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LONG-TERM TUMOR RESISTANCE INDUCED BY LASER PHOTO-IMMUNOTHERAPY

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An ideal treatment modality for metastasizing tumors should eradicate the primary tumor and elicit a systemic, tumor-selective response leading to elimination of metastases and long-term tumor resistance. Also, it should be induced by local treatment at the primary site, to limit adverse systemic effects. A new method for treating metastatic tumors which utilizes a combination of a near-infrared laser, a photosensitizer and an immunoadjuvant has been developed. It involves intra-tumor injection of the sensitizer/adjuvant solution, followed by local non-invasive laser irradiation. It has produced regression and total eradication of treated primary tumors and untreated metastases at remote sites against mammary tumors in rats. Successfully treated tumor-bearing rats showed total tumor resistance to subsequent tumor rechallenge. Our histochemical results showed that sera from cured tumor-bearing rats contained antibodies that bound strongly to the plasma membrane of both living and preserved tumor cells. Western blot analysis of tumor cell proteins using sera from successfully treated rats as the source of primary antibodies also showed distinct bands, indicating induction of tumor-selective antibodies. Our findings indicate that a systemic, long-term effect on metastatic tumors can be induced by local application of laser photo-immunotherapy. *Int. J. Cancer* 81:808–812, 1999.

Systemic cure and long-term resistance to recurrence of malignant tumors of the same type have been elusive goals in the treatment of human cancer. Current systemic treatments, such as chemotherapy, have had limited success due to severe toxic side effects and a high rate of tumor recurrence. Even though single-modality treatments such as radiation and surgery can be curative locally, they lack systemic effects and fail to elicit long-term anti-tumor responses. Conventional immunotherapy depends on either cross reactive tumor-specific antigens or the use of non-specific immunological stimulation. The former has been unsuccessful because the ubiquitous tumor-specific antigens that can be isolated in all human tumors of the same type are not available; the latter, using immunotherapy with adjuvants such as bacille Calmette-Guérin (BCG), *C. parvum* and Freund's adjuvant, has also failed due to lack of specificity.

The use of lasers is gaining widespread acceptance as an effective local treatment due to the precision of energy delivery achieved with the assistance of photosensitizers, as in the case of photodynamic therapy (PDT) (Dougherty *et al.*, 1978, 1989; Fisher *et al.*, 1995). However, the immunological effects of PDT on cancer treatment are unclear. Some groups have reported increased activities of T cells and local macrophages following PDT (Canti *et al.*, 1994; Korbelik and Krosl, 1994; Krosl *et al.*, 1995, 1996), while others have reported immune suppression by PDT (Elmets and Bowen, 1986; Gomer *et al.*, 1988; Lynch *et al.*, 1989; Obochi *et al.*, 1995).

Laser immunotherapy (Chen *et al.*, 1997) was developed to achieve a systemic anti-tumor response in the treatment of cancers. It uses laser-dye photothermal tissue destruction and a co-administered immunoadjuvant for immune stimulation. Initial results indicate success in the treatment of metastatic mammary tumors in rats, including total eradication of primary tumors and untreated metastases. The mechanism of laser immunotherapy has been suggested to involve an induced tumor-selective immune response. The objectives of the current research are to further investigate the systemic effects of this treatment modality on metastatic tumors, to analyze the long-term tumor immunity induced by such treatment and to examine the possible immunological mechanisms using histochemical assays and Western blot analysis.

MATERIAL AND METHODS

Metastatic tumor model

DMBA-4 transplantable, metastatic mammary tumor cells (Kim, 1977) were implanted in young Wistar Furth female rats (Harlan Sprague-Dawley, Indianapolis, IN), ranging in age from 6 to 8 weeks and weighing 150 to 200 g. The tumor line was passed serially through living hosts in our laboratory. Rats were inoculated with 10^5 viable tumor cells s.c. in one of the inguinal fat pads 7 to 10 days before treatment. The primary tumor usually became palpable in 5 to 7 days, and the metastases in the remote inguinal and axillary areas appeared 15 to 20 days after inoculation. Without treatment, tumor-bearing animals had an average survival time of approximately 33 days.

Photosensitizer and immunoadjuvant

The photosensitizer used in our experiments was indocyanine green (ICG) (Becton-Dickinson, Cockeysville, MD). The immunoadjuvant used was glycated chitosan (GC) prepared in our laboratory by incubating an aqueous suspension of chitosan with a 3-fold excess of galactose and subsequent stabilization by borohydride reduction of the Schiff bases. Aqueous ICG solution has an absorption peak near 800 nm. The final aqueous solution contained 0.25% ICG and 1% GC. A volume of 200 µl of the solution was injected directly into the center of each primary tumor before laser treatment, resulting in an ICG dose of 2.5 mg/kg and a GC dose of 10 mg/kg.

Laser photo-immunotherapy in the treatment of metastatic rat mammary tumors

Laser treatment was performed when the primary tumors reached a size of 0.2 to 0.5 cm^3. Two hours after the ICG/GC intra-tumor injection, the tumor was irradiated using an 805-nm diode laser (Diomedics, Woodlands, TX) in a non-invasive mode. The tip of the optical fiber was maintained at a 4-mm distance from the skin overlying the tumor, and the fiber tip was moved smoothly over the entire tumor. A laser spot 3 mm in diameter was produced on the treatment surface. The laser was operated at 2 watts for 10 min, delivering a total energy of 1,200 J to the tumor. The total

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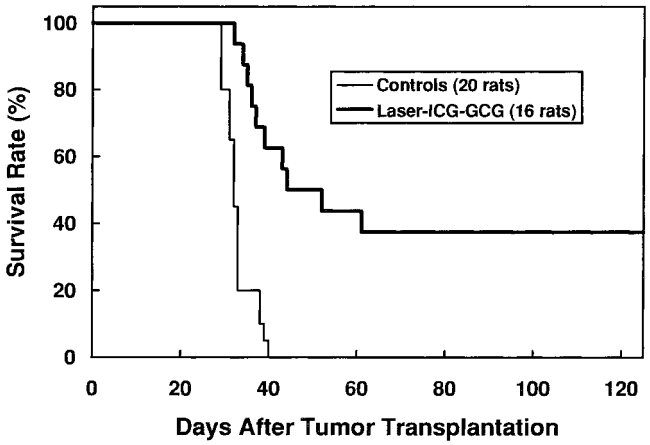

FIGURE 1 – Kaplan-Meier plot of rat survival rate after tumor inoculation. Thick curve represents 16 rats treated with an intra-tumor injection of 200 µl aqueous solution (0.25% ICG and 1% GC), followed by laser irradiation at 2 watts for 10 min. Thin curve represents 20 untreated control tumor-bearing rats, all of which died within 40 days of tumor inoculation, with an average survival time of 32.7 days (±3.5 days SD).

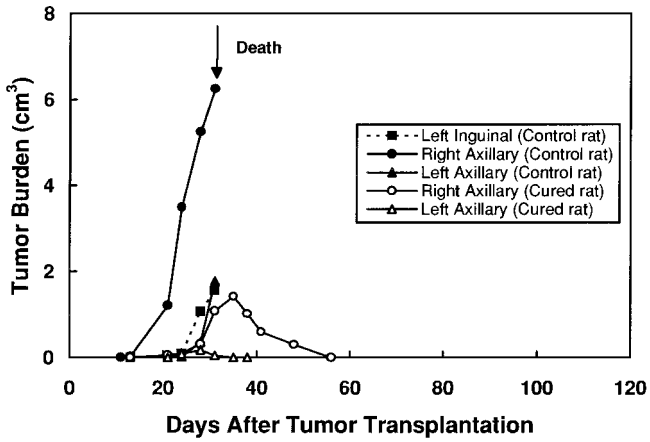

FIGURE 2 – Size of metastatic tumors in unimplanted axillary areas for a successfully treated tumor-bearing rat (open circles and triangles) and in the axillary and inguinal areas of an untreated control tumor-bearing rat (solid squares, circles and triangles). The primary tumor site in both cases was the right inguinal fat pat inoculated with 10^5 viable tumor cells. Laser–ICG–GC treatment took place on day 9. For the treated rat, the metastatic tumor burden continued to increase immediately following treatment, then began to decline between 30 and 35 days; the last metastatic tumor disappeared around day 55, and no recurrence was found throughout 240 days of observation. For the untreated rat, metastases continued to grow until the time of death on day 31, as noted by the arrow.

fluence delivered to each tumor was 68,000 J/cm^2 over the entire surface area.

Fluorescent labeling of living tumor cells

A tumor-bearing rat successfully treated by laser–ICG–GC was rechallenged with 10^6 viable tumor cells 120 days after the initial inoculation. Sera from the rechallenged rat (32 days after rechallenge) and from a control tumor-bearing rat were collected and diluted 1:1,000 in PBS. Freshly collected tumor tissue was dispersed to a single-cell suspension by grinding in a loose-fitting ground glass homogenizer. Approximately 10^6 tumor cells were incubated with 1 ml diluted serum for 1 hr at room temperature and

TABLE I – RECHALLENGE WITH TUMOR CELLS OF RATS PREVIOUSLY CURED BY LASER–ICG–GC TREATMENT

Group	Number of rats	Number of tumor cells	Tumor occurrence	Death rate (in 30 days)	Death rate (in 40 days)	Survival (days)
Cured rats[1]	15	10^6	0%	0%	0%	>120
Age-matched tumor control rats[2]	18	10^6	100%	83%	100%	28.2 ± 2.8
Young tumor control rats[3]	20	10^5	100%	20%	100%	32.7 ± 3.5

[1]Tumor-bearing rats cured by laser–ICG–GC treatment. These tumor-free rats were rechallenged with 10^6 viable tumor cells 120 days after the initial inoculation.–[2]Untreated rats of the same age as the cured rats at the time of inoculation, without previous exposure to tumor.–[3]Same tumor-bearing control rats shown in Figure 1, with tumor inoculation at the age of 8 weeks.

washed 3 times in PBS, which was followed each time by low-speed centrifugation, to remove unbound antibody. Cells were then incubated with secondary fluorescein-labeled goat anti-rat anti-serum (Sigma, St. Louis, MO) for 1 hr at room temperature and rinsed in PBS 3 times. Finally, cells were mounted in an aqueous mounting medium and viewed immediately with a fluorescence microscope.

Tumor tissue immunoperoxidase

Tumor tissue was fixed in 2% paraformaldehyde, then dehydrated and embedded in paraffin. Sections were cut, mounted on glass slides and then rehydrated. Sections were incubated for 1 hr with the diluted serum (1:1,000) from a control tumor-bearing rat and from a successfully treated tumor-bearing rat (32 days after tumor rechallenge), respectively. Sections were then rinsed 3 times in PBS. After the final wash, sections were labeled with peroxidase using an ABC kit (Vector, Burlingame, CA) and viewed by optical microscopy.

Western blot analysis

As the sources of primary antibodies, sera were collected from naive and untreated tumor-bearing rats, as well as from successfully treated tumor-bearing rats at different times after tumor rechallenge. Tumor tissue collected from a control tumor-bearing rat was homogenized to a single-cell suspension. Cells were washed twice in PBS at 4°C and then lysed in Laemmli's sample buffer (Bio-Rad, Hercules, CA) containing 5% β-mercaptoethanol at a final concentration of 8×10^6 cells/ml. Protein extract from 1.6×10^5 tumor cells was loaded into each well of a 10% SDS-polyacrylamide gel and electrophoresed at 100 V for 15 min and then at 200 V for 50 min. Proteins on the gels were transferred to a Hybond-ECL nitrocellulose membrane (Amersham, Arlington Heights, IL) with a 250-mA current at 4°C for 2.5 hr. The membrane was soaked in a blocking solution [50 mM Tris (pH 7.5), 0.9% NaCl, 0.05% Tween-20, 3% non-fat dry milk] for 2 hr at room temperature and incubated in a blocking solution containing rat serum (1:100) overnight at 4°C. After incubation, the membrane was washed 3 times and then incubated with the secondary antibody (anti-rat Ig, horseradish peroxidase–linked whole antibody; Amersham) in a 1:5,000 solution for 1 hr at 4°C. The membrane was then washed 3 times with blocking buffer and rinsed twice with cold PBS. Bands were visualized on X-ray film using a chemiluminescent detection system (Amersham) according to the manufacturer's specifications.

RESULTS

Long-term survival of tumor-bearing rats and regression of untreated metastases after laser immunotherapy

After treatment of primary tumors by laser–ICG–GC, increased survival and total tumor eradication were achieved. A recent experiment yielded 38% long-term survival 120 days after tumor

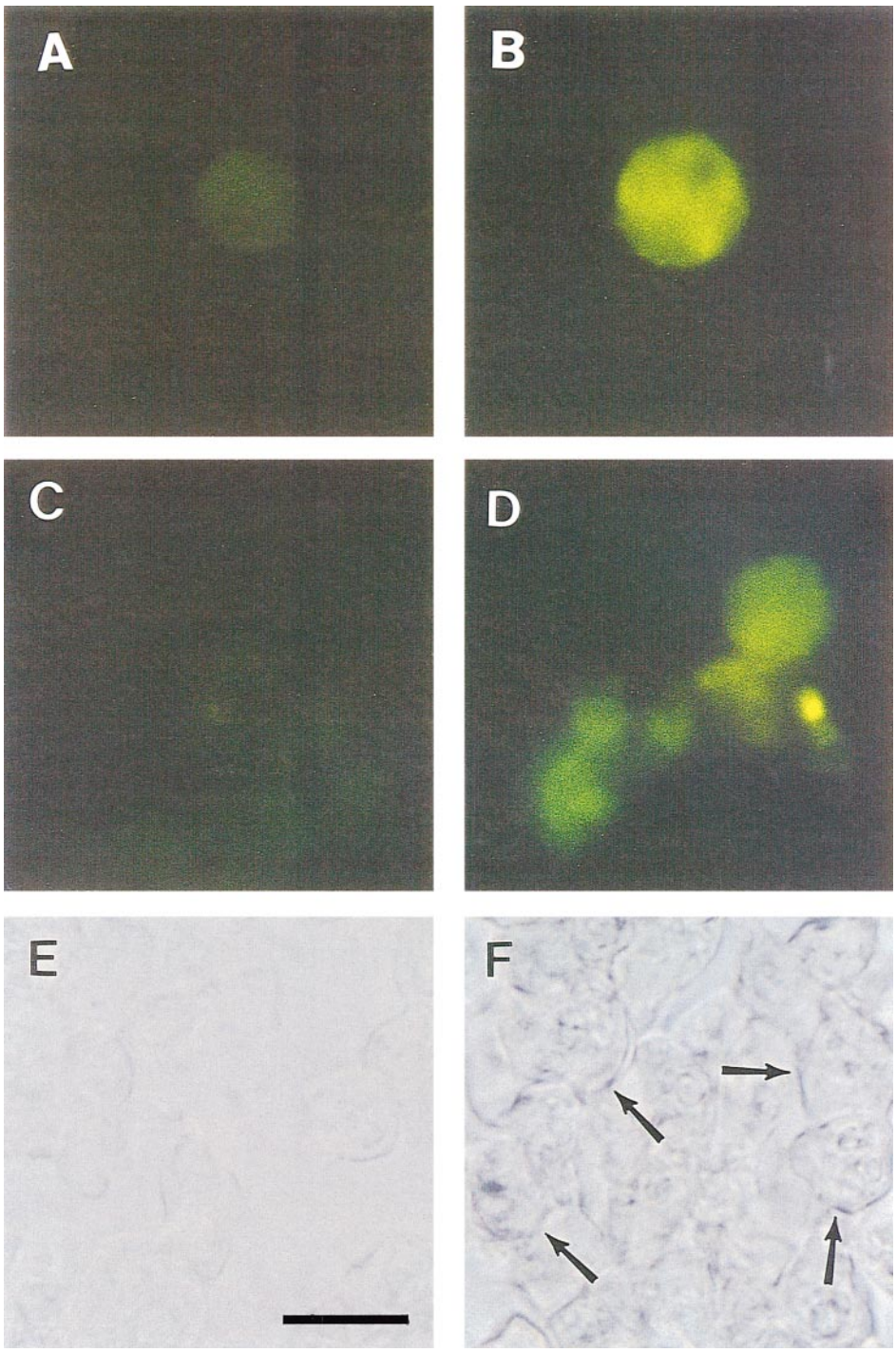

FIGURE 3 – Detection of tumor-selective antibodies in the sera of successfully treated tumor-bearing rats. *(a–d)* Representative photomicrographs of a single living tumor cell and a cluster of living cells incubated with sera from an untreated tumor-bearing rat 32 days after tumor inoculation *(a, c)* and from a successfully treated tumor-bearing rat 32 days after tumor rechallenge *(b, d)*. There is minimal fluorescence in the cell stained by serum from untreated tumor-bearing rats. In contrast, the tumor cell stained with sera from cured tumor-bearing rat shows greater fluorescence intensity and a uniform staining pattern over the plasma membrane. *(e, f)* Photomicrographs of tumor sections incubated with sera from an untreated tumor-bearing rat 32 days after tumor inoculation *(e)* and from a successfully treated tumor-bearing rat 32 days after tumor rechallenge *(f)*. Note in *(e)* the lack of brown reaction product that indicates peroxidase activity. In contrast, intense staining is seen in *(f)*. Note the intense staining at the plasma membrane (arrows) and the lack of staining within the cells. Scale bar: 20 μM.

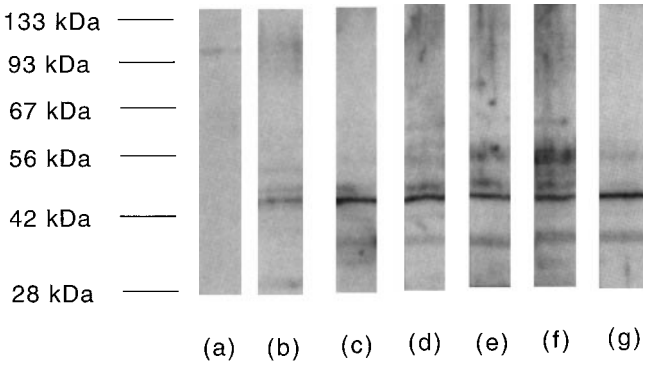

FIGURE 4 – Western blot analysis of tumor cell proteins using sera from different rats as the source of primary antibodies. *(a)* Tumor cell proteins probed using serum of a naive rat. *(b)* Tumor cell proteins probed using serum from an untreated control tumor-bearing rat. *(c–g)* Tumor cell proteins recognized using serum of a tumor-bearing rat cured by laser immunotherapy and 0 hr *(c)*, 1 week *(d)*, 2 weeks *(e)*, 1 month *(f)* and 2 months *(g)* after tumor rechallenge. There was very light antibody binding when the serum from the naive rat was used *(a)*. Note the differences in the 2 bands at approximately 45 and 35 kDa between the tumor control rat *(b)* and the cured rat *(c–g)*. There are also heavy bands at the bottom of the gel below 28 kDa (not shown) recognized by the serum from the cured rat.

inoculation, a 300% increase in the length of survival compared with untreated control tumor-bearing rats (Fig. 1). Although the primary tumors of long-term survival rats usually continued to grow immediately following treatment, after 4 to 6 weeks the tumor burden began to decrease and the tumor disappeared in 9 to 12 weeks. All tumor-bearing rats developed metastases in the remote inguinal and axillary areas 2 to 3 weeks after inoculation of the primary tumor. Untreated metastases of cured rats went through a regression pattern similar to that of the successfully treated primary tumors but with a much smaller burden and much earlier disappearance (Fig. 2). Using laser alone and the laser–ICG combination with the same parameters did not result in tumor eradication (data not shown).

Resistance to tumor rechallenge

To determine the long-term effect of laser immunotherapy, successfully treated rats were rechallenged with 10^6 viable cells, 10 times the number of tumor cells that created the original tumor. None of the successfully treated rats developed tumors. In contrast, all age-matched control tumor-bearing rats died within 35 days (Table I), with multiple tumors in the remote inguinal and axillary areas. Also shown in Table I are the data for 20 young control rats, inoculated with only 10^5 viable tumor cells; all of them developed multiple metastases but had a slightly increased survival time compared with the control rats inoculated with a higher tumor dose.

Antibody binding to tumor cells

After tumor rechallenge with an increased tumor dose (10^6 viable cells), sera obtained from successfully treated tumor-bearing rats were analyzed by 2 histochemical assays for tumor-selective antibodies. The first was an immunofluorescence assay which allows the detection of antibodies that bind to the plasma membrane of isolated live tumor cells. The second assay used preserved tumor tissue and the peroxidase reaction product to determine antibody binding to the plasma membrane and other cellular antigens. Both assays showed strong antibody binding using sera from successfully treated rats when compared with sera from untreated control tumor-bearing rats (Fig. 3).

Antibody binding to tumor cell proteins

Figure 4 shows Western blots of tumor cell proteins probed with sera from different animals: a naive rat, a control tumor-bearing rat and a successfully treated tumor-bearing rat. Apparently, the naive rat does not contain any tumor-selective antibody, as evidenced by the lack of staining in the Western blot (Fig. 4*a*). Sera from rats successfully treated by laser immunotherapy show 2 distinct bands (Fig. 4*c–g*) at approximately 45 and 35 kDa. The 45-kDa band was also observed after probing the blot containing the tumor proteins with serum from the control tumor-bearing rat but with much weaker intensity (Fig. 4*b*). The second band at 35 kDa was absent when the serum from the control tumor-bearing rat was used for probing.

DISCUSSION

Stimulation of a systemic and long-term anti-tumor response following localized tumor treatment was achieved using laser photo-immunotherapy. In this treatment modality, the laser and ICG provide a selective local photothermal reaction (Chen *et al.*, 1995*a,b*, 1996). Although ICG and the laser are known to yield a cytotoxic photochemical product, such as singlet oxygen (Fickweiler *et al.*, 1997; Reindl *et al.*, 1997), our results indicate that photothermal destruction of tumor tissue was the dominant reaction (Chen *et al.*, 1995*a,b*, 1996). Introducing the immunoadjuvant GC adds an immunological component to the treatment since chitosan stimulates an immune response in animals (Maeda *et al.*, 1992; Suziki *et al.*, 1986).

The tumor strain used in these experiments is highly aggressive; 99% of untreated tumor-bearing rats died with multiple metastases approximately 33 days after tumor cells were implanted (Table I). In rats successfully treated using laser–ICG–GC, total eradication of both primary and metastatic tumors and long-term resistance to tumor rechallenge were observed. We attribute these results to an induced immunological reaction. The effectiveness of this treatment is due to the immunoadjuvant GC since the photothermal effect of laser alone and of ICG alone does not result in long-term survival (Chen *et al.*, 1996). The tumor profile of the delayed tumor regression in successfully treated rats indicates an immune response intensified with time (Fig. 2). Furthermore, the regression of untreated metastases at remote sites indicates a systemic reaction. The resistance to tumor rechallenge in cured rats by laser immunotherapy strongly suggests tumor-selective immunity (Table I). Our histochemical assays using both live and preserved tumor cells detected tumor-selective antibodies in the sera of successfully treated tumor-bearing rats, at least a subset of which strongly bound to the plasma membrane of tumor cells (Fig. 3).

The characteristics of antibody binding to the tumor cell proteins in Western blot analysis also suggest a mechanism for the observed anti-tumor immunity induced by laser immunotherapy. Tumor cell proteins probed using the sera from successfully treated rats revealed several distinct bands. Particularly interesting are 2 bands at approximately 45 and 35 kDa (Fig. 4). When sera from tumor control rats were used, the 45-kDa band was observed with less intensity, while the 35-kDa band was absent (Fig. 4*b*). The 45-kDa band was absent when blots were incubated with sera from naive rats; it appeared only when rats were exposed to tumor cells and was enhanced markedly by laser immunotherapy (Fig. 4*c–g*). This may represent a natural host immune response to tumors, but without the laser immunotherapy, it would not be strong enough to control tumor growth. The 35-kDa band, however, suggests that a new antibody was induced in treated rats since this band was absent in naive and tumor control rats (Fig. 4*a,b*). The 35- and 45-kDa bands may represent specific immunodominant antigens.

We hypothesize that this tumor-selective immunity is the result of combined photothermal and photo-immunological interactions. The photothermal reaction reduces the tumor burden and at the same time exposes tumor antigens. The immune system, enhanced by the immunoadjuvant GC, would then recognize the exposed antigens and mount a systemic attack on the remaining cells of the treated tumor and on the untreated metastases. Without laser–ICG photothermal destruction, the immune system may not be able to

recognize the well-masked specific antigens on the tumor cell surface; without the immunoadjuvant stimulation, the host immune system may not react fast enough or reach the required strength to control the remaining mass of the primary tumor and its metastases, especially against the aggressive tumor model used in our studies.

Moreover, this immunity is induced by laser–ICG–GC in individual hosts bearing tumors through local treatment. This treatment could, in effect, produce an *in situ* autovaccine without the cross-reactive tumor antigens required by traditional immunotherapy.

Our previous study showed that without GC laser–ICG photothermal interaction could not have a curative effect (Chen *et al.*, 1996). Using laser alone with the same parameters was also inadequate in treating the metastatic tumors. Although laser–ICG treatment of tumor may have some immunological effect, it did not induce a detectable anti-tumor reaction under the conditions of our studies reported here.

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