Electrochemotherapy with bleomycin and cisplatin enhances cytotoxicity in primary and metastatic uveal melanoma cell lines *in vitro*

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Abstract

Electrochemotherapy (ECT) enhances responsiveness to cytotoxic drugs in numerous cell lines *in vitro*. Clinically ECT is widely applied for skin tumor ablation and has shown efficacy in treating non-resectable colorectal liver metastases. There is limited experience of ECT for ocular tumor therapy. We investigated the cytotoxic effect of bleomycin and cisplatin in combination with electroporation on chemoresistant human uveal melanoma (UM) cell lines *in vitro*. Four UM cell lines (Mel 270, 92-1, OMM-1, OMM-2.5) were treated with electroporation (pulse amplitude 300-1000 V/cm, 8-80 pulses, 100µs, 5 Hz) and increasing concentrations of bleomycin and cisplatin (0-7.5µg/ml). Cell survival was analyzed byMTT viability assay after 36 hours. UM cell lines were resistant to both bleomycin and cisplatin. In combination with electroporation, the effects of bleomycin and cisplatin were increased8–70 fold and 3–15 fold,respectively, in all UM cell lines. At the lowest concentration of bleomycin tested (1µg/ml),viability was maximally reduced in all UM cell lines by ≥69% with electroporation conditions of 750 Volts/cm and 20 pulses. All UM cell lines were more resistant to cisplatin; however, electroporation of 1000Volts/cm and 8 pulses resulted in similar reductions in cell viability of 92-1, Mel270 with2.5µg/ml cisplatin, OMM2-5 cells with 5µg/ml cisplatin and OMM1 cells with 1µg/ml cisplatin.*In vitro*ECT with bleomycin or cisplatin is more effective than the highest concentration of the antineoplastic drug or electroporation alone, opening new perspectives in primary and metastatic UM treatment.

Introduction

Disseminated uveal melanoma (UM) is clinically resistant to many chemotherapy drugs, and indeed the current standard of care, dacarbazine, is effective in <8% of individuals with metastaticUM [1,2]. The mechanisms for the relative innate chemoresistance of UM cells are unclear. Those chemoresistance mechanisms previously described in cancer include: decreased drug accumulation; enhanced anti-apoptotic mechanisms; and increased/altered DNA repair pathways. Electroporation, which is based on the local application of short and intense electric pulses that transiently permeabilise cells, has been used to enhance drug entry into otherwise chemoresistant cancer cells and resulted in their death [3-10]. This process of electrochemotherapy (ECT) is also used currently in clinical practice to treat cutaneous and subcutaneous tumor nodules in patients with progressive disease of different malignancies,e.g.soft tissue sarcomas and carcinomas,cutaneous melanoma [11,12], as well as colorectal liver metastases, located in the vicinity of major hepatic vessels, not amenable to surgery or radiofrequency ablation [13]. The treatment can result in complete responses of the tumors with very limited side effects [11]with drug doses that by themselves have minimal or no antitumor activity.

Amongst the several clinically-approved drugs that have been tested in pre-clinical studies of ECT, bleomycin and cisplatin have been shown to be highly effective [4,8]; exposure of cells to electric pulses increases the cytotoxicity of bleomycin and cisplatin given either intravenously or intra-tumorally [14-16]. Previous studies examining the efficacy of cisplatin in UM cells isolated from primary tumors demonstrated no effect of the drug to reduce cell number in nine cultures tested [17].

In order to determine whether chemoresistance in UM is due to an inability to accumulate drug inside the cancer cell, this study evaluated the cytotoxic effect of cisplatin or bleomycin after electroporation of four UM cell lines; Mel 270, 92-1, OMM-1 and OMM-2.5. The initial electroporation conditions were selected according to the ESOPE protocol [12]. The aim of the study

was to examine the effect of ECT on cell viability after reduction of the voltage/pulses combined with different concentrations of the drug. These parameters would support the hypothesis that ECT could be applied on the eye with minor side effects.

Materials and Methods

Cell lines and culture

The human UM cell lines 92-1and Mel270, derived from primary tumor and the OMM-1 as well as OMM-2.5, derived from subcutaneous and liver metastasis respectively, were kindly provided by Prof. Dr. Martine Jager, Leiden University Medical Centre (LUMC), The Netherlands. All cell lines have been STR profiled and mycoplasma tested. They were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum, 1% L-Glutamine (all from Invitrogen, GIBCO, USA) and 2% Penicillin Streptomycin (Thermo Fisher Scientific, USA). All cell lines were maintained as monolayers in 175 cm^2 tissue culture flasks (Thermo Fisher Scientific, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

In vitro Electrochemotherapy (ECT)

When cells reached 70% confluence they were harvested with 0.05% trypsin, counted and $1x10^6$ cells were re-suspended in 400µl of RPMI,with or without bleomycin or cisplatin, in a 4mmgap electroporation cuvette with parallel aluminum plate electrodes (Geneflow, UK). A range of electroporation conditions were applied to the cell suspensions usingthe voltage pulse generator (Cliniporator™) designed by Igea S.p.A. (Capri, Modena, Italy). Details of all experimental conditions are given below.

All cells were treated with 0, 1 µg/ml, 2.5 µg/ml, 5 µg/ml and 7.5 µg/ml bleomycin or cisplatin combined with all following electroporation settings:

(A) No electroporation;

(B) 80 square wave electric pulses of 300 Volts/cm pulse strength, 100 µs pulse duration, 5 Hz repetition frequency;

(C) 40 square wave electric pulses of 300 Volts/cm pulse strength, 100 µs pulse duration, 5 Hz repetition frequency;

(D) 40 square wave electric pulses of 500 Volts/cm pulse strength, 100 µs pulse duration, 5 Hz repetition frequency;

(E) 20 square wave electric pulses of 500 Volts/cm pulse strength, 100 µs pulse duration, 5 Hz repetition frequency;

(F) 20 square wave electric pulses of 750 Volts/cm pulse strength, 100 µs pulse duration, 5 Hz repetition frequency;

(G) 8 square wave electric pulses of 750 Volts/cm pulse strength, 100 µs pulse duration, 5 Hz repetition frequency;

(H) 8 square wave electric pulses of 1000 Volts/cm pulse strength, 100 µs pulse duration, 5 Hz repetition frequency.

Following treatment, $2x10^4$ cells were pipetted into 6 wells of a 96-well plate for each treatment condition and RPMI was added up to a maximum volume of 100µl. The plates were then incubated for 36 hours.

The protocol was conducted for all fourUM cell lines. Each experiment was performed in triplicate on different dates, giving a total of 18 biological replicates for each ECT setting.

MTT viability assay

RPMI-1640 medium was aspirated from each wellafter 36 hours and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) stock solution (5 mg/ml) was added to each well, equal to one-tenth the original culture volume following the protocol provided by Sigma-Aldrich (90 µl media and 10 µl MTT). All plates were then incubated at 37 °C for 4 hours. Following this, the solution was removed and the formazan formed in the cells was dissolved using 100 µl of a 1:1 solution of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and 2-propanol (isopropanol, Sigma-Aldrich, USA). Absorbance of converted dye was measured with a SPECTRAFLUOR (Tecan, Austria) spectrometer at a wavelength of 570 nm.

Results

Each of the fourUM cell lines were exposed to eight different electrical fields. The duration of 100μs and the pulse frequency of 5Hz remained stable whereas the amplitude and the number of pulses varied.Electroporation alone, reduced cell viability in all cell lines at amplitudes of 500 Volts/cmor higher and this effect was augmented with increasing number of pulses. The greatest reduction in cell viability was noted at 1000 Volts/cm for 8 pulses across all four cell lines ranging from a 29.5% reduction in the most sensitive 92.1 cell line to a 25.0% reduction in the least sensitive OMM-2.5 cell line (Figure 1).

Bleomycin alone had no effect on cell viability in the OMM-1 and OMM-2.5 cell lines and reduced cell viability in the 92.1 and Mel270 cell lines by <10% at the maximum concentration tested (7.5μg/ml; (Figure 2A).When electroporation conditions ≥750 Volts/cm were administered to the UM cells, however, bleomycin cytotoxicity was maximally increased by 8-fold in the 92.1 cell line, 25-fold in the Mel270 cell line and by more than 70-fold in the OMM-1 and OMM-2.5 cell lines (Figure 3). In order to minimize systemic toxicity of bleomycin, we were interested in the electroporation conditions that in combination with the lowest dose of bleomycin tested (1μg/ml) had the maximal effect to reduce cell viability. In the 92.1 and Mel270 cell lines this was achieved at 750 Volts/cm for 20 pulses, reducing cell viability by 74% and 69%, respectively (Figure 3A and 3B). In the OMM-1 and OMM-2.5 cell lines there was little difference between the effectiveness of 1μg/ml bleomycin when combined with electroporation conditions of either 750 Volts/cm for 20 pulses or 1000 Volts/cm for 8 pulses, with a reduction in cell viability of between 76% and 89% (Figure 3C and 3D).

Similar to bleomycin, cisplatin alone had little effect on cell viability at the concentrations tested (Figure 2B), with a maximum 15% reduction in viability of the 92.1 cell line at 7.5μg/ml cisplatin. When electroporation conditions ≥500 Volts/cm were administered to the UM cells, however, cisplatin cytotoxicity was maximally increased by 3, 6, 10 and 15-fold in the 92.1, Mel270, OMM-1 and OMM-2.5 UM cell lines respectively (Figure 4). In combination with electroporation the most

156 sensitive UM cell line was OMM-1, which showed an 80% reduction in cell viability with 1000
157 Volts/cm for 8 pulses and 1µg/ml cisplatin (Figure 4C). In the 92.1, Mel270 and OMM-2.5 cell lines,
158 higher concentrations of cisplatin in combination with electroporation conditions of 1000 Volts/cm
159 for 8 pulses were necessary to achieve similar reductions in viability as noted for the OMM-1 cells.
160 For example, 1000 Volts/cm for 8 pulses with 2.5µg/ml cisplatin was necessary to reduce viability of
161 the 92.1 and Mel270 cell lines by 77% and 70% respectively (Figure 4A and 4B); whilst 1000 Volts/cm
162 for 8 pulses with 5.0µg/ml cisplatin was necessary to reduce viability of the OMM-2.5 cell line by 75%
163 (Figure 4D).

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Discussion

In this novel study we investigated the efficiency of electroporation with bleomycin and cisplatin in fourhuman UM cell lines that demonstrate resistance to these chemotherapeutic drugs at their commonly achieved peak plasma concentrations of 0.5 – 5.0µg/ml and 0.5 – 2.0µg/ml, respectively. We show for the first time that electroporation sensitizes UM cells to doses of either drug within these ranges.

Bleomycin is an anti-tumor antibiotic that causes single and double strand DNA breaks in tumor cells resulting in cell death. It is used to treat a range of malignancies, including head and neck cancer, testicular carcinomas and lymphomas [18-22]. In UM it has been used in the metastatic setting as part of a multicentre study of bleomycin, vincristine, lomustine and dacarbazine (BOLD) in combination with recombinant interferon alpha-2b, although only a modest effect of this regimen against UM at hepatic sites was reported [23]. Cisplatin is another commonly used anti-cancer agent that causes DNA crosslinks resulting in DNA damage, and subsequently inducing apoptosis in cancer cells.It is commonly used in the treatment of lung-, ovarian-, and head-and-neck carcinomas, but has been shown to have little effect in combination chemotherapy for metastatic UM [24].

Bleomycin is a large non-permeant drug, a characteristic that contributes to the resistance of many cell types to this agent [25]. Studies on the Chinese hamster lung cell line (DC-3F) have shown that if bleomycin can enter the cell, <500 molecules of the drug are needed to cause cell death [25,26]. Althoughresistance to cisplatin is considered to be multifactorial, evidence suggests that plasma membrane transporters resulting in the extrusion of cisplatin play a major role in the resistance mechanism(s)[27].

In this study we have shown that by applying an electrical field to UM cells above a threshold amplitude of 500 Volts/cm, sensitivity to bleomycin and cisplatin are greatly increased, and that this is further enhanced by an increased number of pulses as has previously been reported [28.29]. Electroporation creates transient permeable pores in the cell membrane thus enhancing drug entry

and accumulation in the cell [30,31], and indeed ECT has been shown to be effective in a variety of other tumor cell types *in vitro*[3-10]. Furthermore, ECT for skin metastases from tumors of non-cutaneous origin as well as for skin melanoma is currently part of the NICE interventional procedure guidance for these lesions [32].

Small differences in the sensitivity of the cell lines to ECT with both bleomycin and cisplatin were also noted. In particular, the OMM-1 cell line was more sensitive to ECT with cisplatin than the 92.1, Mel270 and OMM-2.5 cell lines. OMM-1 cells are derived from a subcutaneous metastatic UM; whilst 92.1 and Mel270 cells, are derived from primary tumors, and OMM-2.5 is from a hepatic UM metastasis. Previous studies have reported thatcell size, shape, membrane structure, composition and transmembrane potential can affect electroporation [33,34]. In the current study no differences were observed in the response of the four UM cell lines to electroporation despite striking differences in the size and shape of these cells. We did not examine, however, other membrane features, but this will be pursued in primary and metastatic UM cell cultures in the near future. Various preclinical models are available for the study of primary and metastatic UM, and would lend themselves to the examination of new and older chemotherapeutic agents in combination with ECT [35].

In summary, electroporation provides a more targeted pathway into UM cells for bleomycin and cisplatin. The application of this treatment could lead to the shrinkage of large, non-treatable UM in order to enable a further surgical intervention and avoid enucleation as primary treatment. Furthermore the application of ECT could allow a lower drug doses and a reduction of systemic side effects in the treatment of large non-resectable UM hepatic metastases, as has been demonstrated in colorectal liver metastases located close to major hepatic vessels, not amenable to other treatments [13]. The combination of various chemotherapy agents and ECT thus requires further investigation *in vitro* and *in vivo* to investigate the challenges of a clinical application of the protocol in disseminated UM.

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Figure legends

Figure 1 – Effects of electroporation on cell viability 36 hours following exposure. Data are the mean ± SEM of 6 individual experiments for the 92.1 (black bars), Mel270 (Dark grey bars), OMM-1 (white bars) and the OMM-2.5 (hashed bars) cell lines.

Figure 2 – Effects of (A) bleomycin and (B) cisplatin on viability of the 92.1 (solid black line), Mel270 (dotted black line), OMM-1 (solid grey line) and OMM-2.5 (dashed black line) UM cell lines 36 hours after exposure to the drugs. Data are the mean ± SEM of 3 separate experiments.

Figure 3 – Cytotoxic effects of increasing doses of bleomycin on the viability of (A) 92.1, (B) Mel270, (C) OMM-1 and (D) OMM-2.5 UM cell lines following electroporation. Data are the mean of 18 replicates across three separate experiments for the effect of electroporation alone (black bars), 1μg/ml (dotted bars), 2.5μg/ml (grey bars), 5μg/ml (striped bars) and 7.5μg/ml (white bars) bleomycin to reduce cell viability.

Figure 4 – Cytotoxic effects of increasing doses of cisplatin on the viability of (A) 92.1, (B) Mel270, (C) OMM-1 and (D) OMM-2.5 UM cell lines following electroporation. Data are the mean of 18 replicates across three separate experiments for the effect of electroporation alone (black bars), 1μg/ml (dotted bars), 2.5μg/ml (grey bars), 5μg/ml (striped bars) and 7.5μg/ml (white bars) cisplatin to reduce cell viability.