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Effect of Different Components of Laser Immunotherapy in Treatment of Metastatic Tumors in Rats[1]

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ABSTRACT

Induction of a long-term tumor-specific immunity is the ultimate cure of metastatic cancers. Laser immunotherapy is a novel approach that aims at the tumor-directed stimulation of the immune system of the host. It involves an intratumor administration of a laser-absorbing dye and an immunoadjuvant, followed by noninvasive laser irradiation. Previous studies using glycated chitosan (GC) as immunoadjuvant and indocyanine green (ICG) as laser-absorbing dye have shown positive effects of the treatment on metastatic breast tumors in rats. *In vivo* experiments showed promising results such as: (*a*) eradication of treated primary tumors; (*b*) regression of untreated metastases; (*c*) induced antitumor immune response; and (*d*) long-term resistance to tumor rechallenge. In this study, rats bearing metastatic breast tumors and metastatic prostate tumors were treated with various combinations of the three components of laser immunotherapy. The rat survival rates and profiles of primary and metastatic tumors, after treatment by individual components and various combinations of the components, were analyzed. In the treatment of breast tumors, all of the experimental groups without immunoadjuvant showed little or no positive effect. The use of GC, either by itself or in combination with other components, had a noticeable impact on the survival rate of tumor-bearing rats. However, it was the combination of all of the three components that resulted in the highest cure rate. Three different concentrations of GC, 0.5, 1, and 2%, were also used to treat the metastatic breast tumors. The results showed that 1% GC was most effective in laser immunotherapy. In the treatment of metastatic prostate tumors, both the laser-ICG and laser-ICG-GC treatments significantly reduced the growth of primary tumors and lung metastases. Long-term survival of the rats bearing the prostate tumors was also observed after the laser immunotherapy treatment in our preliminary studies. These results revealed the important function of the immunoadjuvant in laser immunotherapy.

INTRODUCTION

The most effective cancer treatment mechanism is the induction of a major, tumor-specific, immune response in the host. Such a response can ultimately provide a systemic cancer cure, eradicating the detectable, treated primary tumors and controlling the undetectable, untreated metastatic tumors at remote sites. Furthermore, the immune response should lead to long-term resistance to the cancer of the same origin.

In an attempt to achieve such an immune response, immunoadjuvant is often used. In traditional cancer immunotherapies, immunoadjuvants have been used as the sole agent or in many cases used in combination with chemotherapy (1–6). Although immunoadjuvants, such as bacille Calmette-Guérin, *Corynebacterium parvum*, and Freund's adjuvants, can function as immune stimulants, they lack specificity to target tumors. Therefore, the current available immunotherapies using adjuvants have only achieved limited effects.

Immunoadjuvants have also been used in nontraditional cancer treatment modalities, such as PDT.[3] Immunological reaction has been noted after PDT treatment (7–11), initiated with induction of inflammatory reaction (12, 13), release of cytokines (14–16), and various other immune activities (17–20). In conjunction with PDT, immunoadjuvants have been used to enhance the immune function of the host (21–24).

Laser immunotherapy was developed to use a nontraditional photothermal laser treatment in combination with immunoadjuvant to treat metastatic tumors (25, 26). The overall goal of laser immunotherapy was to induce an overwhelming tumor-specific immune response. Unlike the photochemical reaction for tumor destruction in PDT, laser immunotherapy uses a selective photothermal reaction as its first line of assault on the tumor. It uses an 805-nm laser light and intratumorally administered ICG to achieve a selective photothermal interaction (27–29). A novel immunoadjuvant, GC, is administered with the laser-absorbing dye to induce an immunological reaction. This method has been used to treat metastatic breast tumors in rats, and the animal experimental results were highly promising (30–32). Not only can the primary tumors be successfully treated, untreated metastatic tumors at remote sites can also be eradicated. In addition, laser immunotherapy cured rats can both withstand repeated tumor challenges with increased tumor doses and provide protection to naive animals against the same tumor through adoptive immunity transfer using immune spleen cells.

The hypothesized mechanism of laser immunotherapy is the photothermal and photoimmunological effects, working in tandem, to induce a host immune response. Such a response can successfully fight the residual tumor cells at the primary sties, as well as allowing the host to establish a long-term defense against cancer of the same origin. Both the photophysical and photobiological interactions play essential roles in laser immunotherapy. However, the mechanism of laser immunotherapy has not been fully understood. The purpose of this present study is to investigate the functions of each individual component used in laser immunotherapy, *i.e.*, the laser light, the laser-absorbing dye, and the immunoadjuvant. In addition, because the immunoadjuvant is a new addition to the laser photothermal treatment of cancer, its role in laser immunotherapy was further investigated by using GC with different concentrations in the treatment of metastatic breast tumors in rats. The understanding of the roles of different components in laser immunotherapy will lead to optimal treatment protocols with increased efficacy.

MATERIALS AND METHODS

Components of Laser Immunotherapy. Laser immunotherapy consists of three main components: a near-infrared laser, a laser-absorbing dye, and an immunoadjuvant. The laser used in our studies is the DIOMED 25 diode laser (DIOMEDICS, The Woodlands, TX). It emits an 805-nm light with a maximum power output of 25 W. The laser-absorbing dye is ICG (Akorn, Inc., Buffalo Grove, IL). Its aqueous solution has a primary absorption peak around 800 nm. The immunoadjuvant used in our experiments is GC. It is prepared in our laboratory by incubating an aqueous suspension of chitosan with a 3-fold

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[3] The abbreviations used are: PDT, photodynamic therapy; ICG, indocyanine green; GC, glycated chitosan.

excess of galactose and subsequent stabilization by borohydride reduction of the mixture of Schiff bases and Amadori products.

Tumor Models. The transplantable, metastatic mammary tumor model, DMBA-4 (33–35), in female Wistar Furth rats was used in the experiments. The rats were purchased from Harlan Sprague Dawley Co. (Indianapolis, IN), at age 5–6 weeks and weight 100–125 g. This tumor line has been maintained in our laboratory through serial tumor transfer using live hosts. Viable tumor tissue were collected from live tumor-bearing rats and diced in medium (RPMI + 10% FCS), followed by grinding in an all glass loose-fitting homogenizer to obtain single cell suspension. The tumor cells were implanted s.c. in one of the inguinal fat pads with 10^5 viable tumor cells/rat. The primary tumors usually emerge 7–10 days after tumor implantation. The tumor metastasizes along the lymphatics and the metastases at remote sites usually become palpable ~2 weeks after the tumor implantation. Without treatment, the tumor-bearing rats have an average survival time of 35 days. The level of immunogenicity of the DMBA-4 model was low based on the results of an immunization experiment using freeze-thaw tumor cell lysate and an experiment of surgical removal of primary tumors.

The transplantable, metastatic prostate tumor model, Met-Lu (36), in male Copenhagen rats was used in our preliminary studies. The rats were purchased from Harlan Sprague Dawley Co. (Indianapolis, IN) at age 5–6 weeks and weight 100–125 g. Viable tumor tissue (1 mm^3) from live tumor-bearing rats was collected and implanted to the inguinal areas of naive rats. These prostatic tumors produce lung metastases. The average survival time of untreated control rats in our laboratory was 55 days.

Treatment Parameters of Laser Immunotherapy. Various combinations of the three components in laser immunotherapy were used to treat the tumors. The detailed permutations and the parameters of the different components are given in Table 1 for the treatment of the metastatic breast tumors in female rats.

For the effect of laser immunotherapy on the tumor burden of the prostatic tumors, two protocols were used. One was the treatment using the combination of laser-ICG, and the other was the combination of laser-ICG-GC. For the survival study, only the combination of laser-ICG-GC was used. The treatment parameters are summarized in Table 2.

To study the impact of immunoadjuvant, three different concentrations (0.5, 1.0, and 2.0%) of GC were used to treat the metastatic mammary tumors; 16 rats were used for each concentration.

Treatment Procedures of Laser Immunotherapy. The tumor-bearing rats underwent treatment when the primary tumor reached 0.2–0.5 cm^3. In the treatment groups without laser irradiation, 200 μl of aqueous solution (ICG, GC or ICG-GC combination) were injected to the center of each primary tumor. The dose and administration of the dye and/or immunoadjuvant are given in Tables 1 and 2. For the groups receiving laser treatment, the solution (ICG, GC, or ICG-GC combination) was administered in the same fashion 2 h before laser irradiation. Before the laser treatment, the rats were anesthetized, and the hairs overlying the tumor were clipped. The laser energy was directed to the treatment sites through optical fibers. In all of the treatments, the laser settings were selected as 2 W and 10 min. The laser fiber tip was maintained at a distance 4 mm from the overlying skin. For detailed treatment procedures, refer to Refs. 27–32.

Posttreatment Observation. After treatment, the rats were housed in individual cages. In the survival studies, the rats bearing breast cancer or prostate cancer were observed daily, and the three dimensions of each tumor were measured weekly. The average survival time of each treatment group was compared with that of the untreated control group.

Table 1 *Permutations and treatment parameters of different components in laser immunotherapy for treatment of metastatic breast tumors in female rats*

Group	Parameters		No. of rats
	Laser	Dye/Adjuvant	
Control			35[a]
ICG injection only		0.25% ICG[b]	12
GC injection only		1% GC[b]	12
Laser only	2 W; 10 min		12
Laser + ICG	2 W; 10 min	0.25% ICG	12
Laser + GC	2 W; 10 min	1% GC	12
ICG + GC injection		0.25% ICG/1% GC[b]	12
Laser + ICG + GC	2 W; 10 min	0.25% ICG/1% GC	31[a]

[a] The data were collected from two separate experiments.
[b] The ICG, GC, or ICG-GC solutions (200 μl) were injected directly to the center of the primary tumor.

Table 2 *Treatment parameters using different components in laser immunotherapy for treatment of metastatic prostatic tumors in male rats*

Group	Parameters		No. of rats
	Laser	Dye/Adjuvant	
Control (tumor burden study)			9
Laser + ICG (tumor burden study)	2 W; 10 min	0.25% ICG[a]	6
Laser + ICG + GC (tumor burden study)	2 W; 10 min	0.25% ICG/1% GC[a]	8
Control (survival study)			8
Laser + ICG + GC (survival study)	2 W; 10 min	0.25% ICG/1% GC	9

[a] The ICG, GC, or ICG-GC solutions (200 μl) were injected directly to the center of the primary tumor.

In the tumor burden study using the prostate tumor model, the rats in all three groups (control, laser-ICG treated, and laser-ICG-GC treated) were terminated 49 days after tumor implantation. The primary tumors were collected, and the weight and the volume of each tumor were measured. The metastases in the lung of each rat were also collected, and the total volume of the metastases from each rat was measured.

Statistical Analysis. The statistical analysis was performed using StatView (SAS Institute, Cary, NC). ANOVA was used to test the differences between the continuous parameters such as the volume of metastasis. The survival curves were generated using the method of Kaplan and Meier, and the log-rank test was used to detect significant difference between survival curves. For all of the determinations, $P < 0.05$ was used to indicate statistical significance.

RESULTS

Effect of Different Components of Laser Immunotherapy in Treatment of Metastatic Breast Tumors. The metastatic breast tumor model in Wistar Furth female rats was used for survival studies. The rats were separated into eight groups, one control group and seven groups treated by various permutations of the three components, as shown in Table 1. Twelve rats were used in each group, except for the control and the laser-ICG-GC groups, which contained the rats from two separate experiments. Three groups of rats were treated by single component, *i.e.*, either by injection of ICG or GC solution, or irradiated by the 805-nm laser only. The survival rates of the rats in these three groups are given in Fig. 1. As shown in Fig. 1, the rats in the ICG and laser-only groups died with an average survival time close to that of the control group. One rat in the GC-only group became a long-term survivor, whereas another rat in that group had prolonged survival time (see the *dotted curve* in Fig. 1). Statistical analysis showed that there was no significant difference in median survival times among the four groups shown in Fig. 1. The rat survival rate in the groups treated by the two-component combinations, *i.e.*, laser-ICG, laser-GC, or ICG-GC, are given in Fig. 2. Laser-GC treatment resulted in one long-term survivor, whereas ICG-GC injection resulted in two long-term survivors. However, there was no significant difference between the median survival times among the four groups presented in Fig. 2. The results using the standard treatment procedure of laser immunotherapy (laser-ICG-GC combination) are given in Fig. 3. In two separate experiments of 31 rats, 9 rats had long-term survival after the standard laser immunotherapy treatment, resulting in a 30% cure rate. Furthermore, the median survival time of the laser-ICG-GC-treated rats was significantly higher than that of untreated control rats ($P < 0.0001$).

The regression and total disappearance of untreated metastatic tumors in successfully treated rats were also observed. These secondary tumors in remote areas usually emerged 2 weeks after the implantation of the primary tumor (also after the treatment of the primary tumors) and reached a peak size before the regression. The time course of two such metastases in a successfully treated rat is given in Fig. 4. In comparison, the time course of two metastases in a control tumor-bearing rat is also given in Fig. 4.

Effect of Different Components of Laser Immunotherapy in Treatment of Metastatic Prostate Tumors. The metastatic prostate tumor model in Copenhagen male rats was used for both tumor burden

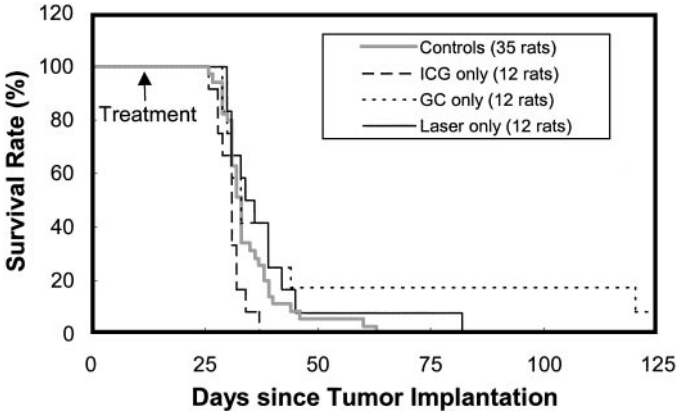

Fig. 1. Survival rates of tumor-bearing rats treated by only one component of laser immunotherapy. The *thick shaded curve* represents 35 untreated control rats. The *dashed curve* represents rats treated with an intratumor injection of 200 μl of solution of 0.25% ICG. The *dotted curve* represents rats treated with an intratumor injection of 200 μl of solution of 1% GC. The *thin solid curve* represents rats treated with laser only at 2 W and 10 min. The only long-term survival rat was observed in the GC-treated group (*dotted curve*). The surviving rates using ICG alone and laser alone treatment were close to that of the control group.

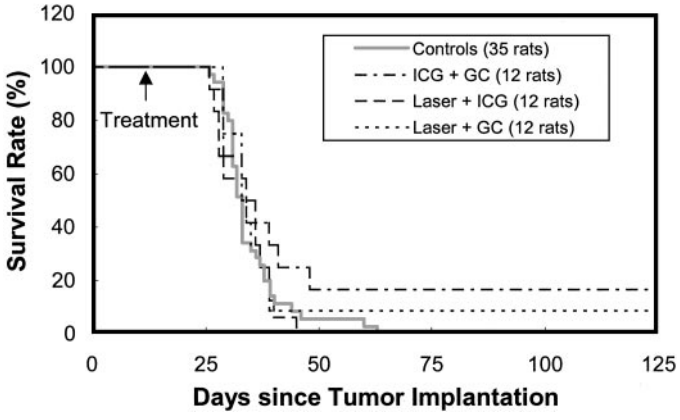

Fig. 2. Survival rates of tumor-bearing rats treated by two-component combinations of laser immunotherapy. The *thick shaded curve* represents 35 untreated control rats. The *dash-dotted curve* represents rats treated with an intratumor injection of 200 μl of solution containing 0.25% ICG and 1% GC. The *dashed curve* represents rats treated with an intratumor injection of 200 μl of solution of 0.25% ICG, followed by laser irradiation at 2 W for 10 min. The *dotted curve* represents rats treated with an intratumor injection of 200 μl of solution of 1% GC, followed by laser irradiation at 2 W for 10 min.

and rat survival studies. In the tumor burden studies, 23 rats were separated into three groups, *i.e.*, the control group, the laser-ICG-treated group, and the laser-ICG-GC-treated group. The primary tumors and lung metastases from rats in all three groups were collected and measured 49 days after the tumor implantation and 34 days after the therapy (laser-ICG or laser-ICG-GC). On average, the burden of the primary tumor and the lung metastasis of laser-ICG treated rats was ~38% of that of untreated control group. On the other hand, the average tumor burden in the laser-ICG-GC-treated group was ~33% of that of the untreated control group. Significant differences in tumor burdens were observed between the laser-ICG-treated group and the control group ($P = 0.0001$) and between the laser-ICG-GC-treated group and the control group ($P = 0.0002$). The tumor burdens of the rats in the two treatment groups did not show significant difference. However, as shown by the data in Table 3, two rats in the laser-ICG-GC group had no metastases, indicating significant tumor regression.

Seventeen rats were used in survival studies, 8 rats in the control group and 9 rats were treated by the injection of ICG-GC solution, followed by the laser irradiation (see Table 2 for the detailed treatment parameters). Two rats in the treatment group survived >120 days, in comparison with the average survival time (~60 days) of the untreated control group, as shown in Fig. 5.

Effect of GC with Different Concentrations. GC was used in laser immunotherapy with three different concentrations. Forty-eight rats were divided into three groups and treated with the laser-ICG-GC combination with the concentration of GC varying from 0.5 to 2%, whereas the parameters of laser and ICG were the same as in the other treatments (see Table 1). The rat survival data are given in Fig. 6. All three concentrations resulted in long-term survivors. However, the 1% GC appeared to be more effective in the treatment, yielding a 38% survival rate *versus* 7% and 19% using 0.5 and 2% GC, respectively.

Statistical analysis showed that there was no significant difference in median survival times between the untreated control rats and the rats treated with laser, ICG, and 0.5% GC. The median survival times of rats treated with 1.0% GC and 2.0% GC were both significantly longer than that of the untreated control rats ($P = 0.001$ and $P = 0.0012$, respectively). However, there was no significant difference between the 1.0% GC-treated group and the 2.0% GC-treated group.

DISCUSSION

Combination therapy using immunoadjuvant has become popular in cancer treatment, not only in the conventional arena such as chemotherapy but also in promising new modalities such as photodynamic therapy. When *Corynebacterium parvum*, bacille Calmette-Guérin, and other immunoadjuvants were intratumorally administered in conjunction with photodynamic therapy treatment, greater tumor response and prolonged survival of tumor-bearing rats were observed (21–24). Long-term impact of immunoadjuvant in combination with PDT has been observed with an enhanced resistance to tumor rechallenges in PDT-cured rats (37).

When used appropriately, immunoadjuvants can significantly improve the efficacy of cancer treatment by stimulating the host immune system. Because tumor-specific immunological response can lead to a long-term cure, its induction has been actively sought in cancer treatment. Laser immunotherapy was developed to target the local tumor mass and at the same time to induce an antitumor immunity (25, 26). It relies on three components in the treatment: a near-infrared laser, a laser-absorbing dye, and an immunoadjuvant. It started with a selective photothermal reaction using the laser-dye combination (27–29). The introduction of immunoadjuvant was an attempt to enhance the laser treatment through the synergism between the photophysical and photobiological reactions. The selective photothermal laser-tissue interaction using the 805-nm laser and

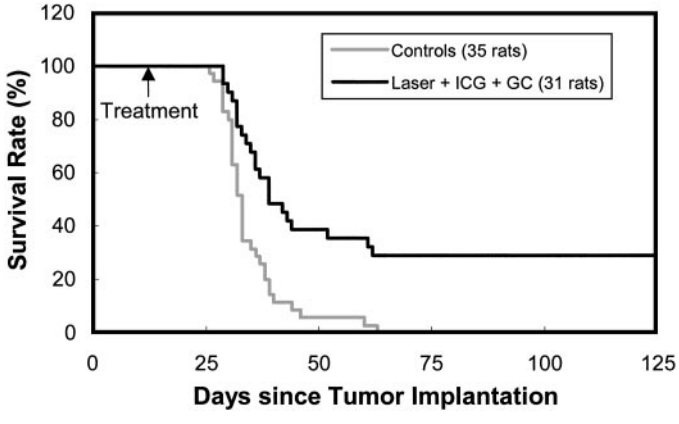

Fig. 3. Survival rates of tumor-bearing rats treated by the combination of three components of the laser immunotherapy. The *thick shaded curve* represents 35 untreated control rats. The *thick solid curve* represents rats treated with an intratumor injection of 200 μl of solution containing 0.25% ICG and 1% GC, followed by laser irradiation at 2 W for 10 min.

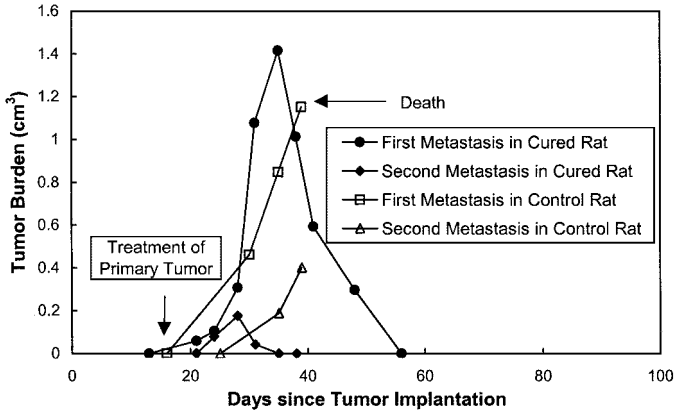

Fig. 4. The metastatic tumors of a successfully treated rat implanted with the breast tumor. The treatment of the primary tumor at the right inguinal fat pad was at day 12. The two secondary tumors emerged after the treatment, with right axillary metastasis (●) first and the left axillary metastasis (♦) ~1 week after the treatment. Both tumors continued to grow and then regressed and totally disappeared within 40 days of treatment. In comparison, the first metastasis (□) and the second metastasis (△) of a control tumor-bearing rat continued to grow until death occurred at day 39 after tumor implantation.

ICG has been demonstrated through *in vitro* and *in vivo* experiments (27–29). The laser-ICG-GC treatment also demonstrated an induced antitumor immunity using the metastatic breast tumor model (30–32). This current research is an attempt to further understand the functions of each component involved in the laser immunotherapy.

In the treatment of the metastatic breast tumor (DMBA-4) using various permutations of the three components, the results can be divided into two categories: groups with and without long-term survival rats. All of the groups treated with GC as one component belong to the first category. In this category, there were long-term surviving rats in each group, which involves GC either through injection only or in combination with the dye or the laser (see the *dotted curve* in Fig. 1, and *dotted* and *dash-dotted curves* in Fig. 2). However, the laser-ICG-GC combination proved to be the most effective, with a survival rate close to 30%, as shown by the results presented in Fig. 3. It is worth noting that most of the surviving rats developed metastases in the early stages and then regressed gradually, as shown in Fig. 4.

All of the groups without GC belong to the second category, in which no successful treatment was achieved. In this category, all rats were treated with either ICG, laser, or a combination of the two. No long-term survival rats were observed, as shown in Figs. 1 and 2 (*dashed* and *solid curves*). The average survival rate in each group showed little or no improvement compared with that of the untreated control group. The rats in these groups developed multiple metastases, and all of the metastatic tumors continued to grow until death.

Statistical analysis showed that the median survival times of treated rats using one and two components were not significantly different from that of untreated control rats. However, the three-component treatment significantly increased the median survival time ($P < 0.0001$) in comparison with that of untreated control rats.

The laser immunotherapy treatment was also effective in our pilot study using metastatic prostate tumor model (Mat-Lu). At the time of termination (49 days after tumor implantation and 34 days after the treatment), the laser-ICG-GC treatment reduced both the primary tumor and lung metastases by ~67%, whereas the laser-ICG treatment yielded a 62% reduction on the primary and secondary tumor burden. Although the difference in the tumor burden reduction between the two groups was not significant, a close examination on the metastatic tumor burdens in Table 3 showed that two rats in the laser-ICG-GC group had no noticeable metastasis, indicating the tumor regression. Furthermore, results in Fig. 5 showed two long-term survivors after laser-ICG-GC treatment. Although the number of animals in our prostatic tumor model study was small, the results indeed showed a trend that supports the enhancement function of GC.

Three different concentrations of GC were used in treating the breast tumors. The results showed a clear differentiation, as shown by the experimental data in Fig. 6. The 1% GC solution in laser immunotherapy provided a 38% long-term survival rate, whereas the 2 and 0.5% GC concentrations yielded 19 and 7% survival rates, respectively. Among the three treatment groups, 1.0% GC and 2.0% GC yielded significantly longer median survival times, compared with that of the control group ($P = 0.001$ and $P = 0.0012$, respectively). However, there was no significant difference between the 1.0 and 2.0% GC-treated groups, suggesting no benefit from increasing GC concentrations >1%.

The immunogenicity of the DMBA-4 model has been tested recently in a preliminary study using a freeze-thaw tumor cell lysate experiment. The immunized rats were challenged by the DMBA-4 tumor cells 3 weeks after the immunization. Tumors, both primary and metastases, were observed in all of the rats. In another experiment, primary tumors were surgically removed; however, both primary and metastases were developed after the surgery. Although our experimental results were not conclusive, we believe that the level of immunogenicity of the tumor model is low. As a consequence, the

Table 3 *Burdens of the lung metastases in rats bearing Mat-Lu prostatic tumors after laser-ICG and laser-ICG-GC treatment*[a]

Group 1 Control	Metastases (cm^3)	Group 2[b] Laser-ICG	Metastases (cm^3)	Group 3[c] Laser-ICG-GC	Metastases (cm^3)
Rat 1	4.4	Rat 1	0.3	Rat 1	1.2
Rat 2	12.0	Rat 2	0.0	Rat 2	0.3
Rat 3	10.9	Rat 3	6.0	Rat 3	5.5
Rat 4	2.6	Rat 4	3.2	Rat 4	0.6
Rat 5	12.0	Rat 5	0.4	Rat 5	0.0
Rat 6	0.8	Rat 6	4.3	Rat 6	4.5
Rat 7	2.9			Rat 7	3.5
Rat 8	4.7			Rat 8	0.0
Rat 9	6.3				
Average	6.3		2.4		2.0
SD	4.3		2.5		2.2

[a] The tumors were collected 49 days after the tumor implantation (34 days after the treatment).

[b] Tumor-bearing rats in group 2 were treated by the laser-ICG combination with an intratumor injection of 200 μl of solution of 0.25% ICG, followed by the laser treatment at 2 W and 10 min, 15 days after tumor implantation.

[c] Tumor-bearing rats in group 3 were treated by the laser-ICG-GC combination with an intratumor injection of 200 μl of solution of 0.25% ICG and 1% GC, followed by the laser treatment at 2 W and 10 min, 15 days after tumor implantation.

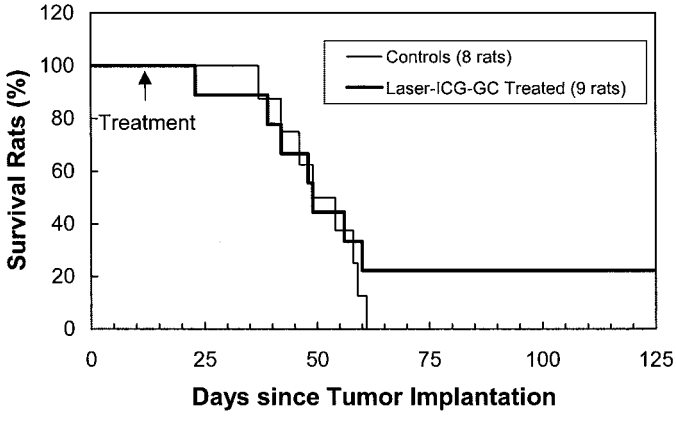

Fig. 5. Survival rates of rats bearing Mat-Lu metastatic prostate tumors. The *thin curve* represents eight untreated control tumor-bearing rats. The *thick curve* represents nine rats, treated by the laser-ICG-GC combination. Two rats survived >120 days, whereas all of the control rats died within 60 days.

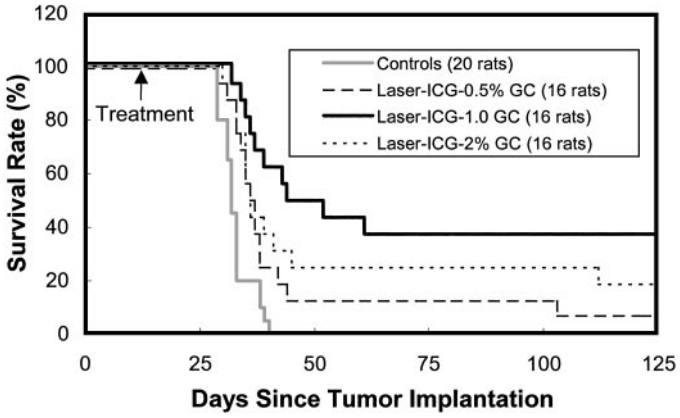

Fig. 6. Survival rates of rats bearing metastatic breast tumors after treatment of laser immunotherapy using different concentrations of GC. The *thick shaded curve* represents 20 untreated tumor-bearing rats. Rats in other groups were treated by intratumor injection of 200 μl of solution (0.25% ICG and GC), followed by laser irradiation (2 W for 10 min). The concentrations of GC used in the experiments were 0.5% (*dashed curve*), 1.0% (*thick solid curve*), and 2.0% (*dotted curve*).

results on the DMBA-4 metastatic tumor model obtained in this study showed a promising clinical potential of laser immunotherapy.

The results shown in Figs. 1–5 and Table 3 clearly demonstrated the essential role of the immunoadjuvant in laser immunotherapy. Without the application of GC, no other components of laser immunotherapy could result in long-term cure of the tumor-bearing rats. Although the laser-ICG combination has shown strong selective tumor destruction on a large scale, the photothermal interaction alone does not appear to be sufficient for total tumor eradication. GC, either through direct injection or in combination with laser irradiation, was necessary for a curative effect to occur. However, the laser-ICG-GC combination appears to be most effective.

The immunoadjuvant is a crucial component in laser immunotherapy. With the help of its immune stimulation, laser immunotherapy has shown many promising features such as local drug injection and noninvasive local laser treatment, while providing a significant and systemic antitumor immunity.

The current investigation has shown the importance of the immunoadjuvant and at the same time indicated that the treatment parameters need to be further optimized. The concentrations of the laser-absorbing dye and immunoadjuvant need to be adjusted to improve the efficacy of laser immunotherapy. Furthermore, the efficacy of this novel modality could be significantly improved by the addition of factors to nonspecifically stimulate the immune system before laser immunotherapy and/or by posttreatment with factors to expand specific immune clones produced by laser immunotherapy.

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