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Multiplexed in vivo fluorescence optical imaging of the therapeutic efficacy of photodynamic therapy☆

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ABSTRACT

In our study we wanted to elucidate a time frame for in vivo optical imaging of the therapeutic efficacy of photodynamic therapy (PDT) by using a multiplexed imaging approach for detecting apoptosis and vascularization. The internalization of the photosensitizer Foslip® into tongue-squamous epithelium carcinoma cells (CAL-27) was examined in vitro and in vivo. For detecting apoptosis, annexin V was covalently coupled to the near-infrared dye DY-734 and the spectroscopic properties and binding affinity to apoptotic CAL-27 cells were elucidated. CAL-27 tumor bearing mice were treated with PDT and injected 2 days and 2 weeks thereafter with DY-734-annexin V. PDT-induced changes in tumor vascularization were detected with the contrast agent IRDye® 800CW RGD up to 3 weeks after PDT. A perinuclear enrichment of Foslip® could be seen in vitro which was reflected in an accumulation in CAL-27 tumors in vivo. The DY-734-annexin V (coupling efficiency 30–50%) revealed a high binding affinity to apoptotic compared to non-apoptotic cells (17.2% vs. 1.2%) with a K_D-value of 20 nM. After PDT-treatment, the probe showed a significantly higher ($p < 0.05$) contrast in tumors at 2 days compared to 2 weeks after therapy (2–8 h post injection). A reduction of the vascularization could be detected after PDT especially in the central tumor areas. To detect the therapeutic efficacy of PDT, a multiplexed imaging approach is necessary. A detection of apoptotic cells is possible just shortly after therapy, whereas at later time points the efficacy can be verified by investigating the vascularization.

1. Introduction

Non-invasive optical imaging of molecular processes in vivo is a promising technology for the therapeutic monitoring in personalized medicine [1]. Among the different oncologic therapeutic approaches available so far, like chemotherapy or radiation, photodynamic therapy (PDT) of tumors uses light along with the administration of a photosensitizing agent as a therapeutic tool [2]. In effect, a direct cell death occurs as a result of the production of reactive oxygen species. Besides the induction of apoptosis in tumor cells, the vasculature is damaged, which leads to an undersupply of the tumor with nutrients and ultimately determines the therapeutic success [3,4].

A central process in the effective elimination of tumor cells is apoptosis [5]. Cells externalize phosphatidylserine on their outer membrane leaflet in the early phase of apoptosis [6]. This process makes the phospholipid accessible to targeted probes where it serves as target for detecting apoptotic cells. Typical examples of phosphatidylserine targeted probes are annexin V based probes [7,8].

In recent studies the use of different modified ^{99m}Tc-labeled annexin V for single photon emission computed tomography (SPECT) or autoradiography have been reported [9,10]. Also targeting of apoptotic cells with biotinylated annexin V followed by administration of ^{64}Cu-labeled streptavidin allowed apoptotic tissues to be imaged by positron emission tomography (PET) [11]. Although the utilization of near-infrared fluorescent (NIRF) probes for the in vivo optical imaging of apoptosis is also very suitable and non-invasive, only few data demonstrated the use of NIRF probes in imaging apoptosis. After conjugation of annexin V to Cy5.5, such probes were shown to image the effect of cyclophosphamide chemotherapy on Lewis lung carcinoma at 24 h post treatment

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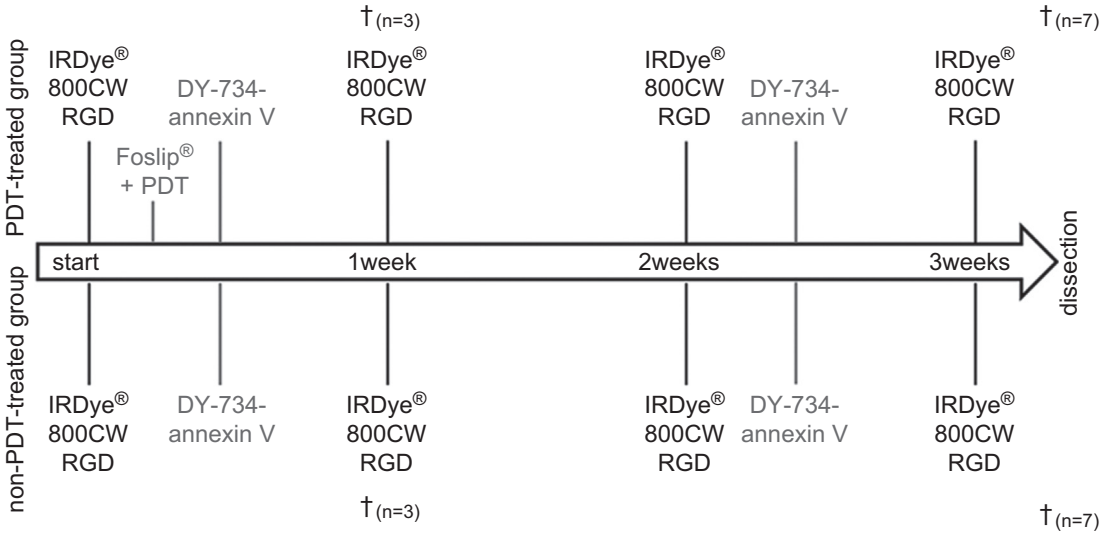

Fig. 1. Schematic presentation of the in vivo experimental design for imaging tumor vascularization and apoptotic cells after PDT. The time points demonstrate injection of the respective probes in PDT-treated and non-PDT-treated mice. †: animals were sacrificed.

[12,13]. In contrast, a failure of annexin-based in vivo apoptosis imaging was demonstrated at day 4 of an anti-angiogenic therapy because of a significant breakdown of the vasculature [14]. These reports reveal that the use of multiplexed methods which enable simultaneous detection of apoptosis and vascularization are inevitable in the reliable monitoring of therapeutic efficacy. Apoptosis is important for detecting cell death as a consequence of the therapeutic efficacy of PDT and vascularization also verifies probe accessibility. The advantage of fluorescence optical imaging over PET is that it allows multiplexed approaches by means of a simultaneous detection of several fluorescent probes which differ in their emission maxima.

In this study, we therefore sought to elucidate the appropriate time frame for in vivo imaging of the therapeutic efficacy of PDT via the use of a multiplexed imaging approach which addresses both the phosphatidylserine molecules on cells at the tumor region as well as the expression of $\alpha_v\beta_3$ integrin on the surface of proliferating endothelial cells as a marker for vascularization. We first examined the internalization of the photosensitizer formulation Foslip® into the tumor cells to corroborate a basic prerequisite for a successful PDT. Then, the binding affinity of a self-designed NIRF annexin V probe to the cells was evaluated in vitro. We subsequently used the probe for detection of apoptosis in mice xenografts together with an integrin-targeted probe to assess the level of vascularization at defined time points after PDT.

2. Materials and methods

2.1. Cells and animals

Tongue-squamous epithelium carcinoma cells (CAL-27, DSMZ, Braunschweig, Germany) were cultured in DMEM-GlutaMAX™ (Gibco®, Darmstadt, Germany) containing 10% FBS, at 37 °C in a humidified 5% CO_2 atmosphere. In vivo experiments were carried out with female athymic nude-Foxn1^{nu} mice (20–25 g, Harlan Laboratories GmbH, Venray, The Netherlands). During experimentation, the animals were anesthetized with isofluorane (Actavis, Munich, Germany). All procedures were approved by the regional animal committee and were in accordance with international guidelines on the ethical use of animals. Mice were housed under standard conditions with food and water ad libitum.

2.2. In vitro characterization and biodistribution of the photosensitizer formulation Foslip®

The photosensitizer formulation Foslip® (liposomal formulation of meta tetrahydroxyphenyl chlorine – mTHPC) was provided by biolitec research GmbH (Jena, Germany) as a lyophilized powder that was reconstituted with sterile water to 1.5 mg mTHPC ml^{-1}. To verify the spectroscopic features of Foslip®, both the absorption and the emission spectrum were determined via spectrophotometry and spectrofluorometry.

To investigate the cellular uptake of Foslip®, CAL-27 cells were incubated with 50 μM of the photosensitizer for 24 h. Nuclei were counterstained with 0.2 μg/ml Hoechst-33258 (Applichem, Darmstadt, Germany). Cells were analyzed using a confocal laser scanning microscope (LSM 510 Meta, Zeiss, Jena, Germany).

The effect of laser treatment alone was investigated by illuminating CAL-27 cells in vitro with different light dosages up to 100 J/cm^2 and measuring caspase 3/7 activity (important enzyme of apoptosis) as well as cell viability by XTT assay at 24 h after illumination.

To study the biodistribution of Foslip®, 2×10^6 CAL-27 cells in Matrigel™ (BD, Heidelberg, Germany) were injected subcutaneously into 8 mice. When the tumors reached a diameter of approximately 5 mm, mice were injected with Foslip® (50 μg/kg, $n = 5$). 3 mice (control group) were left without any photosensitizer to monitor autofluorescence of the organs. 24 h post injection, the animals were sacrificed and the fluorescence intensity of the tumors and organs was measured ex vivo using the Maestro™ in vivo fluorescence imaging system (excitation filter: 570–610 nm, emission filter: 645 nm longpass, CRi Inc., Woburn, MA, USA). The generated composite spectral data were unmixed into the spectrum of the autofluorescence of the mice and the spectrum of Foslip®. For semiquantitative analysis, regions of interest (ROIs) were drawn over the spectrally unmixed image of tumor and organs. For every ROI an averaged fluorescence signal was determined, which represents the area under the curve of the unmixed Foslip® spectrum. Average counts were normalized to exposure time and pixel number.

2.3. Labeling of annexin V with the NIRF dye DY-734

To detect apoptotic cells after PDT via fluorescence optical imaging, annexin V (Abcam, Cambridge, UK) was covalently coupled to the near-infrared dye DY-734 (using a NHS-ester derivative, abs: 736 nm, em: 759 nm, Dyomics GmbH, Jena, Germany): 0.1 mg annexin V was mixed with DY-734-NHS in coupling buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8) and incubated for 2 h (22 °C and 900 rpm). Unbound dye molecules were separated by ultrafiltration on a 10 kDa amicon column (Merck Millipore, Billerica, MA, USA). After washing 3 times with PBS, the fluorescent annexin V probe (DY-734-annexin V) was eluted and the success of coupling as well as the annexin V to DY-734 ratio were determined spectroscopically. Hereto, the absorption maxima of both the protein and the dye were used and the ratio was determined via the Lambert–Beer law.

2.4. Determination of the binding affinity of DY-734-annexin V to apoptotic cells

To induce apoptosis, CAL-27 cells were incubated with etoposide (30 μM, Sigma–Aldrich, Munich, Germany) for 24 h. Afterward, the cells were split into 4 different groups and stained as follows: 1) native control (without staining), 2) apoptosis (staining with 10 μg/ml DY-734-annexin V), 3) necrosis (staining with propidium iodide (PI), Annexin-V-FLUOS Staining Kit, Roche, Grenzach, Germany), and 4) apoptosis and necrosis (staining with DY-734-annexin V and PI). The same procedure was applied to cells without etoposide to control the autofluorescence and the