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ORIGINAL ARTICLE

Effects of pre-radiation exposure to LLLT of normal and malignant cells

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Abstract
Purpose Low-level laser therapy (LLLT) efficacy for the prevention of cancer treatment-induced oral mucositis (OM) has been amply described. However, potential protection of malignant cells remains a legitimate concern for clinicians. We tested LLLT-induced protection from ionizing radiation killing in both malignant and normal cells.
Methods We treated six groups each of normal human lymphoblasts (TK6) and human leukemia cells (HL60) with He-Ne LLLT (632.8 nm, 35 mW, CW, 1 cm^2, 35 mW/cm^2 for 3–343 s, 0.1–12 J/cm^2) prior to exposure to ionizing radiation (IR). Cells were then incubated and counted daily to determine their survival. Optimization of IR dose and incubation time was established prior to testing the effect of LLLT.
Results Growth curves for both cell lines showed significant declines after exposure to 50–200 cGy IR when compared to controls. Pre-radiation exposure to LLLT (4.0 J/cm^2) followed by 1-h incubation blocked this decline in TK6 but not in HL60 cells. The latter cells were sensitized to the killing effects of IR in a dose-dependent manner.
Conclusion This study shows that pre-IR LLLT treatment results in a differential response of normal vs. malignant cells, suggesting that LLLT does not confer protection and may even sensitize cancer cells to IR killing.

Keywords Cancer · LLLT · Cytoprotection · Mucositis

Introduction

Cancer therapy-induced mucositis is a painful, morbid condition that can be dose-limiting, may lead to extensive hospitalization, and is a risk factor for systemic infection [1, 2]. Naturally then, significant efforts have been directed toward finding a prophylactic and/or therapeutic method for this condition. A comprehensive review of the current literature on this topic has been published by the Mucositis Study Group of the Multinational Association of Supportive Care in Cancer [3].

One of the preventive methods for cancer therapy-induced oral mucositis (OM) recommended by the study group is low-level laser therapy (LLLT). A systematic review of clinical trials [4] as well as a meta-analysis [5] has shown that exposure of mucosal tissues to monochromatic light in the red and near-infrared spectrum, either from a laser or from other devices capable of producing monochromatic light, has led to significant benefits in cancer populations treated with cytotoxic methods. Among these benefits, one counts reduction in intensity and duration of pain, and reduced duration and incidence of ulcerative OM. However, despite the voluminous body of literature, LLLT has not penetrated the market of supportive care in cancer and is routinely used in only a handful of cancer centers around the world.

Three important issues are likely to be responsible for the reluctance of clinicians to embrace LLLT:

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1. The incomplete elucidation of the biologic processes at hand,
2. The inconsistency in laser parameters described throughout the literature [6],
3. The concern that LLLT may confer protection or even induce proliferation of malignant cells.

Addressing the 3rd point, we describe here the results of in vitro testing of LLLT effects on normal and malignant cell populations subsequently exposed to ionizing radiation (IR).

Methods

We studied the alteration in survival response after IR of normal and malignant human cells after exposure to LLLT. We compared radiation-induced cell killing in cells exposed to IR alone with cells pre-treated with various laser energy densities prior to IR exposure. We compared these results to control cells unexposed to either laser or IR and to cells exposed to laser alone or IR alone.

The LLLT treatment consisted of exposure of cells to a 35-mW helium-neon (He-Ne) laser operating at a wavelength of 632.8 nm (Spectra-Physics, Inc. Mountain View, CA). Light was transferred via a fiber-optic cable to a collimator with 1.0 cm^2 flat top aperture.

In order to establish the best exposure parameters, cells were laser-irradiated for 3, 29, 57, 114, 229, and 343 s (energy densities of 0.1, 1.0, 2.0, 4.0, 8.0, and 12.0 J/cm^2, respectively). Prior to, and after all, exposures, laser power at the collimator aperture was checked with a wavelength-specific meter. Table 1 below summarizes the parameters used in this study.

Cell lines

We used two lines of cells: (1) normal human lymphoblasts (TK6) and (2) human leukemia cells (HL60) (Coriel Institute for Medical Research, Camden, NJ). These lines were selected in accordance with our aim to study the safety of laser application in bone marrow transplantation patients with hematologic malignancies. Cells were maintained in culture in sterile Petri plates in RPMI 1640 supplemented with 15 % fetal bovine serum (FBS) and 50 μg/ml gentamicin in a humidified incubator at 37 °C, 95 % air, and 5 % carbon dioxide.

Table 1 Summary of laser parameters

Wavelength	632.8	nm
Power	0.035	W
Illuminated area	1.0	cm^2
Time	3, 29, 57, 114, 229, 343	s
Energy density	0.1, 1, 2, 4, 8,12	J/cm^2

Approximately 1.0×10^6 (10^6/ml) cells growing logarithmically in 24-well, untreated, flat-bottom sterile plates (1 ml/well) in a gently stirred buffer suspension were exposed to LLLT and then incubated for 1, 2, or 4 h to allow time for laser-induced cellular processes to occur. The cells were distributed every other well in the 24-well plate in order to allow consecutive LLLT exposure without field crossover. Control cells were handled identically except the laser exposure. Cells were then exposed to 50, 100, 150, or 200 cGy gamma IR (Atomic Energy of Canada, Ltd. Gamma-cell 40, 173 Cs irradiator, 100 cGy/min) and then incubated in complete medium and counted daily for 6 days. Cell survival was determined by a colony survival assay. Nt/No growth curves calculated from the ratio of the number of cells on a given day (Nt) to the number of cells on day 0, prior to laser exposure (No), were analyzed to determine cell survival. We used chi-square and ANOVA (SPSS, Chicago, IL) to analyze differences between Nt/No ratios.

Results

IR dose optimization

Exposure of cells to IR of 50 cGy reduced both cell populations by about 50 %. Higher radiation doses (100 and 200 cGy) resulted in excessive cell killing. Thus, we present the 50-cGy IR exposure experimental results.

Incubation time optimization

Post-LLLT, pre-IR treatment incubation of cells for 1 or 2 h yielded similar results; the difference in cell numbers did not achieve statistical significance. After incubation for 4 h, the benefit for laser-treated TK6 cells that was achieved with 1 and 2 h of incubation was lost (IR killing was similar to control). Thus, results presented below are for cells treated with LLLT or sham and incubated at 37 °C for 1 h prior to exposure to 50 cGy IR.

Cell survival

Compared to non-IR exposed controls, the Nt/No value declined twofold after exposure of both TK6 and HL60 to 50 cGy IR. There were no significant differences in killing rates between the two cell lines. Pre-radiation exposure of TK6 (normal) lymphoblasts to He-Ne laser at 4 J/cm^2 followed by a 1-h incubation time blocked this decline ($p < 0.001$) (Table 2; Fig. 1). Laser energy densities below or above 4.0 J/cm^2 had no significant protective effect on TK6 cells ($p > 0.05$ when compared to cells unexposed to laser). Results remained consistent for the 6-day observation period (Fig. 1).

Table 2 TK6 cells response to treatment

Cell treatment	Relative cell number (Nt/No)					
Control (no IR)	2.1 +/- 0.2	4.5 +/- 0.2	10.6 +/- 0.6*	18.9 +/ 2.7*	57.0 +/- 0.9*	111 +/- 1.4*
Laser (4J/cm^2) + IR	1.6 +/- 0.2	3.9 +/- 0.2	7.2 +/- 0.3	18.2 +/- 2.1*	53.0 +/- 7.5*	100.5 +/- 1.8*
IR	1.3 +/- 0.1	3.0 +/- 0.6	5.5 +/- 0.1	9.6 +/- 1.3	28.2 +/- 0.6	52.4 +/- 1.9

*$p < 0.001$ compared to IR

HL60 leukemia cells pre-exposed to LLLT were sensitized to IR killing in an energy dose-dependent manner and showed Nt/No values lower than gamma-irradiated-only HL60 controls ($p < 0.01$). LLLT alone (4.0 J/cm^2) resulted in a day 6 Nt/No of 19.1 ± 1.3 as compared to 49 ± 0.8 for control HL60 cells ($p < 0.001$; Table 3). Measurements were consistent over the 6-day observation period (Fig. 2).

Discussion

Exposure of mammalian cells to IR results in cell death due to their inability to process radiation damage. Thus, cell survival (measured by their ability to reproduce) is widely used as a measure of radiation sensitivity/resistance. The current study confirmed the cytoprotective effects on normal lymphoblasts while addressing the issue of LLLT protection of leukemia cells from killing by IR. Our results suggest that not only there is no protection but LLLT exposure may sensitize leukemia cells to the killing effects of IR. Moreover, exposure of HL60 to LLLT alone induced cell death in a dose-dependent manner, which is consistent with previous results [8]. In contrast, normal lymphoblasts treated with 4 J/cm^2 were significantly protected by pre-IR LLLT exposure. Lower and higher energy densities did not show the same effect, suggesting that protective cellular processes are active in a fairly narrow range of receptor saturation.

Diminished duration/intensity or outright prevention of radiation and/or chemotherapy-induced mucositis is an important goal for increased cancer treatment efficacy and reduction of related morbidity. Exposure of tissues to light within the red or near-infrared spectrum has been clinically proven to reduce incidence, duration, and severity of oral mucositis, leading to a recommendation for use of LLLT for prevention of mucositis in cancer patients treated with chemotherapy [3–5]. The parameters used in the current study are within those suggested by the Multinational Association for Supportive Care in Cancer/International Society of Oral Oncology in their recommendation [3]. However, cellular mechanisms for this clinical effect have not been completely elucidated. Hence, extension of cytoprotection to malignant cells has been a natural concern. This in vitro study was designed in response to such concern.

Our selection of cell lines was based on the concept of LLLT use in bone marrow transplantation patients treated with myeloablative regimens [7]. Hence, we examined normal lymphoblasts and leukemia cells. Our results may not be generalizable to other cell lines, and we strongly encourage replication of our study with solid tumor cells, particularly squamous cell carcinoma (SCC).

In one such published report [8], SCC25 cell line growth was reduced by exposure to LLLT. Conversely, another in vitro study [9] suggested that light therapy (660 and

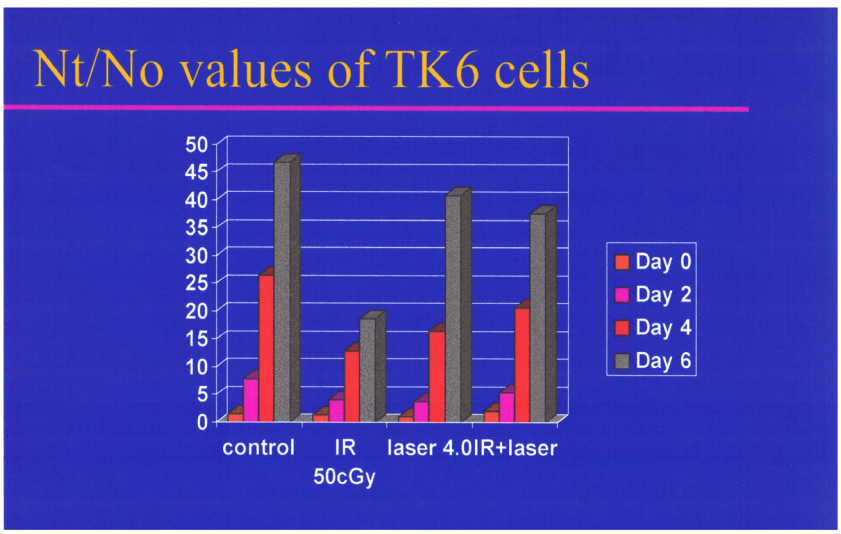

Fig. 1 Nt/No values for TK6 (normal lymphoblast) cells

Table 3 HL60 response to treatment

Cell treatment	Relative cell numbers				
	Day 1	Day 2	Day 3	Day 4	Day 6
Control (no IR)	1.49 +/- 0.1	3.54 +/- 0.2	9.92 +/- 0.2	16.3 +/- 0.3	48.98 +/- 0.8
IR (50 cGy)	1.57 +/- 0.1	1.71 +/- 0.1	4.83 +/- 0.3*	6.50 +/- 0.3*	28.8 +/- 1.5*
Laser (4 J) + IR	1.46 +/- 0.2	1.09 +/- 0.1*	3.14 +/- 0.1*	5.35 +/- 0.5**	20.45 +/- 1.2**
Laser (4 J)	1.04 +/- 0.1	2.36 +/- 0.2	5.17 +/- 0.2*	5.90 +/- 0.6**	19.10 +/- 1.3**

*$p < 0.05$ compared to control

**$p < 0.001$ compared to control

780 nm) may induce progression to and/or invasion of existing head and neck cancer cells. In this experiment, dysplastic and malignant squamous cells showed increased expression of proteins associated with invasion or progression of tumor (pAkt, pS6, and Cyclin D1). However, results on SCC25 were consistent with the study above [8], as apoptosis was significantly increased as a result of exposure to LLLT. Thus, this study suggested that both more aggressive behavior and increased apoptosis are possible after exposure to LLLT, depending on cell line, light parameters, and time of evaluation. Another study found growth in numbers of ex vivo laryngeal cancer cells after exposure to larger energy densities then typically used with near-infrared LLLT [10]. These studies did not test cell response to cytotoxic therapy or general cell survival. We are aware of no other study that exposed cells to cytotoxic treatments after LLLT. Hence, further work in this area is necessary.

Our study did not provide any useful data for further elucidation of LLLT mechanisms of action. However, the protection of lymphoblasts detected in our study is consistent with some described cellular mechanisms [activation of mitochondrial cytochrome c oxidase [11], NF-kB [12], or TGF-beta [13]; decrease in COX-2 receptors [14]]. Continued research is required in order to provide a clear mechanism for clinically described LLLT effects.

The results presented here are also consistent with recent clinical data of a prospective, randomized clinical trial of LLLT for the prevention of radiochemotherapy-induced mucositis in head and neck cancer patients [15]. The investigators followed patients for a median of 18 months and reported better locoregional progression-free survival for the laser-treated group. Results for mucositis incidence, severity, and pain were all significantly better for the laser treatment group as well. It is worth noting that this study also employed red light (660 nm) at an energy density of 4 J/cm^2. These results support our findings of protection of normal cells and sensitization of malignant cells by LLLT.

The current study has some limitations that require cautious interpretation of these data. We only used two cell lines and none was squamous cell carcinoma of the head and neck. Additionally, in vitro cell behavior does not necessarily replicate clinical behavior. Hence, it may be useful if our experiment were replicated with other malignant lines and the effects also be tested for cytotoxic chemotherapy. Nevertheless, the fact that LLLT did not protect leukemia cells but rather sensitized them to the killing effects of radiation is encouraging and supports the use of LLLT as a low-cost, effective method for the prevention and treatment of mucositis and potentially other morbid effects of therapy in cancer patients.

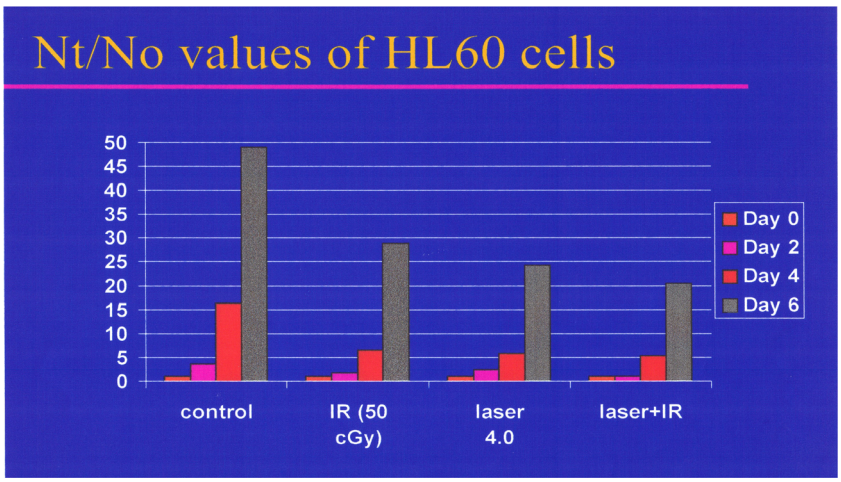

Fig. 2 Nt/No values for HL60 leukemia cells

Conclusion

LLLT exposure of leukemia cells prior to IR did not protect but sensitized these cells to the killing effects. Normal human lymphoblasts pre-treated with He-Ne light at 4 J/cm^2 were able to deflect the IR kill. This study supports the finding that there is a differential effect of LLLT on normal vs. malignant tissues.

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Compliance with ethical standards

Conflict of interest James Carroll is the CEO of Thorlaser Co.

There is no other conflict of interest to report.

The authors had full control of the primary data and agree to allow review of said data, if requested by the Journal.

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