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Doxorubicin delivery enhanced by electroporation to gastrointestinal adenocarcinoma cells with P-gp overexpression

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ABSTRACT

Electroporation (EP) can effectively support the penetration of macromolecules from the extracellular space into cells. Electropores induced by the influence of electromagnetic field generate additional paths of transport for macromolecules. The aim of this study was evaluation of the electroporation effect on doxorubicin transport efficiency to human colon (LoVo and LoVo/DX) and gastric (EPG85-257/P and EPG85-257/RDB) adenocarcinoma cells with overexpression of P-glycoprotein and murine macrophage cell line (P388/D1). In our EP experiments cells were placed into a cuvette with aluminum electrodes and pulsed with five square electric pulses of 1300 V/cm and duration of 50 μs each. Cells were also treated with low doxorubicin concentration ([DOX] = 1.7 μM). The ultrastructure (TEM) and changes of P-glycoprotein expression of tumor cells subjected to electric field were monitored. The mitochondrial cell function and trypan blue staining were evaluated after 24 h. Our results indicate the most pronounced effect of EP with DOX and disturbed ultrastructure in resistant gastric and colon cells with decrease of P-gp expression. Electroporation may be an attractive delivery method of cytostatic drugs in chemotherapy, enabling reduction of drug dose, exposure time and side effects.

1. Introduction

Cancers of the gastrointestinal (GI) tract are the most common cause of death and regarded as civilizational diseases. Genetic predisposition, age, diet, and stomach disease can affect the risk of developing a gastric cancer. Commonly used methods of treating gastrointestinal cancer include surgery, chemotherapy and radiation therapies. Chemotherapy is currently one of the main standard treatments for progressive neoplastic diseases, including GI cancers, particularly following surgical exclusion of tumors. One of the main reasons for ineffective chemotherapeutic treatment is resistance of the cancer cells to cytotoxic drugs. There are many mechanisms responsible for the resistance of cancer cells. The most recognized mechanisms involve activity of ABC-transporters, apoptosis disorders, DNA repair defects, overexpression of protooncogenes, and also diminished expression of tumor suppressors [1,2]. Due to therapeutic problems oncology researchers focus on new strategies for application of cytotoxic drugs. One of the potent solutions is drug delivery supported by electroporation, which can improve the efficiency of applied chemotherapy. Electrochemotherapy (ECT) is a physical procedure that enables cytotoxic drugs to be efficiently delivered in tumor cells by induced permeabilization of the plasma membrane. The process of membrane electro-permeabilization is still not fully understood, and in different cancer types separate metabolic pathways can be involved in ECT with regard to the sensitivity to electric field, calcium homeostasis and drug resistance [3–5]. This method has been successfully applied in several animal models and clinical trials with various types of cancers: squamous cell carcinoma, basal cell carcinoma, melanoma and liver tumors [5–18]. The main anticancer drugs used in ECT are bleomycin (non-permeant) and cisplatin (weakly permeant). The first clinical trial on liver metastasis of colorectal tumors with bleomycin was performed by Edhemovic et al. with good treatment effectiveness even in difficult-to-reach locations [19]. There have also been published data on electroporated colorectal cell lines in vitro treated with bleomycin, whose authors indicated electropermeabilization enhanced cytotoxicity of bleomycin and cisplatin [20].

In the present study we investigate the effect of membrane electropermeabilization applied in combination with doxorubicin. Doxorubicin (DOX), an anthracycline antitumor antibiotic is a hydrophilic drug, used in a broad range of cancers. Generally available pharmacokinetic data show that doxorubicin is not suitable for oral administration because only less than 5% of the drug is absorbed. In the present research we

Abbreviations: EP, electroporation; ECT, electrochemotherapy; DOX, doxorubicin; MDR, multidrug resistance; MDR1, multidrug resistance protein 1; P-gp, permeability glycoprotein; TEM, transmission electron microscopy; CLSM, confocal laser microscopy; rER, rough endoplasmic reticulum.

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applied short time incubation with a cytostatic drug (10 min) assisted by electroporation in order to check drug absorbance yield into the cell. This was an attempt to simulate local drug administration in low concentration and short time of exposure. On the basis of this information tumor cells were selected with regard to the potential methods of drug administration (oral/rectal), which may allow delivery of sufficient amount of the cytostatic drug to the targeted gastric and colon carcinoma. The next criterion for cell line selection was strong drug resistance of both types of adenocarcinoma to all standard pharmacological therapies in clinical practice [21]. The aim of this study was evaluation if EP can increase the cytotoxic effects of DOX on multidrug resistant cell lines. As experimental models, multidrug resistant (MDR) human gastric adenocarcinoma cells (EPG85-257/P and EPG85-257/RDB) and colon adenocarcinoma cell lines (LoVo and LoVo DX) were applied. Both adenocarcinoma cell types: EPG85-257/RDB and LoVo DX exhibit strong drug resistance to any pharmacological therapy in clinical practice [21]. Additionally, the same experiments were performed on P388/D1 cells, which are macrophage-like cells suitable for analysis of the immune system response.

2. Experimental section

2.1. Cell lines

The studies were performed on human adenocarcinoma cell lines: doxorubicin or daunorubicin — sensitive (LoVo: ATCC CCL-229, EPG85-257P), doxorubicin or daunorubicin resistant (LoVoDX, EPG85-257 RDB). LoVoDX cell line indicates resistance to doxorubicin and EPG85-257 RDB cell line resistance to daunorubicin. Both cell lines overexpress MDR1 (known as P-gp) mechanism involvement [1]. For a comparison, P388/D1 cells, which are the mouse macrophage-like cell line, were also used.

Colon adenocarcinoma cell lines and P388/D1 (ATCC CCL-46) were obtained as a kind gift from the Institute of Immunology and Experimental Therapy in Wroclaw. P388/D1 cell line was isolated from a methylcholanthren-induced lymphoid neoplasm of a DBA/2 mouse and it exhibits characteristics typical of macrophages [22]. The macrophages were grown in RPMI/Dulbecco's (1:1; Sigma, Poland) medium, and colon adenocarcinoma cells (LoVo and LoVoDX) were grown in Ham F-12 medium (Lonza, Poland) with addition of 10% fetal bovine serum (FBS, Biowhittaker) and supplemented with antibiotics (penicillin/streptomycin; Sigma). Gastric cell lines were grown in Leibovitz L-15 medium (Sigma, Poland) supplemented by 10% fetal bovine serum (FBS, Lonza, Poland), 1 mM Ultraglutamine (Sigma, Poland), 6.25 mg/L fetuin (Sigma, Poland), 2.5 mg/mL transferrin (Sigma, Poland), 0.5 g/L glucose, 1.1 g/L $NaHCO_3$, 1% minimal essential vitamins (Mem-Vit, Sigma, Poland). For the experiments, the cells were removed by trypsinization (Trypsin 0.025% and EDTA 0.02%; Sigma) and washed with PBS (BioShop, Lab Empire Poland). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

2.2. Electroporation protocol

EP alone and EP with doxorubicin (EP + DOX) were performed using ECM 830 Square Wave Electroporation System (BTX Harvard Apparatus, purchased from Syngen Biotech, Poland). After trypsinization and centrifugation (5 min, 1000 rpm, Centrifuge MPW Med. Instruments MPW-341 with stable rotor), cells were counted ([cells density] = 3×10^6/ml) and resuspended in 100 μl of EP buffer with low electrical conductivity of 0.12 S/m (10 mM KH_2PO_4/K_2HPO4, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) [23]. In case of DOX (Sigma, Poland) treatment, cells were suspended in EP buffer with [DOX] = 1.7 μM (1 μg/ml). Cell suspension was pulsed in a cuvette with two aluminum plate electrodes with electrical field of E(appl) = 1300 V/cm (Eq. (1)), which was previously proved as effective [24].

$$E(appl) = \frac{U(appl)}{d\_electrode} \quad (1)$$

U(appl) = 520 V (electrical field);
d_electrode = 0.4 cm (distance between electrodes in the cuvette);

Five rectangular electrical pulses were delivered by the electroporator, pulse duration of 50 μs and frequency of 1 Hz. Control cells were not permeabilized, only incubated with EP buffer, or with DOX in EP buffer. After pulsation, before further experiments, the cells were left for 10 min at 37 °C, centrifuged, and resuspended in cell culture medium.

2.3. Cells viability

Cells viability was determined by the MTT assay (In Vitro Toxicology Assay, Sigma, Poland). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed 24 h after the end of experiments to evaluate cells mitochondrial dehydrogenase activity (NADH) as a viability marker. The cells viability assay was performed according to the manufacturer's protocol. The absorbance was measured at 570 nm using multiwell plate reader (EnSpire Multimode Reader, Perkin Elmer, Poland). Three samples per experiment were prepared. Additionally each experiment was performed in 3 independent repetitions. Mean values and standard deviations of all results were calculated. The final results were expressed as the percentage of mitochondrial function relative to untreated control cells.

2.4. Trypan blue staining

To observe cell necrosis, after experiments performed according to the protocol described in Section 2.2, the samples containing cell suspension needed to be placed on a basic slide and incubated overnight to allow adhesion. Afterwards, 0.4% trypan blue solution (concentration 1:1 in PBS; Sigma Aldrich, Poland) was added intravitally to cover the whole cell surface. Cells had to be observed immediately afterwards, otherwise the dye could start to penetrate the living cell membranes. Samples were examined with Olympus BX51 optical microscopy (Olympus, Poland). Results were determined by counting stained cells within 100 cells in 3 randomly selected fields. Stained dark blue nuclei were judged as necrotic cells. The percentage of necrotic cells was determined as the ratio of trypan blue negative cells to the total number of cells according to Eq. (2):

$$NC\% = \frac{nNC \times 100\%}{nTC} \quad (2)$$

NC — percentage of necrotic cells
nNC — number of necrotic cells
nTC — number of total cells (at least 100)

2.5. Expression of P-glycoprotein by immunofluorescence

Confocal laser scanning microscopy (CLMS) was used for evaluation of doxorubicin treatment effects, with regard to MDR1 distribution in different cell lines. LoVo, LoVoDX, EPG85-257 P, EPG85-257 RDB, and P388/D1 cells were prepared for immunofluorescence experiments. After EP alone, [DOX] = 1.7 μM, or EP + DOX (1.7 μM), cells were grown on coverslips for 24 h. Then samples were fixed with 4% paraformaldehyde (Roth, Linegal Chemicals, Poland) in PBS, permeabilized with 0.5% Triton X-100 (Sigma, Poland) in PBS (v/v) (for 5 min at room temperature) and blocked with 1% BSA in PBS (for 30 min at room temperature). All washing steps were done with PBS. The following antibodies were used: primary monoclonal anti-MDR1 antibody produced in mouse (overnight incubation at 4 °C; 1:100; Santa Cruz Biotechnology); secondary antibody goat anti-mouse IgG FITC