Bioelectrochemistry 123 (2018) 248–254

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Electrochemotherapy with cisplatin or bleomycin in head and neck squamous cell carcinoma: Improved effectiveness of cisplatin in HPV-positive tumors

Ajda Prevc [a,b], Martina Niksic Zakelj [a,b], Simona Kranjc [a], Maja Cemazar [a,c], Janez Scancar [d], Tina Kosjek [d], Primoz Strojan [b,e,*], Gregor Sersa [a,f,*]

[a] Department of Experimental Oncology, Institute of Oncology, Ljubljana, Zaloska 2, SI-1000 Ljubljana, Slovenia
[b] Faculty of Medicine, University of Ljubljana, Vrazov trg 2, SI-1000 Ljubljana, Slovenia
[c] Faculty of Health Sciences, University of Primorska, Polje 42, SI-6310 Izola, Slovenia
[d] Department of Environmental Sciences, Jozef Stefan Institute, Jamova cesta 39, SI-1000 Ljubljana, Slovenia
[e] Department of Radiation Oncology, Institute of Oncology, Ljubljana, Zaloska 2, SI-1000 Ljubljana, Slovenia
[f] Faculty of Health Sciences, University of Ljubljana, Zdravstvena pot 5, SI-1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 29 December 2017
Received in revised form 9 May 2018
Accepted 5 June 2018
Available online 06 June 2018

Keywords:
Electrochemotherapy
HPV infection
Cisplatin
Bleomycin
Pharyngeal squamous cell carcinoma

ABSTRACT

Human papillomavirus (HPV) is an important etiological factor in head and neck squamous cell carcinomas (SCCs). Standard treatment of HPV-positive tumors with platinum-based radio(chemo)therapy results in a better outcome than in HPV-negative tumors. Electrochemotherapy is becoming an increasingly recognized mode of treatment in different cancers; thus, its use in the management of head and neck SCC is of considerable interest. However, response to electrochemotherapy according to HPV status of the tumors has not been evaluated yet. Thus, our aim was to compare the effect of electrochemotherapy with cisplatin or bleomycin between HPV-negative and HPV-positive human pharyngeal SCC derived cell lines and tumor models.

HPV-positive cells and tumors were found to be more sensitive to electrochemotherapy with cisplatin than HPV-negative ones, whereas sensitivity to electrochemotherapy with bleomycin was similar irrespective of the HPV status. The higher sensitivity of HPV-positive cells and tumors to electrochemotherapy with cisplatin is likely due to the higher level and slower repair of DNA damage. In HPV-negative tumors, a higher number of complete responses was recorded after bleomycin-based rather than cisplatin-based electrochemotherapy, while in HPV-positive tumors electrochemotherapy with cisplatin was more effective.

1. Introduction

Electrochemotherapy is a promising local ablative treatment that has already been used in the clinics for the treatment of melanoma, basal and squamous cell carcinoma (SCC) and other types of tumors [1]. It combines intravenous or intratumoral application of chemotherapeutic drugs (cisplatin or bleomycin) and local application of short electric pulses (electroporation). The latter are used to increase the permeability of the cell membrane, leading to an increased uptake of chemotherapeutic drugs [2]. The effectiveness of electrochemotherapy is high, with 60–70% complete response and about 80% objective response rate [3, 4]. It depends on several factors, among them on the concentration of the chemotherapeutic agent in the tumor at the time when the electric pulses are applied and on the electric field distribution over the tumor which further depends on pulse parameters. It has also been found that tumor type and previous treatments with other modalities are important factors determining the effectiveness of electrochemotherapy [4, 5].

Human papillomavirus (HPV) is a well-established etiological factor in different cancers, also in oropharyngeal SCC [6]. HPV-positive oropharyngeal SCC was found to respond better to radio(chemo)therapy than HPV-negative tumors [6–8]. The reason for the increased sensitivity of HPV-positive tumors to ionizing radiation and some systemic drugs is thought to be due to HPV viral proteins E6 and E7 which interfere with the regulatory mechanisms of the cell cycle and DNA repair mechanisms [6, 9, 10]. Also, it is speculated that E6 and E7 viral proteins increase the immunogenicity of these tumors, which also contributes to better responsiveness of tumors to radio(chemo)therapy [6, 11].

In patients with oropharyngeal SCC, the rate of local and/or regional tumor re-appearance is in the range of 50% [12], and

Abbreviations: HPV, human papillomavirus; SCC, squamous cell carcinoma; PI, propidium iodide.

* Corresponding authors at: Zaloska ulica 2, SI-1000 Ljubljana, Slovenia.
E-mail addresses: pstrojan@onko-i.si, (P. Strojan), gsersa@onko-i.si (G. Sersa).

https://doi.org/10.1016/j.bioelechem.2018.06.004
1567-5394/

electrochemotherapy has a potential to become a valid treatment option in selected patients with recurrent tumors.

The impact of HPV status of the oropharyngeal SCC on the effectiveness of electrochemotherapy has not been tested yet. Thus, we hypothesized that HPV infection might predispose these tumors to greater sensitivity to electrochemotherapy. The aim of the present study was to compare the effectiveness of electrochemotherapy using either cisplatin or bleomycin, in an HPV-negative and an HPV-positive human head and neck SCC derived cell lines and tumor models. The possible underlying mechanisms that could be responsible for eventual differences observed, *i.e.* drug uptake and DNA damage repair capacity of the cells, were explored as well.

2. Materials and methods

2.1. Cell lines

Pharyngeal SCC cell line FaDu (American Type Culture Collection, VA, USA) and HPV-positive pharyngeal SCC cell line 2A3 (derived from the FaDu cell line, a gift from prof. Dadachova [13]) were used. They were cultured in advanced Dulbecco's Minimum Essential Medium (ADMEM, Gibco, Thermo Fisher Scientific, MA, USA) supplemented with 5% fetal bovine serum (FBS, Thermo Fisher Scientific), 10 mM/l L-glutamine (Thermo Fisher Scientific), 100 U/ml penicillin (Germany) and 50 mg/ml gentamicin (Krka, Slovenia). For 2A3 cell line, the medium was additionally supplemented with 1 mg/ml G418 disulfate salt solution (Sigma-Aldrich, MO, USA). Cell lines were cultured at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. In vitro electrochemotherapy

The cells were trypsinized, centrifuged for 5 min at 1500 rpm (470 ×g) and resuspended in electroporation buffer (125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4, 2 mM $MgCl_2 \times 6H_2O$). A suspension with the concentration of 2.2×10^7/ml was prepared, 90 μl (2×10^6 cells) was taken out and 10 μl of the chemotherapeutic agent in a concentration 10 times higher than the final concentration (ranging from 5 to 50 μM for cisplatin and 10^{-5}–10 μM for bleomycin) or 0.01 M phosphate buffered saline (PBS) (for control) was added. For electrochemotherapy, 50 μl of this suspension was placed between two parallel stainless steel plate electrodes with 2 mm gap in-between and exposed to electric pulses generated by the electric pulse generator GT-01 (Faculty of Electrical Engineering, University of Ljubljana, Slovenia). Eight square wave pulses with amplitude over distance ratio 1300 V/cm, duration 100 μs at frequency 1 Hz were applied. The other 50 μl was used as a control without the electric pulse application. After the treatments, cells were incubated for 5 min at room temperature (21 °C), thereafter diluted in 1 ml of medium and plated for clonogenic assay.

2.3. Clonogenic assay

After the treatment, cells were plated in 6-cm Petri dishes in 4 ml medium at different densities (depending on the therapy intensity, ranging from 400 to 2400). Nine (for FaDu) or fourteen (for 2A3) days after the treatment, when the colonies were formed (groups of at least 50 cells were considered a colony), they were stained with crystal violet solution (Sigma-Aldrich) and counted. The plating efficiency and surviving fraction were calculated. Plating efficiency was defined as the ratio of the number of colonies to the number of cells plated and the surviving fraction as the ratio of plating efficiencies between the treated group and the control [14].

2.4. Electropermeabilization

Cell electropermeabilization was measured by propidium iodide (PI) uptake. The cells were prepared for electroporation as described above. Before electroporation, 7.5 μl of 100 μM PI (Sigma-Aldrich) was added to 67.5 μl cell suspension, then, the cells were immediately exposed to electric pulses as described above. After electroporation, the cells were incubated at room temperature for 5 min. Then, 25 μl of the cell suspension was resuspended in 1 ml PBS, and PI uptake was measured immediately by FACSCanto II flow cytometer (BD Biosciences, CA, USA). Fluorescence was detected with the bandpass filters 488/10 for forward and side scatter (FSC and SSC) and 585/42 for PI.

2.5. γH2AX foci immunofluorescent staining

The cells were plated in a 6-well plate with coverslips on the bottom of the wells. After 24 h incubation, the chemotherapeutic agent (1 μg/ml cisplatin or 5 μM bleomycin) was added for 2 h. The medium with the chemotherapeutic was removed, the cells washed with PBS, and fresh medium added. At two different time points (6 and 24 h for cisplatin or 2 and 24 h for bleomycin) after the chemotherapeutic exposure, the cells were fixed in 4% paraformaldehyde and 0.1% Triton X-114 mixture for 20 min at room temperature, washed in PBS three times, permeabilized in 0.5% Triton and PBS mixture for 15 min at room temperature, washed with PBS three times and then blocked in 5% BSA. Coverslips were incubated overnight in mouse monoclonal anti-gamma H2A.X (phospho S139) antibody [9F3] (ab26350, Abcam, USA) in a 5% BSA and 0.1% Triton X-114 mixture at 4 °C. After the cells were washed five times in PBS, coverslips were incubated in secondary donkey anti-mouse IgG H&L (Alexa Fluor 488) antibody (ab150105, Abcam) diluted in 0.1% Triton X-114 for 1 h at room temperature. The cells were then washed three times with PBS and once in distilled water, coverslips were mounted on microscope slides with Fluoroshield with DAPI (Sigma-Aldrich) for nuclei counterstaining, dried and viewed under the fluorescence microscope Olympus BX-51 (Olympus, Japan) equipped with a camera DP72 (Olympus) using filters U-MWIB (Olympus) for γH2AX foci and U-MWU2 (Olympus) for nuclei counterstaining at 100× magnification. The number of γH2AX foci per nuclei was evaluated by image analysis using Fiji software [15].

2.6. In vivo electrochemotherapy

In the experiments, 6–8 week old female SCID mice (C·B-17/IcrHsd-Prkd^{cscid}, Envigo, Italy) were used. The mice were maintained in a 12 h light/dark cycle under specific pathogen-free conditions at constant room temperature and humidity. Food and water were provided *ad libitum*.

Treatment protocols were approved by the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (Permission No. 34401-1/2015/23) based on the approval of the National Ethics Committee for Experiments on Laboratory Animals and conducted in accordance with the guidelines for animal experiments of the EU Directive.

For induction of subcutaneous tumors, a 100 μl suspension of 2×10^6 FaDu or 5×10^6 2A3 cells, prepared from cell cultures *in vitro,* was injected subcutaneously into the flanks of the mice. When the tumor volume reached 40 mm^3, the mice were divided into the following experimental groups: control (CTRL), cisplatin or bleomycin chemotherapy, application of electric pulses alone and in combination with cisplatin or bleomycin (electrochemotherapy). Experimental groups consisted of 11–12 mice.

In the experiments, the drug dosage that can result in complete responses in different tumor models was selected (4 mg/kg cisplatin or 5 mg/kg bleomycin) [16, 17]. The drugs, cisplatin or bleomycin, were injected intravenously (i.v.) in the orbital sinus of the mice in 0.2 ml of physiological saline, alone or in combination with pulse application. Electric pulses (8 pulses (4 in one direction and then 4 in a