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ORIGINAL ARTICLE

Photobiomodulation effects on head and neck squamous cell carcinoma (HNSCC) in an orthotopic animal model

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Abstract

Background Photobiomodulation (PBM) has shown efficacy in preventing and treating cancer therapy-induced mucositis and dermatitis. However, there is contradictory information regarding the effect of PBM on (pre)malignant cells, which has led to questions regarding the safety of this technique. We address this issue using an orthotopic mouse model (Cal-33) with human squamous cell carcinoma of the oral cavity.

Methods Mice with actively growing orthotopic Cal-33 head and neck carcinoma tumors were divided into 4 groups: control, PBM only, radiation therapy (RT) only, and PBM + RT. We performed three experiments: (1) PBM at 660 nm, 18.4 J/cm^2, and 5 RT × 4 Gy doses delivered daily; (2) PBM at 660 nm, 18.4 J/cm^2, and 1 × 15 Gy RT; and (3) PBM at 660 nm + 850 nm, 45 mW/cm^2, 3.4 J/cm^2, and 1 × 15 Gy RT. Mice were weighed daily and tumor volumes were evaluated by IVIS. Survival time was also evaluated.

Results Animals treated with RT survived significantly longer and had significantly smaller tumor volume when compared with the control and PBM-only treatment groups. No significant differences were noted between the RT alone and PBM + RT groups in any of the experiments.

Conclusion Our results suggest that PBM at the utilized parameters does not provide protection to the tumor from the killing effects of RT.

Keywords Photobiomodulation · Radiation therapy · Head and neck cancer · Orthotopic mouse model

Introduction

Mucosal damage is a common side effect of cytotoxic cancer therapies [1]. To date, the pathobiological processes at work in cancer therapy-induced mucositis have not been completely elucidated, but it is complex and dependent on multiple variables [2, 3]. Given the cost and morbidity of this side effect, its prevention has been a research priority [4]. However, with the notable exception of cryotherapy for bolus drug infusion regimens [5], and keratinocyte growth factor 1 (Palifermin) for

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hematopoietic cell transplant patients [6, 7], there are no established effective means for prophylaxis against cancer therapy-induced mucositis [7].

More recently, studies have demonstrated the clinical efficacy of PBM for prevention and/or amelioration of oral mucositis [7–11] induced by both chemo- and radiation therapy. However, discrepant information exists regarding the safety of PBM, particularly with regard to the possibility that the same mechanism that prevents integument breakdown may also protect or enhance the growth of the tumor cells. Some in vitro studies suggested that PBM stimulates malignant cell growth and invasion [12, 13], while others showed sensitization of malignant cells to killing by RT [14–16]. One clinical study that followed PBM-treated head and neck cancer patients for 8 years described a significant survival advantage for these patients when compared with controls [17]. Nevertheless, no conclusive data exist to determine whether or not PBM may impart protection from cytotoxic treatments to malignant tissues in the field of exposure. This issue is particularly relevant for head and neck cancer. Thus, in the current study, we evaluated tumor effects of PBM used prior to RT in an animal model of human head and neck cancer.

Materials and methods

All in vivo studies were conducted according to the Memorial Sloan Kettering Research Animal Resource Center-approved protocols.

Orthotopic HNSCC model

Cal-33 cells were maintained as a monolayer culture in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified incubator at 37 °C in an atmosphere containing 5% CO_2. After the cells had reached confluence, they were transected with a plasmid encoding for both GFP and luciferase for labeling. Cells were then collected and injected into the ventrolateral aspect of the tongues of SCID/nude mice. Approximately 5×10^5 Cal-33 cells were injected in each mouse and the viability of the injected cells was verified with bioluminescence imaging following intraperitoneal injection of D-luciferin (50 mg/ml). Tumors were allowed to grow until day 7. For the survival experiment, mice were divided randomly into groups and treated with either PBM, RT, PBM + RT, or not treated. After treatment, mice were monitored daily for 30 days and subsequently on a bi-weekly schedule. Mice were killed using CO_2. The Kaplan–Meier method was used to evaluate survival.

Study design

We conducted three distinct experiments, in which we altered both the PBM and the RT delivery. For all studies, we used SCID/nude mice with human head and neck cancer cells, Cal-33. Our laboratory developed and tested this orthotopic model, which was found to replicate the behavior of oral cancer in humans [18].

Experiment 1 Four groups (control, PBM, RT, and PBM + RT) of 5 mice each with growing tumors on their tongue were included. RT consisted of 4 Gy/session (we used a X-RAD 225 Cx Micro-irradiator-Precision X-Ray), given over 5 consecutive days, for a total of 20 Gy per animal, delivered at a 50-cm source to skin distance. PBM consisted of exposure to red light (660 nm wavelength), at a power of 75 mW continuous wave emission, through a flat 8-mm aperture hand piece in direct contact with the animal, over 75 s (the illuminated $1/e^2$ area was 0.260 cm^2, the $1/e^2$ irradiance was 245 mW/cm^2, energy was 5.6 J, and energy density was 18.4 J/cm^2) (PBM1). We used a Thor LX2 machine (Thor Photomedicine Ltd., Chesham, UK). PBM exposure was completed 30 min prior to RT.

Experiment 2 As described above, four groups of 5 animals each with growing tongue tumors were treated with a single radiation dose of 15 Gy. Animals in the PBM and RT + PBM received the same light exposure as above (660 nm wavelength at a power of 75 mW continuous wave emission, through a flat 8-mm aperture hand piece in direct contact with the animal, over 75 s, 5.6 J/cm^2), but only once, 30 min prior to RT.

Experiment 3 Animals in groups described above were exposed to one 15 Gy RT dose. The PBM2 in this experiment consisted of an array of 5 mm LEDs made up of a combination of wavelength (56×10 mW at 660 nm and 48×30 mW at 850 nm with a combined total power of 2 W), delivered simultaneously from a flat surface hand piece of 75 mm diameter at a distance of approximately 1 cm from the animal skin.

All animals were housed in cages and fed ad lib normal chow. Tumor growth was measured using IVIS imaging following intraperitoneal injection of D-luciferin (50 mg/ml). After treatment, mice were monitored daily for 30 days. Mice were killed using CO_2.

The outcome measures included tumor volume (TV) fluorescence stereoscopy (SteREO Lumar.V12, Carl Zeiss, Göttingen, Germany), body weight (measured every other day), and survival time. We used the Kaplan–Meier method to evaluate survival and ANOVA to determine differences in animal weight and TV. All calculations were two-tailed and significance was established at 0.05.

Results

In the first set of experiments, we used a fractionated schedule of RT: 4Gy × 5 given on consecutive days, Monday–Friday, similar to the clinic scheduling, and the PBM1 (660 nm wavelength at 75 mW power over 75 s through a flat 8-mm aperture hand piece in direct contact with the animal, energy of 5.6 J/cm^2 and energy density of 18.4 J/cm^2) was given 30 min before each RT dose.

PBM1 alone had no significant effect on tumor growth as measured by IVIS up to 8 weeks post-treatment. Control and PBM1-only animals had steady tumor growth and died at an average of 18 and 26 days, respectively (Fig. 1). All RT-treated animals had a significant slowing of tumor growth ($p < 0.001$) and longer median survival time (61 days, $p = 0.001$). There was no statistically significant difference in tumor response to RT between the PBM+ vs. PBM− groups ($p > 0.05$). Figure 2 shows the fluorescence at day 7 obtained in this experiment.

We next decided (experiment 2) to test the effect of PBM1 on single high-dose radiation therapy (SDRT), since this procedure is becoming more commonly used in the clinic for tumors that are resistant to fractionated RT (Fig. 3). We used the same orthotopic animal model with one dose of 15 Gy with or without pre-treatment with PBM1 (660 nm), at 75 mW power over 75 s through a flat 8-mm aperture hand piece in direct contact with the animal (energy density 18.4 J/cm^2). PBM1 was given 30 min before the RT treatment. Control mice and mice exposed to PBM1 experienced unacceptable weight loss and therefore needed to be sacrificed, while the mice treated with either RT or RT + PBM1 lost initially between 15 and 25% body weight but recovered within 13–17 days of treatment and lasting to the end of the observation period ($p = 0.032$ at day 7, $p = 0.051$ at day 13, and $p = 0.041$ at day 17) (Fig. 5).

There were no significant differences in tumor volume between RT and RT + PBM groups as measured by IVIS (Figs. 4 and 5). Both treatments induced a significant but transient decrease on tumor volume, which grew back by week 5. All animals exposed to 1 × 15Gy RT ± PBM1 had significantly longer survival than control-treated and PBM1-only treated animals. However, there was no difference in survival between RT vs. RT + PBM1 animals (Fig. 3).

Subsequently (experiment 3), we tested the effect of PMB2 which consisted of a combination of wave lengths (56 × 10 mW at 660 nm and 48 × 30 mW at 850 nm) for a total power of 2.0 W, delivered concomitantly from a flat surface hand piece of 75 mm diameter at a distance of approximately 1 cm from the animal's skin, on the RT effects on the tumor volume. The RT regimen was the same as in experiment 2 (single 15 Gy exposure). Again, control mice and mice exposed to PBM2 experienced unacceptable weight loss and therefore needed to be sacrificed, while the mice treated with either RT or RT + PBM2 lost initially about 20% body weight but recovered within 20 days of treatment. Similar to experiment 2, there was no significant difference between the RT-exposed groups in any of the assessed variables. Both treatments induced a significant but transient effect on tumor volume, which grew back by week 4. Unlike the previous set of experiments, here, there was a non-significant difference in survival (the RT group survived longer than the RT + PBM2). No significant differences in any of the variables between the RT alone and RT + PBM2 groups were observed.

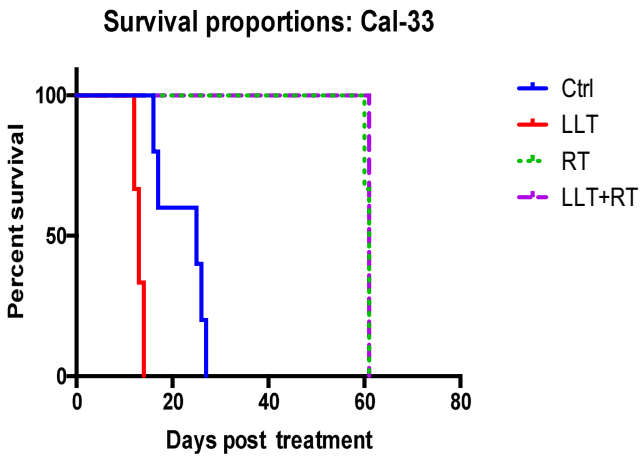

Fig. 1 Survival of animals in experiment 1. The control and PBM-only animals had significantly shorter survival than those exposed to RT. There were no significant differences in survival of PBM+ vs. PBM− RT-exposed animals. LLT, low-level light therapy; RT, radiation therapy; Ctrl, control

Discussion

The relatively new field of PBM in oncology clearly requires significant and intensive study to elucidate mechanisms of action, basic effects, and the specific parameters at which these effects occur. In the current pilot study, we aimed to examine primarily the effect of PBM on human head and neck tumors in an orthotopic animal cancer model that closely resembles head and neck cancer behavior in humans [18]. We used PBM parameters that have been in clinical use for prevention of mucosal and dermal toxicity in head and neck cancer patients, in a quest to determine whether or not there was a PBM-induced protective effect on tumors subsequently exposed to RT. This specific mouse model did not allow for assessment of clinical mucositis. However, the exposed tissues were saved and studied for potential mechanistic developments, which will be published under different heading.

In this pilot project, we used a limited number of variants for PBM and RT delivery. It is important to note that results of our three distinct experiments were not significantly different. Animals treated with 5 RT sessions of 4 Gy each had better

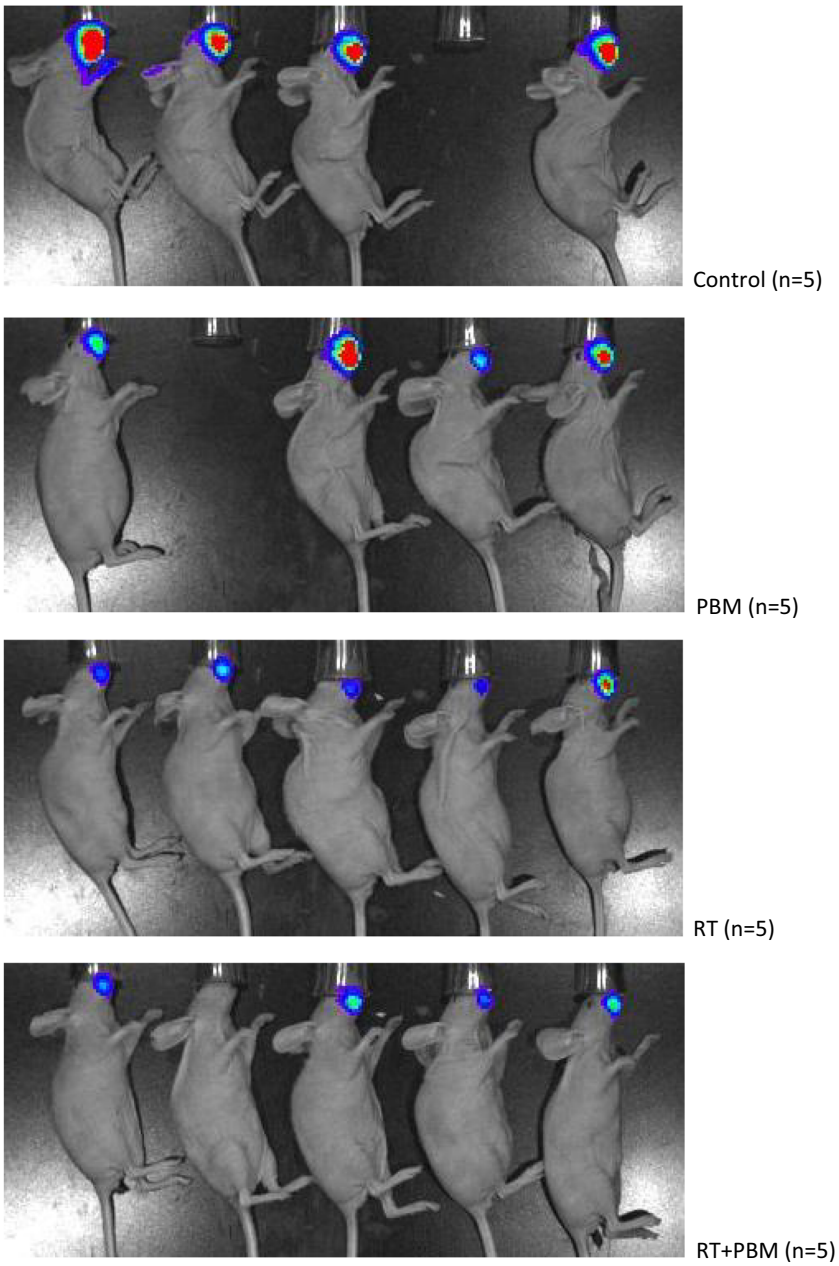

Fig. 2 Tumor volume at day 7 after initiation of treatment: Control and PBM-only animals had significantly larger tumor volume as measured by fluorescence than RT-exposed animals. One animal each from the control and PBM-only groups had died. **a** Control ($n = 5$). **b** PBM ($n = 5$). **c** RT ($n = 5$). **d** RT + PBM ($n = 5$)

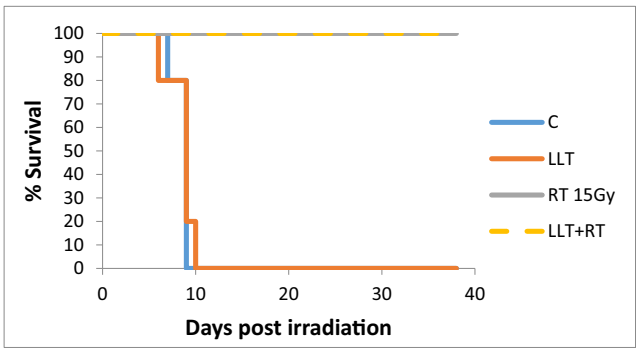

Fig. 3 Survival after 15 Gy RT (experiment 2). Animals exposed to RT ($n = 10$) had significantly longer survival regardless of the PBM status ($p < 0.001$). LLT, low-level light therapy (PBM); C, control; RT, radiation therapy

survival, which did not reach statistical significance. Neither PBM wavelength nor delivery method changes showed any protection of the tumor. These results suggest strongly that there is no protective effect conferred to human head and neck cancer (Cal-33) cells by PBM at the utilized parameters, which are the most commonly used parameters for prevention of therapy-induced mucositis in cancer patients [10].

We could not detect any PBM killing or sensitizing effects of the malignant cells, as suggested by others [14, 15, 19]. Although in experiment 2 there was a significant tumor volume difference in favor of PBM-exposed animals at day 20, this difference vanished by day 30. We believe that this lack of sensitization may be due to the fact that the used SCID

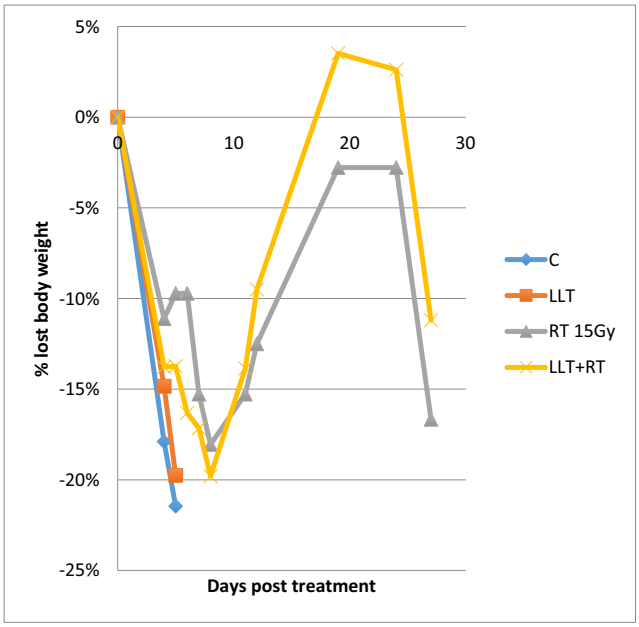

Fig. 5 Weight maintenance (experiment 2). There was a statistically significant difference in weight between animals in the control (C) and PBM-only (LLT) groups vs. those exposed to RT (RT 15 Gy and LLT + RT). No overall significant difference was noted between the RT-treated groups except at day 20, when animals treated with RT + PBM had higher weight

animals did not have a working immune system, which may be necessary for sensitization to occur [19]. Further studies are needed to elucidate this issue.

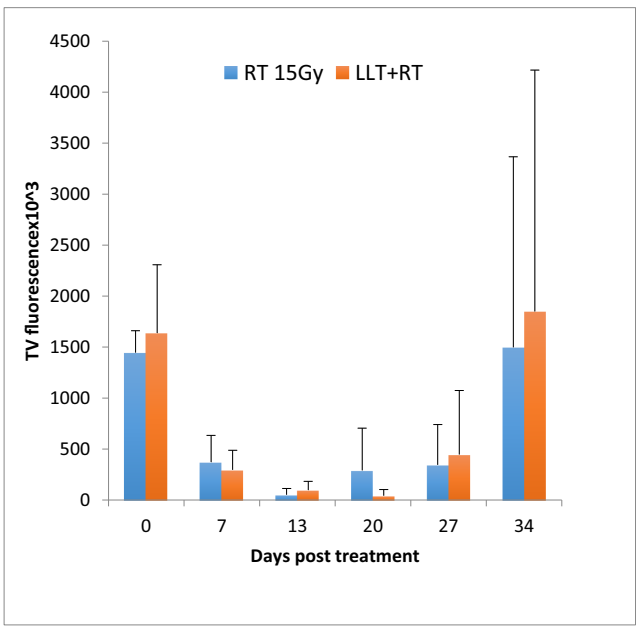

Fig. 4 Tumor volume fluoroscopy after 15 Gy (experiment 2) for radiation-only ($n = 5$) vs. radiation plus PBM ($n = 5$) groups. There is no overall statistically significant difference between RT + PBM vs. RT-alone groups except at day 20 when the tumor volume was lower for the PBM group. LLT, low-level laser therapy (PBM)

The literature on effects of PBM on malignant cells in vitro is broad and diverse. Hence, our results confirm some, but contradict other findings. A study by Liang et al. [16] used PBM at wavelength of 810 nm and energy density of 10–60 J/cm^2 on oral cancer cells vs. gingival fibroblasts. They found that malignant cell growth was inhibited while normal cells were not affected. Another study [15] described the effect of PBM at 685 nm at 5–20 J/cm^2 on cervical cancer cells and found that while PBM itself was not cytotoxic, pre-exposure before RT resulted in significant sensitization. These results were supported by data from our group [14], with the exception that we found PBM to be cytotoxic to leukemia cells while protecting normal lymphoblasts. A similar study was described by Ramos Silva et al. [20], who used PBM post-RT and showed that it improved survival of normal cells but did not affect the exposed malignant cells. The light energy doses used in this study were quite large (30–150 J/cm^2). Liu et al. [21] showed that PBM at 808 nm, 6–8 J/cm^2, inhibited growth of hepatoma cells by more than 70%, while Schartinger et al. [22] showed differential response to PBM of normal (increased proliferation of fibroblasts) vs. malignant (decreased proliferation of SCC 25).

In vivo studies also provide support for our findings. Zacchinga et al. [23] tested PBM at 660 nm (3 J/cm^2) and 970 nm (180 J/cm^2) on two distinct mouse models with squamous cell carcinoma and melanoma tumors, respectively. All experiments showed beneficial effects on tumor tissues consisting on less spread and lower infiltration of cancer in treated animals. Ottaviani et al. [19] studied the effects of three PBM protocols (660 nm, 3 J/cm^2; 800 nm, 6 J/cm^2; and 970 nm, 6 J/cm^2) on oral cancer or melanoma mouse models. The authors report decreased tumor growth in all PBM-exposed animals compared with controls. We note that the PBM parameters used are similar to those we used in the current study. A similar study [24] used PBM (670 nm, 5 J/cm^2) on UV-induced melanoma in mice and reported results consistent with ours: there was no measurable effect on 330 tumors examined over 37 days.

Other studies have supported the opposing view: Gomes Henriques and colleagues [25] used PBM (660 nm, 0.5–1 J/cm^2) on squamous cell carcinoma cells (SCC 25) and reported increased proliferation in vitro. Similarly, Sperandio et al. [13] described increased expression of p-Akt, pS6, and Cyclin D1 after SCC9, SCC25, and dysplastic cells was exposed to 660 nm or 780 nm, 2–6 J/cm^2. Rhee and colleagues [12] performed the only in vivo study we could identify, which showed increased proliferation of thyroid cancer cells after exposure to PBM at 15 or 30 J/cm^2. These authors also reported increases in p-Akt and HIF1A.

These divergent results are difficult to explain, particularly when similar PBM variables and cell populations are used. We believe that our animal model is superior to others described in the literature, as the tumor behavior replicates head and neck SCC in humans. We also performed our study with rigorous methodology. We believe that, regardless of the in vitro observed phenomena, the results obtained in vivo are more relevant and should guide future projects and clinical applications. Further study with a broader set of variables must also be undertaken to elucidate potential PBM tumor sensitization.

Conclusions

Our results obtained in an orthotopic animal model with human oral cancer suggest that malignant tissues exposed to specific PBM parameters prior to ionizing radiation are not protected from the killing effects. If confirmed in further studies, these findings indicate the safety of PBM used for prevention and treatment of radiation-induced collateral damage.

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Compliance with ethical standards

Conflict of interest James Carrol is the CEO of Thor Laser. None of the other authors declare any conflict of interest.

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