Published in final edited form as:

Int J Hyperthermia. 2013 August ; 29(5): 474–479. doi:10.3109/02656736.2013.817615.

Hyperthermia-enhanced Indocyanine Green delivery for laser induced thermal ablation of carcinomas

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Abstract

Purpose—Intravenous administration of indocyanine green (ICG) dye can effectively convert near infrared laser light into heat and enhance thermal injury of blood vessels; however, there is no selective uptake of ICG by the tumor compared to the other tissues which impacts the therapeutic ratio of this strategy unless tumor uptake is somehow increased in tumor tissue. Here, we investigated the use of local hyperthermia prior to intravenous ICG administration to enhance ICG uptake in tumor tissue and thereby laser thermal ablation of solid tumors.

Materials and Methods—Female A/J and C3H mice with SCK or SCCVII tumors were treated with 755-nm laser light using 70 J/cm² radiant exposures and a 3 ms pulse. The laser therapy was performed either alone or with prior intravenous administration of 4 mg/kg ICG with and without local tumor hyperthermia at 42.5°C for 60 minutes. Retention of ICG was quantified using a near-infrared animal imaging system. Tissues were examined for damage histologically.

Results—The ICG uptake and retention in the heated tumors was 1.23-fold greater on average compared to non-heated tumors, in both models. In SCK tumors, animals receiving either laser irradiation alone or in conjunction with ICG had 1.86 and 3.91-fold increase in tumor growth delay, respectively. The addition of local hyperthermia before ICG injection resulted in complete regression of SCK tumors. Although uptake of ICG was increased in SCCVII tumors, little change in tumor growth delay was observed.

Conclusion—Using local hyperthermia may improve the delivery of ICG to the tumor and thereby increase the extent of laser thermal ablation of smaller superficial malignancies that can be effectively exposed to laser therapy.

Keywords

Cancer; Perfusion; laser therapy

INTRODUCTION

Minimally invasive laser thermal therapy, in combination with nanoparticles or drugs, is among the most promising of emerging technologies to arrest expansion of cancerous growths with minimal morbidity and reduced toxicity [1, 2]. One approach is to use systemic injection of laser absorbing agents or dyes to increase laser induced thermal

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damage in the tumor. Indocyanine green (ICG) is a water-soluble tricarbo-cyanine dye (775 g/mol) that was first approved for clinical use by the US Food and Drug Administration in 1956 [3]. After intravenous injection, ICG binds tightly to plasma proteins and is rapidly cleared by the liver. The absorption spectrum of ICG dissolved in plasma exhibits a strong peak from 750–830 nm with a maximum at 800–810 nm [4, 5]. The absorption maximum of ICG at about 800 nm promotes absorption of NIR laser light delivered by a diode laser [6]. In addition, preliminary studies by our group have demonstrated that ICG can induce marked thermal damage to solid tumors after laser irradiation compared to laser alone [7]. These studies were conducted in one of the same tumor cell lines used in this paper, A/J mice with SCK tumors.

High magnification of necrotic tumors demonstrates cellular features of necrosis, including loss of cytoplasmic and nuclear detail. Significant damage to the vascular wall in peritumoral vessels, including endothelial cell necrosis and loss of endothelial cells and fibrin deposition has also been observed [7].

ICG-enhanced diode laser photocoagulation appears to be superior to diode treatment alone in achieving vessel closure, but is limited by clot resolution due to both excessive vascular damage and an accompanying inflammatory response [8]. These results suggest that more durable feeder vessel closure may be achievable by the use of hyperthermia prior to ICG administration allowing for more interstitial cell death. However, the damage/thermal ablation was not complete as evidenced by re-growth of the tumors. In general, there has been no study showing evidence of preferential accumulation of ICG in tumor tissue compared to other tissues which calls into question whether or not the appropriate amount and distribution of ICG is obtained before laser therapy for small tumors. In the current investigation we attempted to increase ICG accumulation at the tumor site by exposing tumors to local hyperthermia. Accumulating experimental data adds support to the idea of using local moderate temperature hyperthermia to improve drug delivery via an increase in tumor blood flow and tumor vessel permeability [9–11]. Our previous studies have suggested that for a short term perfusion/permeability increase, 42.5°C may be an optimal temperature. Above this temp there is vascular damage, while below this temperature, while there may be beneficial effects, they might take hours or even days to be expressed. Therefore, for the current study we selected 42.5°C. We hypothesized that local hyperthermia would enhance delivery of ICG to the tumor site and interstitium thereby allowing a greater thermal ablation effect of laser therapy on the tumor vasculature and surrounding tumor cells to induce tumor regression.

METHODS

Animal Preparation and Treatment

SCK mammary or SCC-VII squamous cell carcinoma tumor cells (2×10^5) were subcutaneously inoculated in the rear limb of A/J or C3H mice, respectively. A/J mice are an inbred, albino strain produced by Jackson Laboratories used to grow SCK mammary carcinomas.

C3H mice are an inbred, agouti strain produced by Jackson Laboratories used for SCCVII squamous cell carcinoma growth. The SCK cell line is a mouse mammary carcinoma which grows rapidly and has significant microvascular dysfunction and the SCC-VII is a squamous cell carcinoma that grows slightly slower and more uniformly. Cells were cultured and harvested by a short addition of trypsin and then suspended in serum free medium. These cells were then injected at 2×10^5 cells per 0.05 mL of serum-free medium into the right rear limb. Tumors were allowed to grow until sizes reached 4–8 mm in diameter, at which time the mice were randomized and divided into a control group ($n=5$ for A/J and $n=5$ for C3H).

and two study groups (n=5 for ICG/laser and n=5 for the hyperthermia/ICG/laser for both murine strains). In the two study groups, the mice were treated with NIR laser preceded by an intravenous administration of sterile ICG solution and laser (group 1) or hyperthermia, ICG, and laser (group 2), as described below. No treatment was applied to the mice in the control group.

Two minutes prior to laser irradiation, a single dose of 4 mg/kg of ICG solution was administered into the lateral tail vein of each mouse in the first study group and all groups receiving ICG hereafter. In mice, we observed a maximum concentration of ICG in the blood and at the tumor site as soon as 2 minutes. However, this window is very short in that the ICG is being cleared quickly by the liver. If we were to wait until the sixth minute, we may miss this window. We feel it best to treat as soon as this window becomes open. In our earlier studies, we had to use multiple laser pulses to cover tumor area and therefore began at 2 min after i.v. injection as well. For these reasons, we chose to treat at this particular time after ICG administration. This was followed by treatment with one pulse from a near-infrared laser. In the second study group, hyperthermia was administered at 42.5°C for one hour. A single dose of 4 mg/kg of ICG was injected into the tail vein of each mouse followed by one pulse from a near-infrared laser.

A NIR laser system (GentleLASE, Candela, Wayland, MA) emitting 755 nm light was used in this study. The laser beam diameter was 8 mm, the pulse time was 3 ms and the laser radiant exposure was 70 J/cm². Epidermal cooling was accomplished by using cryogen spray cooling with two 30 ms spurts before and 10 ms after the laser pulse. The laser hand-piece was positioned directly above and perpendicular to the heated platform on which the mouse was placed. The platform was attached to a micrometer-driven translation stage to ensure the same orientation was used for each laser exposure. The entire tumor and margins were irradiated using a single pulse while the laser beam was stationary. This pulse resulted in complete coverage of the exposed surface of the tumor.

During laser treatments, the mice were anesthetized with 1–2% isoflurane by inhalation. The body temperatures of the mice were maintained by individually placing them on a 37°C heated platform during treatment. Following laser treatment, the mice were allowed to recover and returned to the colony. The mice were monitored for behavior and tumor volumes for up to 10 days post treatment. Tumor sizes were measured every one to two days with a Vernier caliper. Tumor volumes were calculated using the formula $a^2b/2$, where a and b are the shorter and longer diameters of the tumor, respectively.

One mouse from each study group was sacrificed at day 3 after laser treatment for histological analysis. Immediately following euthanasia, the tumors were excised and were processed and embedded in paraffin blocks for routine histological analysis. Tissue sections (5 µm thick) were stained with hematoxylin and eosin (H&E) and examined under a light microscope. Quantitative pathological analysis hardware and software, Aperio Scanscope T2 and ImageScope software (Aperio, Vista, CA), was used to determine the relative necrotic area in each tissue section.

ICG Uptake

Indocyanine green was obtained from Pulsion (Pulsion Medical Systems, Munich Germany) in vials of 25 mg. The ICG powder was dissolved in 5 ml sterile water and further diluted in sterile saline to a concentration of 1.25 mg/mL. A single dose of ICG solution (4 mg/kg body weight) was administered to each mouse, for imaging only. Kodak *in-vivo* imaging system (Carestream Health Molecular Imaging, New Haven, CT) was used to excite and monitor the ICG fluorescence intensity within the anesthetized mouse. The fluorescence intensity was recorded by taking consecutive images of the mice immediately before and up

to 15 minutes post ICG administration. The change of the fluorescence intensity (FI) was determined by calculating the ratio between the FI at each time point and the FI prior to the ICG administration, as previously described [7].

RESULTS

ICG Accumulation

In the ICG uptake assay, hyperthermia was administered at 42.5°C for 60 minutes, ICG at 4mg/kg bodyweight and a single pulse from a laser emitting at a wavelength of 755 nm. Examples of fluorescence intensity measured across the tumor region in a control and heated tumor are shown in figure 1.

Our results demonstrated an increase in maintained ICG retention over 30 minutes after hyperthermia as compared to tumors receiving no hyperthermia prior to ICG administration. The area under the curve was determined for each animal in each treatment group. The average of each treatment group is seen in figures 2 and 3.

In SCK tumors, ICG retention was assessed when the laser pulse was given two minutes after ICG administration and/or hyperthermia exposure. The retention of ICG was greater over time when hyperthermia was added before ICG and laser (P Value = 0.0010). In SCCVII tumors, hyperthermia was administered for varying times of 60 or 90 minutes to determine the optimal duration for this tumor model since our original tumor response was not found to be as substantial as in the SCK model (Figures 2–3).

At 90 minutes, ICG was retained in the tumor at higher concentrations than at 60 minutes or with no hyperthermia (P Value = 0.03). However, when ICG was administered just after 60 minutes of local hyperthermia, there was an increase in ICG retention but it was not statistically significant (P Value = 0.35) compared to animals receiving ICG alone in SCCVII tumors. The statistical significance of the retention assessment was determined by comparing the ratio between the FI at each time point and the FI prior to the ICG administration followed by an analysis of the area under the curve value this graph produced using a two tailed, t-test.

Tumor growth delay

Treatment groups in the SCK tumor model were untreated control, laser alone, ICG and laser, and hyperthermia, ICG and laser. Animals receiving laser irradiation alone had a tumor growth delay 1.86 fold greater than untreated control tumors and tumors treated with laser irradiation in conjunction with ICG had a 3.91 fold increase in growth delay. When SCK tumors were treated with hyperthermia for 60 minutes at 42.5°C followed by ICG administration and laser thermal therapy, a complete regression was seen in 4 of 4 tumors monitored for tumor growth (Figure 4). Healthy tissue was spared and no major skin damage was observed during or post-treatment.

The same treatment groups were used in the SCCVII tumor model (Figure 5) with the same parameters. The tumor volume was approximately 1.5 fold greater on average than that in the SCK tumors on the day of treatment. Control animals grew to four-fold the starting volume in approximately 7 days. Tumors exposed to laser alone also grew four-fold in volume in 7 days. When mice were treated with ICG with or without hyperthermia before laser exposure, the mean tumor volume increased 4 fold in approximately 8 days, a tumor growth delay of 1.15 days.

Histological assay

Seventy-two hours after treatment, 2 SCK tumors were harvested, one from the ICG with laser group and one from the hyperthermia, ICG with laser. Both tumor tissue samples were fixed and stained with Hematoxylin and Eosin (figure 6, A–B). These sections were converted to grayscale in Image J, and a threshold intensity value was set using the ‘image-adjust-threshold’ menu to exclude background fluorescence of normal epithelial structures in a non-ablated control sample. Using the black and white method of digital mark-up previously developed by our group, normally fluorescent structures (collagen and keratin) remain white, while viable epithelial cells and tumor cells are black [12]. Tumor margins were outlined in red as illustrated in Figures 6, C and D. When tumors were treated with hyperthermia, ICG and laser, a marked decrease in tumor tissue viability was observed at 72 h post treatment compared to tumors treated with ICG and laser irradiation.

SCCVII tumors were not studied with histology because there was no noticeable difference in tumor response between the treated and control groups.

DISCUSSION

The purpose of this study was to determine the effects of hyperthermia on the anti-tumor effect of ICG in conjunction with laser therapy in two murine tumor types. We hypothesized that the addition of heat via local hyperthermia would create increases in tumor perfusion and vascular permeability which would, in turn, lead to increased ICG concentration within the tumor tissue. We expected that an increase in ICG concentration would result in higher absorption of the laser light and maximize temperature elevation within the target tissue, based on our previous observations with hyperthermia and ICG-induced thermal ablations [7, 13].

Tumor vasculature is formed from normal arterioles which become incorporated in the tumor tissue [14]. These vessels dilate upon heating at mild temperatures probably due to smooth muscle relaxation via stimulation by nitric oxide synthesized by endothelial cells [15]. The increase in tumor blood flow and vascular permeability caused by mild temperature hyperthermia is demonstrated to increase delivery of indocyanine green in our investigation. This result agrees with other studies that have demonstrated that as other treatment agents such as drug-containing liposomes, immunotherapeutic agents and genetic constructs can be delivered more effectively when hyperthermia is applied [16].

From our results, we can conclude that SCK tumors in A/J mice respond in a therapeutically favorable manner to the addition of local hyperthermia before ICG administration and laser irradiation as evidenced in the full tumor regression observed (Figure 4). Since this tumor type is a model of a mammary carcinoma, it appears feasible that patients presenting with superficial mammary tumors or chest wall recurrent nodules at a relatively shallow depth or those that could be exposed to laser irradiation interstitially or intra-operatively may have improved tumor control by adding ICG-based laser therapy.

In the other model studied, a head and neck cell line (SCC-VII), the tumors did not respond as favorably over time. These tumors were 1.5 fold larger on average than the SCK tumors at the time of treatment and possibly did not receive a homogenous laser dose, which is always a limiting factor for laser-based treatments. The ICG uptake results also indicated a less statistically significant increase in ICG accumulation after heating. Interestingly, we noted an obvious treatment effect in the top layer of the tumors (data not shown), especially after the combined heat, ICG and laser exposure which suggests that a portion of the tumor responded favorably, but the remainder of the tumor volume continued to grow and therefore overshadowed the damage caused in the top 3 mm of tumor tissue. This result is to

be somewhat expected as these tumors generally grew to diameters near the boundary of the treatment area (8 mm) by the day of treatment. This may have accounted for the poor response we saw in this tumor model overall. Regardless, as proof of principle, our results suggest that in a clinical application with optimized lasers there may be improved tumor control if superficial head and neck tumors could be locally heated before ICG infusion and laser irradiation.

From a clinical standpoint, in-vivo monitoring of ICG accumulation after hyperthermia may be used for determining the optimal time for delivery of laser irradiation and the likelihood of success for that treatment. The fluorescent nature of ICG when excited by laser light offers a means of visualizing the peak time for laser exposure as denoted by ICG accumulation and thereby may elicit a more favorable therapeutic response. For example, a photoacoustic flow cytometry method was developed for real-time detection of ICG and other contrast agents to characterize the kinetics of ICG in a mouse ear [12] and there are clinical systems already in use to determine clearance time of ICG in the blood [17]. Therefore, a similar approach to monitor tumor concentration may be useful in the further development of individually specialized laser therapy in conjunction with ICG injection and local hyperthermia to maximize tumor response.

Acknowledgments

This study was supported in part by NIH/NCI 3R01CA044114-21S1 and CA44114. RJG and GS were co-PIs of this study. We thank Pulsion Medical Systems AG for providing the ICG.

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Figure 1.

SCK tumors were treated in-vivo with A) ICG at 4 mg/kg followed by laser thermal therapy or B) local hyperthermia at 42.5°C before ICG administration at 4 mg/kg followed by laser thermal therapy. Near-infrared imaging was performed for 30 minutes following therapy with snapshots taken every 60 seconds to assess fluorescent intensity of ICG in the tumor.

Figure 2. Hyperthermia increases fluorescent intensity of ICG in SCK tumors

Hyperthermia was administered at 42.5C for 1 60 min prior to ICG (4mg/kg bodyweight) administration and whole body near-infrared imaging. Each treatment group consisted of 5 animals.

Figure 3. Hyperthermia increases fluorescent intensity of ICG in SCCVII tumors
Hyperthermia was administered at 42.5C for 1 60–90 min prior to ICG (4mg/kg bodyweight) administration and whole body infrared imaging.

Figure 4. Mean SCK tumor volume as a function of time (days) after treatment

Tumors were treated with hyperthermia for 60 minutes prior to IG administration. Thereafter, one laser pulse was delivered to the tumor region. Tumor diameters were measured every day and tumor volume was calculated by the formula $a^2b/2$, where a and b are the shorter and longer diameters of the tumor, respectively. Measurements were taken every 24 hours.

Figure 5. Mean SCCVII tumor volume as a function of time (days) after treatment

Tumors were treated with hyperthermia for 60 minutes prior to IG administration. Thereafter, one laser pulse was delivered to the tumor region. Tumor diameters were measured every day and tumor volume was calculated by the formula $a^2b/2$, where a and b are the shorter and longer diameters of the tumor, respectively. Measurements were taken every 48 hours.

Figure 6. H&E stain after treatment

All tumors were harvested 72 hours post-treatment and subsequently treated with hematoxylin and eosin stain, fixed and mounted. **A)** SCK tumors were treated with ICG and laser. **B)** SCK tumors were treated with hyperthermia at 42.5C for 60 minutes, ICG and laser. **C)** SCK tumors were treated with ICG and laser. The portion of this slide that has been outlined in red denotes the tumor tissue. Within this region, the white area is dead while the black portion is live tissue. **D)** SCK tumors were treated with hyperthermia at 42.5C for 60 minutes, ICG and laser. The portion of this slide that has been outlined in red denotes the tumor tissue. Within this region, the white area is dead while the black portion is live tissue.