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Research Paper

Laser Therapy Inhibits Tumor Growth in Mice by Promoting Immune Surveillance and Vessel Normalization

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ABSTRACT

Laser therapy, recently renamed as photobiomodulation, stands as a promising supportive treatment for oral mucositis induced by oncological therapies. However, its mechanisms of action and, more importantly, its safety in cancer patients, are still unclear. Here we explored the anti-cancer effect of 3 laser protocols, set at the most commonly used wavelengths, in B16F10 melanoma and oral carcinogenesis mouse models. While laser light increased cell metabolism in cultured cells, the in vivo outcome was reduced tumor progression. This striking, unexpected result, was paralleled by the recruitment of immune cells, in particular T lymphocytes and dendritic cells, which secreted type I interferons. Laser light also reduced the number of highly angiogenic macrophages within the tumor mass and promoted vessel normalization, an emerging strategy to control tumor progression. Collectively, these results set photobiomodulation as a safety procedure in oncological patients and open the way to its innovative use for cancer therapy.

1. Introduction

The number of clinical and preclinical studies, assessing the use of laser light in the prevention and treatment of oral mucositis (OM) in oncological patients undergoing chemo and/or radiotherapy, is rapidly growing (Migliorati et al., 2013). Despite the exact mechanisms by which laser light impacts on biological tissues have not been clarified, the remarkable reduction in local inflammation and promotion of wound healing (Lins et al., 2010), eventually results in a rapid analgesic effect and in a net improvement in the quality of life of the patients (Chung et al., 2012).

Since 2009, we are successfully exploiting Class IV laser light in our clinical practice for both the prevention and the treatment of radio/chemo-induced OM and dermatitis (Chermetz et al., 2014, GOBBO et al., 2014, Ottaviani et al., 2013, Gobbo et al., 2016), constantly obtaining a faster wound healing and a reduced relapse frequency. We have recently compared the efficacy of low-power and high-power laser therapy (LPLT and HPLT) approaches, differing in their wavelength (635 nm for the LPLT and 970 nm for the HPLT) and thus in tissue penetration capacity. We found that both protocols, but mostly HPLT, are able to stimulate the formation of new arterial vessels and the proliferation of vascular smooth muscle cells (Ottaviani et al., 2013).

These encouraging results also opened additional, relevant questions. In particular, considering that the laser therapy, recently named as photobiomodulation (PBM) (Anders et al., 2015), is often applied to head and neck oncological patients, what could be the consequence of promoting angiogenesis and cell proliferation on either dysplastic or neoplastic lesions within the oral cavity of the patients? A few studies have so far assessed the effect of laser light on cancer cell metabolism and proliferation, supporting the hypothesis that PBM could foster the development and the growth of neoplastic lesions (De Castro et al., 2005, Sperandio et al., 2013). However, studies investigating the effects of laser irradiation on different tumor cell lines in vitro have generated conflicting results, and very few of them considered the behavior of tumor cells in vivo, using different protocols and obtaining inconsistent data (Frigo et al., 2009).

Based on these considerations, here we explored the effect of PBM both in cultured cells and in various in vivo models of cancer. In

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particular, we compared the activity of 3 different laser protocols (L1, L2 and L3), based on the wavelengths most commonly used in pre-clinical and clinical studies (660, 800 and 970 nm, respectively).

2. Material and Methods

2.1. Laser Devices and Protocols

A gallium arsenide (GaAs) + indium gallium aluminium arsenide phosphide (InGaAlAsP) diode laser device (class IV, K-Laser Cube series, K-laser d.o.o., Sežana, Slovenia) was employed to irradiate cultured cells and animals. To provide a uniform irradiation to multiwell plates, the device was equipped with an adapted prototype probe, specifically designed by Eltech S.r.l. Cells were seeded on sterile 24-well plates (well area: 2 cm^2) in 500 μl of medium without cover during irradiation. The emission tip was hold perpendicular above the culture media and the irradiation was carefully timed and carried out in a dark room. The diode area laser source consisted of equal diodes, which emitted an elliptic laser field with a Gaussian distribution of irradiance (Zacchigna et al., 2014). The emitted light completely covered the irradiated field of each culture plate, as assessed using an optical power meter. The control group was not exposed to laser, but during the laser treatment dishes were removed from the incubator, the cover was removed and cells were kept at room temperature (RT). Three different laser protocols were employed:

- L1: λ 660 nm, laser power 100 mW, irradiance 50 mW/cm^2, fluence 3 J/cm^2, time 60 s, continuous wave
- L2: λ 800 nm, laser power 1 W, irradiance 200 mW/cm^2, fluence 6 J/cm^2, time 30 s, continuous wave
- L3: λ 970 nm, laser power 2.5 W, irradiance 200 mW/cm^2, fluence 6 J/cm^2, time 30 s, continuous wave

The same protocols were applied in vivo, once a day for 4 consecutive days, keeping the laser pointer perpendicular above the affected tissues (tumor area 2 cm^2).

2.2. Cell Lines and Primary Cells

Mouse B16F10 melanoma cells (CRL-6475; ATCC) and human bone osteosarcoma cells (U2OS, HTB-96; ATCC) were grown in complete medium with DMEM 1 g/L glucose supplemented with 10% of FBS, 2 mM L-glutamine, penicillin and streptomycin.

Human skin fibroblasts (HSF, PCS-201-012; ATCC) were plated in complete medium (PCS-201-041; ATCC). Commercial human umbilical vein endothelial cells (HUVEC, CRL-1730; ATCC) were seeded onto gelatin pre-coated acrylic plates, in complete medium (EGM, CC-2517; Lonza). Primary bone marrow-dendritic cells (BM-DCs) were isolated by flushing femura and tibiae of C57BL/6 male mice with RPMI-1640, followed by red blood cell lysis with Ammonium-Chloride-Potassium (ACK) buffer and supplementation with murine recombinant granulocyte macrophage-colony stimulating factor (GM-CSF, 800 U/ml). Aggregates of BM-DCs, evident upon removal of granulocytes and residual lymphocytes, were dislodged by gentle pipetting, counted and plated. mDCs were harvested after 7 days, when the number of $CD11c^+$ was higher than 80%. DCs were also treated with lipopolysaccharide (LPS, 200 ng/ml) endotoxin to promote the secretion of pro-inflammatory cytokine.

2.3. In Vitro ATP Production Assay

Cells plated on 24-well plates were exposed to the various laser protocols and cell proliferation was measured at 24 and 48 h using the ATPlite Luminescence Assay System (PerkinElmer, Waltham, MA), according to manufacturer's instructions. Experiments were performed on 3 biological replicates, each one consisting of 3 technical replicates.

2.4. Animal Models

Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 12, 1987) and upon approval by the Institutional Animal Care Use Committee and by the Italian Minister of Health. *Ifnar1*$^{-/-}$ (IFNAR KO) mice, lacking the α chain of the type IFN α/β receptor. on a C57BL/6 background were provided by U. Kalinke (Twincore, Hannover Germany).

Animals (n = 48 C57BL/6 and n = 24, IFNAR KO 6-week-old female mice) were housed under controlled environmental conditions for 5 days with a 12-hour light/dark cycle. Each animal was injected with 1×10^6 B16F10 melanoma cells at the dorsal subcutaneous level. After 10 days, when all masses were clearly detectable, C57BL/6 and IFNAR KO mice were homogenously divided according to tumor size into 4 and 2 groups, respectively. C57BL/6 mice were treated with L1, L2 and L3 protocols, while IFNAR KO mice with L3 protocol from day 11 to 14, whereas one group was used as a control. Tumor volume and mice weight were daily evaluated using a caliper and calculated applying the following formula: $V = \pi/6 * (d^2_{max} * d_{min}/2)$ (Tomayko and Reynolds, 1989). At the end of the experiment (day 15) tumor masses were eventually harvested for accurate measurement of their volume and weight, prior to histological or flow cytometry analysis.

For the oral carcinogenesis model, 50 6-week-old C57BL/6 female mice were used, following the same guidelines described above. The 4-NQO carcinogen (Sigma-Aldrich) was dissolved in propylene glycol (4 mg/ml), diluted in the drinking water to a final concentration of 50 μg/ml, administered to mice for 16 weeks and replaced by regular water starting from the 17th week. All mice were weighed every 4 weeks (Chang et al., 2010, Hawkins et al., 1994). Mice were randomized to receive only the 4-NQO carcinogen (n = 25, control) or to be also exposed to the L3 laser protocol (n = 25, laser group) for 4 consecutive days during the 20th week. Dysplastic and neoplastic oral lesions, mostly located on the ventral side of the tongue, were diagnosed and processed to both macroscopic and microscopic examination by week 21.

The minimal number of animals compatible with obtaining valid scientific results was calculated by a statistical design of the sample size using the software http://homepage.stat.uiowa.edu/~rlenth/Power/, setting a variation coefficient (s) of 30%, alpha of 5%, and a power of 80% (p).

Randomization was performed using a random number schedule and the main experimenter was blind to group assignment.

2.5. Evaluation of Thermal Effect

The temperature of the medium inside the cell culture wells and on the body surface of mice was monitored at baseline, during and after PBM using the Ti20 Thermal Imager (Fluke), as graphically described in Supplementary Fig. S1.

2.6. Histology and Immunofluorescence

For histopathological evaluation, 5 μm tissue sections were stained with haematoxylin and eosin and analyzed by three independent reviewers. The observed lesions were classified into 5 different types: mild dysplasia (D1), moderate dysplasia (D2), severe dysplasia (D3), in situ squamous cell carcinoma (SCC) or invasive SCC. Histopathological diagnosis and grading were based on established criteria (Akhter et al., 2011, Tang et al., 2004) to obtain the following measures: i) the extension of each lesion (D1, D2, D3, in situ SCC, invasive SCC), expressed as a percentage of the total perimeter of the tongue; ii) the

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