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Non-thermal near-infrared exposure photobiomodulates cellular responses to ionizing radiation in human full thickness skin models

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ABSTRACT

Ionizing and near-infrared radiation are both part of the therapeutic spectrum in cancer treatment. During cancer therapy ionizing radiation is typically used for non-invasive reduction of malignant tissue, while near-infrared photobiomodulation is utilized in palliative medical approaches, e.g. for pain reduction or impairment of wound healing. Furthermore, near-infrared is part of the solar wavelength spectrum. A combined exposure of these two irradiation qualities – either intentionally during medical treatment or unintentionally due to solar exposure – is therefore presumable for cancer patients. Several studies in different model organisms and cell cultures show a strong impact of near-infrared pretreatment on ionizing radiation-induced stress response. To investigate the risks of non-thermal near-infrared (NIR) pretreatment in patients, a human in vitro full thickness skin models (FTSM) was evaluated for radiation research. FTSM were pretreated with therapy-relevant doses of NIR followed by X-radiation, and then examined for DNA-double-strand break (DSB) repair, cell proliferation and apoptosis. Double-treated FTSM revealed a clear influence of NIR on X-radiation-induced stress responses in cells in their typical tissue environment. Furthermore, over a 24 h time period, double-treated FTSM presented a significant persistence of DSBs, as compared to samples exclusively irradiated by X-rays. In addition, NIR pretreatment inhibited apoptosis induction of integrated fibroblasts, and counteracted the radiation-induced proliferation inhibition of basal keratinocytes. Our work suggests that cancer patients treated with X-rays should be prevented from uncontrolled NIR irradiation. On the other hand, controlled double-treatment could provide an alternative therapy approach, exposing the patient to less radiation.

1. Introduction

During cancer therapy patients can be exposed to a variety of radiation qualities. Low doses of ionizing radiation for instance are applicable for medical imaging techniques (1–10 mGy), while high doses are an important curative component of cancer radiotherapy, in order to devastate malignant tissue (fractionated up to a local dose of 80 Gy). Water-filtered near-infrared can be applied for the reduction of pain as well as for the improvement of wound healing, e.g. in case of superficial cancerous lesions [1,2]. Notably, near-infrared makes up 40% of the energy from solar radiation reaching the earth's surface. Therefore, an unintentional combined exposure to both radiation qualities will often occur. Furthermore, a positive impact of near-infrared photobiomodulation on radiotherapy of cancer patients has been reported [3,4]. Because of their different physical properties, the impact of radiation on tissues varies widely. The high energetic X-rays have the ability to induce ionizing events which in turn can lead to long lasting disruptive molecular effects. Therefore, therapy-relevant doses of ionizing radiation are utilized to reduce malignant tissue non-invasively by inducing a large number of cellular damages, eventually leading to cell death. The most harmful effects are DNA-double strand breaks (DSBs) because, if not correctly repaired, such damage can lead to persistent mutations of the genome. DSBs arise from direct interaction of ionizing radiation with the DNA as well as indirectly via emerging reactive oxygen species [5]. The induction of DNA damages in proliferative cells normally results in cell cycle arrest, preventing cells from passing on damages during cell cycle progression [6]. During cell cycle arrest cells activate phase-specific repair mechanisms [7]. If the repair of cellular damages fails for any reason, cells will induce cell death signalling [8]. Contrary, near-infrared radiation does not result in long-term changes of biological matter. Near-infrared primarily affects tissues via absorption of the radiation energy through different molecular

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chromophores, a process called photobiomodulation. The main cellular absorber of near-infrared is the cytochrome *c* oxidase (COX), located in the inner membrane of mitochondria. Activation of COX increases respiratory metabolism associated with an increase of ROS in certain cells [9–12]. Based on changes in mitochondrial metabolism near-infrared radiation might regulate gene transcription via activation of the so called retrograde signalling pathway between mitochondria and the nucleus [10,13,14]. Different wavelengths of the near-infrared spectrum have been shown to influence besides cell proliferation also cell metabolism, and angiogenesis in skin, bone and skeletal muscles [2,9,11,15–22]. Furthermore, protective effects of near-infrared irradiation against heat treatment and UV-induced apoptosis have been reported recently [23–25]. In contrast to this preserving impact, near-infrared in combination with X-rays worsens the cellular damages induced by ionizing radiation. Comparable to early findings in plants and *Drosophila*, for different mammalian cells we could document a repair delay of ionizing radiation-induced DNA damages [11,26–30]. Also, our study revealed that pretreatment with non-thermal near-infrared (NIR) counteracts cell cycle arrest in X-irradiated cells and increases the number of mitotic catastrophes caused by therapy relevant doses of X-rays [11]. These in vitro observations can help to establish improved radiotherapy options by combining these two types of radiation, but also could lead to unexpected risks for this group of patients, especially because the selected NIR parameters approximate with an outdoor solar exposure as reached already within 15–30 min (depending on season, daytime and geographic location: 22 year average in June: 14 min Rome, Italy; 18 min New York, USA; 24 min Frankfurt, Germany; 30 min Sydney, Australia; data from the Atmospheric Science Data Center of NASA). Due to the high likelihood of combined exposure of cancer patients, an investigation of possible negative effects of NIR on radiotherapy outcome is of great clinical relevance. In order to examine this possible risk for patients, we first evaluated our human cell-based in vitro full thickness skin models (FTSM) for radiation research [31]. Subsequently we treated FTSM with our previously established treatment regime, comprising pretreatment with NIR and irradiation with therapy-relevant X-ray doses. Our analyses focused on DSB repair, proliferation, cell death as well as cell morphology.

2. Material and Methods

2.1. Cell Culture

2.1.1. Isolation

Normal human fibroblasts as well as normal keratinocytes were isolated from infantile foreskins. Donor tissue was washed several times with PBS supplemented with a 2% (v/v) penicillin/streptomycin solution (Gibco, Germany). For separation of the dermal and epidermal part skin specimens were treated with thermolysin (Sigma-Aldrich, Germany) over-night at 4 °C. Supportive epidermis was mechanically removed from dermal tissue using sterile forceps. To obtain fibroblasts from the dermis the extracellular matrix was digested by 0.25% collagenase I solution (Worthington, USA) within 4 h at 37 °C. For keratinocyte isolation, epidermal sheets were incubated in a 0.05% trypsin/EDTA solution (Gibco) for 20 min at 37 °C. During the incubation both tissue parts were vigorously pipetted. Resulting cell suspensions were filtered through sterile gauze and centrifuged. Keratinocytes were then seeded at a density of 2×10^6 cells/ml in keratinocyte DermaLife K Complete-Medium (CellSystems, Germany). Fibroblasts were seeded at a density of 2×10^6 cells/ml in DMEM (Gibco) supplemented with 5% (v/v) fetal calf serum (PAA, Germany), 1 mM ascorbic acid 2-phosphate (Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin solution [32,33].

2.1.2. Culture of FTSM

Primary fibroblasts were cultured in MatriDerm® (MedSkin Solutions Dr. Suwelack AG, Germany) at a density of 4×10^5 cells per cm^2 in above-named medium to create the dermal part of the FTSM. Next keratinocytes were seeded on top at a density of 4×10^5 cells per cm^2 in DermaLife K Complete-Medium. After a certain period of submerse cultivation the tissue-like culture was lifted up to the air-liquid interphase to induce epidermal cell differentiation. Medium was changed to AirLiquidInterphase-Medium (DMEM (Gibco) supplemented with 30% (v/v) Ham's F12 (Gibco), 2% (v/v) L-glutamine (Lonza Group AG, Switzerland), 2% (v/v) bovine serum albumin (Roth, Germany), 1% (v/v) ascorbic acid 2-phosphate, 1% (v/v) penicillin/streptomycin solution 0.3% (v/v) insulin (Gibco) and 0.2% (v/v) hydrocortisone (Sigma-Aldrich). Before FTSM were used for irradiation experiments the differentiation status was controlled via haematoxylin/eosin staining (H/E) of untreated control models [31]. For immunofluorescence staining and TUNEL-analysis FTSM were fixed in 4% paraformaldehyde in PBS at 4 °C for 24 h and cryoprotected overnight in 25% (v/v) sucrose/dH_2O. Tissue was embedded in RAS Neg 50 (Tissue Tek, Thermo Fisher Scientific, USA) and sectioned at a thickness of 12 µm. Differentiation and morphology of FTSM post-treatment were evaluated by H/E. All studies were conducted according to the Declaration of Helsinki Principles and in agreement with the Local Ethics Commission/Institutional Review Board.

2.2. Irradiation

Photobiomodulation with non-thermal near-infrared (NIR) was performed as described earlier [11]. Cells were irradiated in irradiation medium (DMEM, free of carbonate, supplemented with 10 mM HEPES). During irradiation culture dishes were placed on a water-cooled tray to maintain sample temperatures between 20 and 25 °C. To ensure homogeneous irradiation exclusively with the wavelength spectra of water-filtered near-infrared (600–1400 nm, non-thermal) with cutting off the specific water absorption bands of 944 and 1180 nm, a 4 mm thick frosted glass and a 20 mm ice-cooled water filter were interposed between the IR radiator (Philips IR250RH, Germany) and the sample plane. The cell reaching irradiance of 20 mW/cm^2 was measured with a SPECTRO 320 D spectroradiometer (Instrument Systems GmbH, Germany). Cells were irradiated for 30 min, equivalent to 360 kJ/m^2 NIR or were light protected as sham-control (S). X-radiation (90 kV, 33.7 mA, 5.23 Gy min^{-1}) was carried out in AirLiquidInterphase medium. When necessary, medium was supplemented with 25 µM BrdU (Sigma).

2.3. Immunofluorescence and Histochemical Analysis of FTSM

Antigene retrieval was performed in citrate buffer for 1 h at 95 °C. Paraformaldehyde-fixed (4%) cryosections were incubated with primary antibodies (53BP1 1:500, Bethyl/Biomol, USA; γH2AX (Ser139) 1:400, Merck Millipore, Germany, BrdU 1:500, G3G4, DSHB, Germany; Ki-67 1:500, Abcam, UK) in blocking solution (PBST/0.2% Triton X-100/1:10 RotiBlock) for 5 h at 37° or overnight at 4 °C. Secondary antibodies (Cy3-, Alexa488- or Alexa 594-conjugated, Dianova, Germany) were diluted 1:400 in blocking solution and incubated for 2.5 h at room temperature. Cell nuclei were counterstained with DAPI. Images of immunofluorescence-stained tissue sections were taken on a confocal microscope (Leica TCS SP5 II, Germany) with LAS AF Lite software (Leica, Germany). Apoptotic cells in cryosections were determined via TdT-mediated dUTP nick end labeling (TUNEL), according to manufacturer's manual (Promega, Germany). Cell nuclei were counterstained with DAPI. $TUNEL^+$ cells were detected via Axio Observer.D1 (Zeiss, Germany). For morphological analysis cryosections were stained with haematoxylin/eosin. Image processing and arrangement of all microscopic images were done with ImageJ [34].

2.4. Image Analysis and Statistics

Quantification of 53BP1-foci, BrdU- and $Ki\text{-}67^+$ cell nuclei was performed on confocal images. Foci were determined either in 50