferation, signal transduction
E2F3 (NM_001949)	1.4 ± 0.1	Transcription factor E2F3	Cell cycle, transcription
INSM1 (NM_002196)	1.4 ± 0.2	Insulinoma-associated protein 1	Cell differentiation, anatomical structure development, transcription
HMGB2 (NM_002129)	1.4 ± 0.2	High-mobility group protein 2	DNA metabolic process, chromosome organization, signal transduction, transcription

[a]Values represent mean fold change in expression and standard error of pooled data from three independent experiments relative to control untreated cells.

lesser extent than without this barrier. This was expected because other factors, such as proteolytic enzymes, play an important role in degrading Matrigel layer. In contrast, our results are not in agreement with the study showing that SK-MEL28 cells penetrate neither Matrigel nor collagen type I barriers [35]. It is possible that this difference is because of shorter incubation period (4 h compared with 24 h) and/or different thickness of Matrigel layer used. In our study, we prepared Matrigel layer with a concentration of 0.4 mg/filter in comparison with 10 μg/filter used by Wach *et al.* [35].

As different migration and invasion activities can be affected by the differing ability of cells to attach to membranes [35], we tested the ability of the cells that were viable 48 h after CDDP treatment, ECT, or application of EPs alone to attach to Matrigel. Adhesion of SK-MEL28 to Matrigel did not differ significantly between CDDP treatment, ECT, or application of EPs alone. Interestingly, there was a trend that cell adhesion to Matrigel was lower after application of EPs. Adhesive interactions play a central role in cell migration and they have a dynamic nature enabling cells to attach and detach to different substrates [36]. Migration and invasion activities can be influenced by cell death as well [13]. To avoid this problem, the number of seeded cells was always the same for all experimental groups. In addition, we showed that there was no difference in cell proliferation observed during the 24 h interval of the migration and invasion assays.

The metastatic potential of tumour cells is believed to be regulated by interactions between the tumour cells and their extracellular environment. These interactions can be modified by the accumulation of genetic changes and by the transient alterations in gene expression induced by the local tumour microenvironment [14]. In ECT, because of insufficient drug distribution, suboptimal electroporation and size of tumours, some cells could have acquired genetic alterations that would promote metastatic behaviour of tumour cells. Therefore, differentially expressed genes were investigated by microarray analysis to evaluate the effect of ECT on gene expression. The number of differentially expressed genes after treatment with CDDP, ECT, or application of EP alone, was very low. This is in agreement with the studies carried out on muscles, showing that application of EPs alone to mouse muscle does not induce significant changes in gene expression. It was confirmed that DNA electrotransfer to mouse muscle induces only small changes in the expression of cytoskeletal and intracellular transport proteins, and that no significant changes in gene expression profiles of proteins involved in stress, cell death and inflammation or muscle regeneration were observed in response to EP delivery [37,38]. In addition, EPs used in either ECT or electrogene therapy did not significantly change the expression profile of genes involved in the carcinogenesis, including metastasis of malignant melanoma cells [39]. Furthermore, it was shown that EPs used in ECT did not increase the number of metastasis of liver tumours in rabbits [16]. This study also showed that the application of EPs alone did not affect migration and invasion activities in melanoma SK-MEL28 cells *in vitro*.

Differentially expressed genes after CDDP treatment, ECT, or application of EP alone, were found to be involved in different cellular processes, such as apoptosis, cell cycle, cell proliferation, cell–cell signalling, metabolism, response to stress, signal transduction, transcription and translation. None of the genes downregulated or upregulated after treatment with CDDP were involved in

Table 2 Down-regulated and up-regulated genes after application of electric pulses

Gene symbol (RefSeq)	Fold change[a]	Protein product	Process
Down-regulated genes			
RPL31 (NM_000993)	3.0 ± 0.8	Ribosomal protein L31	Translation
RPS17 (NM_001021)	2.3 ± 0.9	40S ribosomal protein S17	Homeostatic process, translation
TBCA (NM_004607)	2.2 ± 0.5	Tubuline specific chaperone A	Protein folding, tubulin complex assembly
PPIA (NM_021130)	2.0 ± 0.5	Peptidylprolyl cis-trans isomerase A	Interspecies interaction between organisms, protein folding, DNA metabolic process, viral infectious cycle
S100B (NM_006272)	2.0 ± 0.5	S100 protein	Cell proliferation, anatomical structure development, response to stimulus
MYL9 (NM_006471)	1.9 ± 0.3	Myosin regulatory light chain 2	Muscle contraction, heart contraction, anatomical structure development, cell growth
RPA3 (NM_002947)	1.9 ± 0.5	Replication protein A	DNA metabolic process
NQO1 (NM_000903)	1.9 ± 0.3	NAD(P)H dehydrogenase 1	Nitric oxide metabolic process, oxidation reduction, response to stimulus, cell–cell signalling, xenobiotic metabolic process
RPS6 (NM_001010)	1.8 ± 0.1	40S ribosomal protein S6	Translation
H4FN (NM_175054)	1.7 ± 0.1	Histone H4	Chromosome organization
ITGB4 (NM_000213)	1.7 ± 0.2	Integrin β 4 precursor	Cell adhesion, signal transduction
EEF1A1 (NM_001402)	1.7 ± 0.1	Elongation factor-1 α1	Translation
CD28 (NM_006139)	1.7 ± 0.1	CD28 antigen precursor	Signal transduction, immune response, cell proliferation, apoptosis, cytokine production, cell cycle, viral infectious cycle
H3F3A (NM_002107)	1.7 ± 0.2	Histone H3.3	Chromosome organization
CASP9 (NM_001229)	1.7 ± 0.1	Caspase 9 precursor	Apoptosis, protein metabolic process
TNFRSF14 (NM_003820)	1.6 ± 0.1	TNF receptor superfamily member 14 precursor	Apoptosis, signal transduction, immune response
CGB5 (NM_033142)	1.6 ± 0.1	Choriogonadotropin β chain precursor	Apoptosis, cell–cell signalling, female gamete generation, signal transduction
RPH3AL (NM_006987)	1.5 ± 0.06	Rabphilin 3A like	Exocytosis, intracellular protein transport
TFDP1 (NM_007111)	1.5 ± 0.07	Transcription factor Dp-1	Cell cycle, cell proliferation, transcription
CST3 (NM_000099)	1.5 ± 0.06	Cystatin C precursor	Anatomical structure development, collagen metabolic process, response to stimulus, elastin metabolic process, extracellular matrix organization, tissue remodelling, regulation of peptidase activity
Up-regulated genes			
IL6 (NM_000600)	2.0 ± 0.5	Interleukin 6 precursor	Response to stress, signal transduction, cell–cell signalling, immune response, apoptosis, cell proliferation, cytokine production, cell differentiation, protein metabolic process, transcription, translation, response to hormone stimulus
HSPA1B (NM_005346)	1.9 ± 0.1	Heat shock 70 kDa protein 1	Apoptosis, RNA metabolic process, response to stress
RBL2 (NM_005611)	1.7 ± 0.2	Retinoblastoma like protein 2	Transcription, cell cycle, chromosome organization, regulation of kinase activity
CCNF (NM_001761)	1.7 ± 0.1	G2/mitotic specific cyclin F	Cell cycle, cell division, anatomical structure development
CRABP2 (NM_001878)	1.7 ± 0.1	Retinoic acid binding protein II	Anatomical structure development, transcription, hormone metabolic process, signal transduction, transport
GLIPR1 (NM_006851)	1.7 ± 0.2	Glioma pathogenesis related protein	Apoptosis
AD7c-NTP (NM_014486)	1.7 ± 0.2	Neuronal thread protein	Cell death
CDC25C (NM_001790)	1.6 ± 0.03	M phase inducer phosphatase 3	DNA metabolic process, cell cycle, cell division, cell proliferation, interspecies interaction between organisms
RBBP4 (NM_005610)	1.6 ± 0.1	Chromatin assembly factor 1 subunit C	DNA metabolic process, cell cycle, chromosome organization, cell proliferation, transcription
HOXA4 (NM_002141)	1.6 ± 0.1	Homeobox protein HOXA4	Transcription, multicellular organismal development, anatomical structure development
MATR3 (NM_018834)	1.5 ± 0.02	Matrin 3	Transcription
DNAJB1 (NM_006145)	1.5 ± 0.05	DNAJ homolog subfamily B member 1	Protein folding, response to stress
RIN2 (NM_018993)	1.5 ± 0.04	Ras and Rab interactor 2	Endocytosis, signal transduction
RB1 (NM_000321)	1.5 ± 0.02	Retinoblastoma 1	Cell cycle, signal transduction, cell division, cell differentiation, interspecies interaction between organisms, cell growth, cell proliferation, transcription, regulation of kinase activity

[a]Values represent mean fold change in expression and standard error of pooled data from three independent experiments relative to control untreated cells.

metastatic processes. In contrast, after ECT, two genes involved in metastatic processes were downregulated. These were LAMB3, coding for β3 chain of laminin-5 and CD63, coding for CD63 antigen, also known as melanoma-associated antigen ME491.

Laminin-5 consists of three subunit polypeptide chains, α3, β3 and γ2, and has been shown to promote cell growth, migration, invasion and metastasis of tumour cells [40–43]. It was shown that in melanomas, some tumour cells spread along the abluminal side of the small vessels,

Table 3 Down-regulated and up-regulated genes after electrochemotherapy with CDDP

Gene symbol (RefSeq)	Fold change[a]	Protein product	Process
Down-regulated genes			
TNFRSF14 (NM_003820)	2.6 ± 0.7	TNF receptor superfamily member 14 precursor	Apoptosis, signal transduction, immune response
H4FN (NM_175054)	2.1 ± 0.1	Histone H4	Chromosome organization
NQO1 (NM_000903)	2.1 ± 0.2	NAD(P)H dehydrogenase 1	Nitric oxide metabolic process, oxidation reduction, response to stimulus, cell–cell signalling, xenobiotic metabolic process
CYP2A7 (NM_000764)	2.0 ± 0.4	Cytochrome P450 2A7	Oxidation reduction
SGK (NM_005627)	1.8 ± 0.2	Serine/threonine protein kinase SGK	Apoptosis, protein metabolic process, response to stress, ion transport, homeostatic process
EEF1A1 (NM_001402)	1.8 ± 0.03	Elongation factor-1 α1	Translation
LAMB3 (NM_000228)	1.8 ± 0.3	Laminin β3 chain precursor	Cell adhesion, anatomical structure development
CD63 (NM_001780)	1.8 ± 0.1	CD63 antigen	Cell motility, cell growth, signal transduction, cell development
RPL31 (NM_000993)	1.5 ± 0.06	Ribosomal protein L31	Translation
ALDOA (NM_000034)	1.5 ± 0.07	Fructose bisphosphate aldolase A	Carbohydrate metabolic process, anatomical structure development nucleotide metabolic process, actin filament organization, homeostatic process
BRD1 (NM_014577)	1.5 ± 0.04	Bromodomain containing protein 1	Unknown function
Up-regulated genes			
HSPA1B (NM_005346)	1.7 ± 0.1	Heat-shock 70 kDa protein 1	Apoptosis, RNA metabolic process, response to stress
BAG3 (NM_004281)	1.6 ± 0.08	BCL2-associated athanogene 3	Apoptosis, protein folding

[a]Values represent mean fold change in expression and standard error of pooled data from three independent experiments relative to control untreated cells.

and these cells were positioned along the sites staining β2 chain of laminin [40]. Over-expression of the laminin γ2 chain by tumour cells, and lowered laminin α3 and/or β3 chains, may contribute to the loss of basement membrane structures in invasive carcinomas [41]. In our study, gene expression of laminin β2 and γ2 chains was not altered, whereas gene expression of laminin β3 chain was down-regulated after ECT. These results, together with the results showing that migration and invasion of SK-MEL28 melanoma cells were not affected after ECT, support our hypothesis that ECT does not increase metastatic potential in SK-MEL28 melanoma cells.

CD63 antigen, also known as melanoma-associated antigen ME491, is a marker for the early stages of tumour progression of human melanoma [44,45]. CD63 is expressed in several normal tissues, but not in normal tissue melanocytes, and in most cultured melanoma cell lines and is strongly expressed in early stages of melanoma, but its expression is weaker or absent in late malignant stages, suggesting some role of CD63 in malignant progression of melanoma [45,46]. Interestingly, experimental data on CD63 cellular functions are very contradictory. Expressed CD63 antigen reduced the number of metastasis [47] and the decrease in CD63 antigen expression promoted the invasion process of melanoma [46]. However, it was also shown that expression of CD63 antigen dramatically suppressed random tumour cell motility and enhanced migration toward the β_1-integrin substrates such as fibronectin, laminin and collagen [48]. Contradictory effect on cell migration is also apparent for other tetraspanins, as their expression seems to reduce migration when no extracellular matrix component is added, whereas motility seems to increase in the presence of some β_1-integrin substrates [49]. In our study, expression of CD63 was downregulated after ECT, and together with our results on migration and invasion of SK-MEL28 melanoma cells after ECT, this further supports our hypothesis that ECT does not increase metastatic potential of SK-MEL28 melanoma cells.

To conclude, our study showed that metastatic potential of human melanoma SK-MEL28 cells was not affected by ECT with CDDP. ECT did not induce significant changes in gene expression as only a very low number of genes was differentially expressed. In addition, expression of metastasis promoting genes was not increased after ECT and some of them were even downregulated, which further supports the hypothesis that ECT does not affect metastatic potential. However, more detailed analysis of specific gene expression is needed to clearly define the causal relation between metastatic potential and differential gene expression.

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