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Nano Res. Just Accepted Manuscript • DOI 10.1007/s12274-015-0884-x
http://www.thenanoresearch.com on August 20, 2015

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The combination of gold nanorods (GNRs), near-infrared laser light and controlled surface cooling minimizes skin damage during photothermal therapy of tumors in lymph nodes.
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Received: day month year / Revised: day month year / Accepted: day month year (automatically inserted by the publisher)
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ABSTRACT
Photothermal therapy (PTT) using near-infrared (NIR) laser light and gold nanorods (GNRs) shows promise as a novel cancer treatment. However, the laser intensity required to destroy tumor cells located beneath the skin is larger than the threshold intensity that produces skin damage; thus, irradiation with laser light causes damage to the skin as well as the tumor. Here, we show that a temperature control system allows metastatic lymph nodes (LNs) to be treated by PTT using NIR laser light and GNRs without the occurrence of skin damage. A mouse model of LN metastasis was developed by injection of tumor cells, and the tumor-bearing proper axillary LN was treated with NIR laser light after injection of GNRs. The skin temperature was maintained at 45°C during irradiation by means of a temperature control system. Bioluminescence imaging revealed that tumor progression was inhibited in LNs exposed to NIR laser light and GNRs, compared to LNs exposed to NIR laser light alone or controls (no irradiation or GNRs). Furthermore, the skin and LN capsule were macroscopically intact on day 9 after irradiation with NIR laser light, whereas tumor cells within the LN showed necrosis. A numerical analysis demonstrated that the high-temperature zone and LN region showing subsequent damage were localized to an area up to 3 mm in depth. The proposed novel PTT technique, using NIR laser light and GNRs with controlled surface cooling, could be applied clinically to treat metastatic LNs located inside and outside a LN surgical dissection area.

KEYWORDS
lymph node metastasis, photothermal therapy, gold nanorods, temperature control

1. Introduction
Metastasis to regional lymph nodes (LNs) is related to tumor aggressiveness. LN status is an important

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predictor of patient survival, and is used as an indicator in disease evaluation and the selection of therapy. The use of LN resection is limited to those patients that are in a sufficiently good physical condition to tolerate surgery, and in who the risk of metastatic foci invading other major organs is minimal [1]. LN dissection is invasive, but unfortunately it does not destroy micrometastases located outside the dissection area; thus, adjuvant treatments such as radiotherapy and chemotherapy are required. However, since adjuvant treatments cause side effects, there is a genuine need for a new non-invasive therapy for LN metastasis that could be a viable alternative to currently used cancer management strategies.

Optical transparency in the near-infrared (NIR) region (700−1000 nm) is high. Thus, NIR laser light has the ability to penetrate deep into the body but cause only minimal adverse effects to the irradiated region [2]. Gold nanorods (GNRs) show a strong longitudinal surface plasmon resonance mode along the elongated direction when irradiated with NIR laser light. In addition, the absorption wavelength of GNRs can be tuned by changing the GNR aspect ratio [3], resulting in effective heat production. Thus, photothermal therapy (PTT) using GNRs and NIR laser light represents a potentially attractive new treatment for localized cancer [4-6]. However, it is difficult to determine the distribution of laser light inside a biological tissue because the tissue has strong light scattering properties. The scattering and absorption of light in a biological tissue strongly affect the penetration depth of the laser light, and these effects have to be taken into consideration when evaluating the temperature distribution in a biological tissue at different depths.

Most previous studies of PTT using GNRs and NIR laser light utilized experimental systems in which tumor cells were injected subcutaneously and GNRs systemically or locally, and in which laser light was typically applied to the skin at an intensity of ~3 W/cm² [6, 7]. However, this laser intensity is larger than the threshold of ~420 mW/cm² for the induction of skin damage [8]. Indeed, skin damage was reported in all of these published papers. Thus, a new PTT methodology is required that utilizes NIR laser light and GNRs but does not induce skin damage.

A damage function has been defined to evaluate thermal injury [9]. According to this function, damage is defined by time and temperature. Thus, if a PTT technique using NIR laser light and GNRs was developed that resulted in skin exposure below the threshold defined by the damage function, skin damage would not be induced.

In the present study, we developed a new PTT protocol for the treatment of LN metastasis using NIR laser light, GNRs and a skin temperature control system. An experimental model of a tumor-bearing (metastatic) LN was developed in MXH10/Mo-lpr/lpr (MXH10/Mo/lpr) inbred mice [10] by the injection of a mixed solution of tumor cells and GNRs into the proper axillary LN (PALN). Skin temperature was maintained at 45°C during irradiation with NIR laser light by means of a temperature control system. The temperature distribution and region of the LN showing damage were estimated by numerical analysis after taking into account the degree of light scattering. We found that PTT using NIR laser light, GNRs and controlled surface cooling elicited an anti-tumor effect in the metastatic LN but did not induce macroscopic damage to the skin or PALN capsule. The novel technique described in this study could be developed into a new clinical treatment method for LN metastasis that would avoid the adverse effects of LN dissection and adjuvant therapies.

2. Numerical analysis. Introduction

2.1. Temperature distribution generated after irradiation of a LN with NIR laser light

The LN was assumed to be an isotropic bio-simulated material that did not include GNRs, and it was assumed that there was ‘glass’ on the isotropic bio-simulated material. The temperature distribution in the bio-simulated material generated by irradiation with NIR laser light was calculated...
using the Pennes bioheat transfer equation [11] and a radiative transfer equation. The temperature distribution in the bio-simulated material was analyzed in a two dimensional axisymmetric model.

The Pennes bioheat transfer equation is given by:

$$\rho C_v \frac{\partial T}{\partial t} = \nabla \cdot (k \nabla T) + q_{\text{met}} + q_{\text{blood}} - \rho C_v \frac{dT}{dt}$$  \hspace{1cm} (1)

where $\rho$ [kg/m$^3$] is the density of the biological tissue, $C_v$ [J/(kg·K)] the specific heat, $k$ [W/(m·K)] the thermal conductivity, $T$ [K] the temperature, $t$ [s] the time, $r$ [m] the radius, $z$ [m] the depth, $q_{\text{met}}$ [W/m$^3$] the metabolic heat generation rate, $q_{\text{blood}}$ [W/m$^3$] the effect of blood perfusion heat generation, and $q_r$ (W/m$^3$) the radiative heat flux (obtained by solving the radiative transfer equation described below). This equation describes the energy conservation in the biological tissue with heat generation by laser heating, with $q_{\text{blood}}$ being determined by:

$$q_{\text{blood}} = \rho_b C_v \alpha_b (T_a - T)$$  \hspace{1cm} (2)

where $\rho_b$ [kg/m$^3$] is the density of blood, $C_v$ [J/(kg·K)] the specific heat of blood, $\alpha_b$ [1/s] the blood perfusion rate and $T_a$ [K] the temperature of the blood. Equation 1 was solved by a finite volume method [12].

The radiative transfer equation in the bio-simulated material is derived as follows:

$$\frac{dt}{ds}(r,\tilde{s}) = \beta \left[-I_s(\tilde{r},\tilde{s}) + (1-\omega)I_a(\tilde{r},\tilde{s}) \right] + \omega \left[ \frac{\Theta}{4\pi} \Phi(\tilde{r},\tilde{s}) d\Omega \right]$$  \hspace{1cm} (3)

where $\beta$ [1/m] is the extinction coefficient, and $\omega$ [-] is the albedo (i.e., the proportion of incident light or radiation reflected by a surface) inside the bio-simulated material. The 1$\omega$, 2$\omega$, and 3$\omega$ terms on the right side of Eq. 3 denote the extinction of light intensity due to absorption and scattering, the amplification by self-emission, and the amplification due to scattered light coming from unspecified directions. Thus, this equation can practically describe the variation in light intensity due to absorption, emission and scattering. Equation 3 can be solved by the radiation element method using a ray emission model (REM$^2$) [12], permitting the distribution of light intensity $I$ to be determined. The radiative heat flux $q_r$ can be calculated from the distribution of the light intensity $I$.

The thermal and optical properties are summarized in Tables 1 and 2. The initial conditions were: temperature of the bio-simulated material, 37°C; coolant temperature, 10°C (constant); laser beam radius, 3 mm; laser beam power, 10 W; laser beam wavelength, 1,064 nm; and $q_{\text{met}}$ set to 0. The boundary conditions were as follows: the heat transfer coefficient was constant (3rd kind boundary condition) at the skin. The other 3 surfaces were adiabatic. The NIR laser light was irradiated directly onto the bio-simulated material.

### 2.2. The damage function

The damage function $\Omega_{td}$ can be described by [9]:

$$\Omega_{td}(x,y,t) = \int_0^t A_r \exp \left( -\frac{AE_{\text{w}r}}{RT(r,t)} \right) dt,$$  \hspace{1cm} (4)

| Table 1. Thermal and optical properties of the simulated lymph node. |
|---------------------------------|-----------------|
| **Thermal conductivity [W/(m·K)]** | 0.51 [28] |
| Specific heat [J/(kg·K)] | 3,720 [28] |
| Density [kg/m$^3$] | 1,060 [28] |
| Initial temperature [°C] | 37 |
| Blood perfusion rate [1/s] | 1.26×10$^{-2}$ [28] |
| Absorption coefficient for 1,064 nm [1/m] | 50 [29] |
| Scattering coefficient for 1,064 nm [1/m] | 1,830 [29] |
| Anisotropy factor [-] | 0.9 [29] |

Since data for the optical properties of lymph nodes were not available, those of phantom tissue were used [29].

| Table 2. Thermal properties of the quartz glass. |
|---------------------------------|-----------------|
| **Thermal conductivity [W/(m·K)]** | 1.38 [30] |
| Specific heat [J/(kg·K)] | 740 [30] |
| Density [kg/m$^3$] | 2,190 [30] |
| Heat transfer coefficient [W/(m$^2$·K)] | 2,128 |

### 2.2. The damage function

The damage function $\Omega_{td}$ can be described by [9]:

$$\Omega_{td}(x,y,t) = \int_0^t A_r \exp \left( -\frac{AE_{\text{w}r}}{RT(r,t)} \right) dt,$$  \hspace{1cm} (4)
where \( R \) [8.314 J/(mol·K)] is the universal gas constant, \( A_f \) [2.98 \times 10^{90} \text{s}] the frequency factor and \( \Delta E_a \) [506,400 J/mol] the activation energy [13]. The damage, \( \Omega_{rd} \), is dimensionless, exponentially dependent on temperature, and linearly dependent on the exposure time. In the present study, we assumed that irreversible thermal damage would occur when \( \Omega_{rd} \geq 3 \) [14].

3. Materials and methods

All in vivo studies were approved by the Institutional Animal Care and Use Committee of Tohoku University.

3.1. GNRs

Neutravidin polymer-conjugated GNRs (aspect ratio, 6.7; surface plasmon resonance peak, 1,065 nm; axial diameter, 10 nm; length, 67 nm) were used (Nanopartz). The zeta potentials of the GNRs (6.0 \times 10^{10} \text{ particles/mL in distilled water}) were measured (ELSZ-2 analyzer; Otsuka) to be -13.79 ± 1.33 mV (\( n = 3 \)) [15].

3.2. Cell culture

KM-Luc/GFP cells [16], which stably expressed a fusion of luciferase (Luc) and enhanced-green fluorescent protein (EGFP) genes, were cultured in Complete Medium consisting of Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum containing 1% L-glutamine-penicillin-streptomycin (Sigma-Aldrich) and 0.5% geneticin/G418 (Wako). The absence of Mycoplasma contamination was confirmed using a Mycoplasma detection kit (R&D Systems).

3.3. Mice

MXH10/Mo/lpr mice, established by intercrossing MRL/Mpj-lpr/lpr and C3H/HeJ-lpr/lpr strains [10], were bred and maintained at the Institute for Animal Experimentation, Graduate School of Medicine, Tohoku University. Seventy-six mice were used (weight, 25–35 g; age, 11–14 weeks) [15].

3.4. Metastatic LN model

Ten mice were used. KM-Luc/GFP cells (final concentration, 3.3 \times 10^5 \text{ cells/mL}) were suspended in a mixture of 10 \muL phosphate-buffered saline (PBS; Ca^{2+} and Mg^{2+} free) and 20 \muL of 400 mg/mL Matrigel (Collaborative Biomedical Products). This mixture was injected, either with GNRs (1.08 \times 10^{11} \text{ particles added to the mixture; } n = 5) or without GNRs (\( n = 5 \)), into the PALN. Injections were performed under the guidance of a high-frequency ultrasound imaging system (Vevo770, VisualSonics) with a 25 MHz transducer (RMV-710B). The day of inoculation was defined as day 1.

3.5. Light transmittance of the skin

The skin (normal thickness, 0.34 mm) of a mouse was removed, uniformly extended and placed between two glass slides (thickness, 1.3 mm). The slides were secured together using two springs and then placed on a calorimeter (AC2500, Scientech). The slides were irradiated vertically with NIR laser light (wavelength, 1064 nm; laser power, 0.1 W; beam radius, 3 mm). The light transmittance of the skin was found to be circa 65%.

3.6. Skin temperature control system

The system used to control the surface temperature of the skin consisted of a rectangular channel (width, 15 mm; length, 42 mm; height, 1 mm), 2 quartz glasses (thickness, 1 mm) that were light-transmitting windows for the NIR laser light, and a closed forced-circulation refrigerant fluid (water) system (Fig. 1A). The lower quartz glass was placed on the skin surface and NIR laser light was irradiated vertically onto the upper surface of the upper quartz glass. The light transmittance through
Skin temperature control system and laser irradiation system.

A. Skin temperature control system. The skin surface temperature control system consisted of a rectangular channel (width, 15 mm; length, 42 mm; height, 1 mm), 2 quartz glasses (thickness, 1 mm) that were light-transmitting windows for the NIR laser light (1,064 nm), and a closed forced-circulation refrigerant fluid (water) system. The flow rate was 760 mL/sec. The lower quartz glass was located on the skin surface. NIR laser light was irradiated vertically onto the upper glass. The light transmittance, measured by a calorimeter, was 75%.

B. Laser irradiation system. Laser light from a continuous Nd:YVO4 air-cooled laser (wavelength, 1,064 nm; TEM$_{00}$ beam diameter, 0.6 mm) was delivered to the PALN on day 3 and day 4 after tumor cell inoculation, under the operation of the skin surface temperature control system [15].

The temperature control system was 75%, measured by a calorimeter (AC2500, Scientech). The laser intensity was varied according to the preset temperature of the skin.

3.7. Treatment of tumor-bearing PALNs by irradiation with NIR laser light and controlled surface cooling of the skin

Laser light from a continuous Nd:YVO4 air-cooled laser (wavelength, 1,064 nm; TEM$_{00}$ beam diameter, 0.6 mm; CYD-010-TUBC, Neoarc) was delivered to the PALN on day 3 and day 4 after tumor cell inoculation, with the skin surface cooled using the temperature control system (Fig. 1B). The laser beam was delivered through an optical fiber (fiber diameter, 400 μm; diameters of the two collimators, 6 mm) [15]. The temperature at the irradiated site was measured using a K-type thermocouple (Ishikawa Trading). Before irradiation, the skin temperature was cooled to < 20°C by the skin temperature control system (cooling system water temperature, 12°C; water flow rate, 760 mL/sec). The skin was then irradiated with NIR laser light through the skin temperature control system and once the skin temperature had reached 45°C, it was maintained at 45°C for 300 sec by varying the intensity of the laser.
Figure 2. Skin temperature and damage during laser irradiation in the presence and absence of GNRs, with skin surface cooling using the temperature control system. Initially, the skin temperature was cooled to < 20°C. After irradiation with NIR laser light, the skin temperature increased to 45°C within 100 sec. The skin temperature was maintained at 45°C for 300 sec by varying the laser light power; the initial laser power was 2.55 ± 0.45 W in the presence of GNRs (n = 5) and 3.16 ± 0.33 W in the absence of GNRs (n = 5). The water flow rate (12°C) was 760 mL/sec. The heat transfer coefficient was 2,128 W/m²·K. The value of the damage function increased with increasing irradiation time. The value at 400 sec was less than the damage threshold of 3, indicating that only minimal skin damage would be induced.

3.9. Histological analysis

Harvested LNs were fixed overnight in 10% formaldehyde at 4°C (Rapid Fixative, Kojima Chemical Industry), dehydrated, embedded in paraffin and sectioned (2–4 µm serial sections). The sections were stained with hematoxylin and eosin (H&E) and analyzed using a microscope (BX51, Olympus) coupled to a digital camera (DP72, Olympus).

3.10. Statistical analysis

Data are presented as the mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical comparisons were made using the Kruskal-Wallis test. P < 0.05 was considered to be statistically significant.

4. Results

A temperature control system was used to cool the skin to < 20°C before irradiation with NIR laser light. Irradiation of the tumor in the LN was carried out while the skin temperature was maintained at 45°C for 300 sec. We assumed that irreversible thermal damage would occur when $\Omega_{irr} \geq 3$. Figure 2 shows the changes in skin temperature and the damage estimated by the damage function. Before irradiation, the skin was cooled to < 20°C using the temperature control system. The skin temperature increased from 18°C to 45°C within 100 sec both in the presence and absence of GNRs, when the applied laser power was 2.55 ± 0.45 W in the presence of GNRs (n = 5) and 3.16 ± 0.33 W in the absence of GNRs (n = 5). The damage increased with increasing irradiation time both in the presence and absence of GNRs. The damage function $\Omega_{irr}$ was calculated to be < 3 even if the tissue was irradiated for 300 sec, suggesting that damage would not be induced (in theory).

Next, we numerically estimated the axisymmetric 2D temperature distribution (Fig. 3, right) and subsequent distribution of the damage to the LN (Fig. 3, left), when the LN was treated as an
Figure 3. Numerical analysis of the distributions of temperature and damage in the LN after irradiation. The LN was treated as an isotropic bio-simulated material. The coolant temperature was 10°C, the heat transfer coefficient 2.128 W/(m²·K), the radius \( r \) of the laser beam 3 mm, the laser power 10 W, and the initial temperature of the bio-simulated material 37°C. The surface temperature was cooled to around 14.5°C at 100 sec before irradiation. Temperature (°C) is color-coded; the zone of damage is shown in grayscale. A. 0 sec, immediately after the LN had been cooled for 100 sec. B. 10 sec after irradiation. Damage occurred 1 mm below the surface. C. 50 sec after irradiation. The region of damage had a distribution that was somewhat similar to that of temperature. D. 100 sec after irradiation. The system appeared to be in a state of thermal equilibrium.

isotropic bio-simulated material. The surface temperature was cooled to 14.5°C for 100 sec before irradiation with NIR laser light. The skin temperature was maintained at 14.5°C during irradiation and a high-temperature field was generated in a region that excluded the area adjacent to the skin. Subsequently, damage was detected in the high-temperature field region.

The distributions of temperature and damage in the bio-simulated material along the vertical axis at radius \( r = 0 \) mm are shown in Fig. 4. The maximal temperature was generated at a depth of 1 mm from the skin surface. The temperature reached 70°C for an irradiation time of 100 sec (Fig. 3D). The temperature decreased asymptotically to body temperature (37°C) along the depth direction. Maximal damage was generated 1 mm from the skin surface, consistent with the temperature distribution. Assuming that damage occurred when \( \Omega_{TD} \geq 3 \), skin damage would not be induced at 10 sec, but would occur in the area just below the skin at 100 sec. Damage was induced to a depth of 3 mm from the skin surface, i.e., the damage induced by the laser light was restricted to a region that reached a depth of 3 mm from the surface.

Next, we quantified whether irradiation in the
Figure 4. Distributions of temperature and damage in the bio-simulated material along the vertical axis at radius \( r = 0 \) mm. The peak temperature occurred about 1 mm below the surface. The temperature decreased gradually, asymptotically approaching 37°C. The calculated damage function 1 mm below the surface was < 1 at 10 sec after irradiation. However, the value reached > 1,000 after irradiation for 100 sec. Since it was assumed that damage occurred when \( \Omega_{TD} \geq 3 \), damage occurred up to a depth of 2.8 mm below the skin, excluding the region near the surface.

The presence or absence of GNRs exerted any anti-tumor effects. Figure 5 shows temporal changes in the luciferase activity of the PALN, measured using an \textit{in vivo} bioluminescence imaging system. The data in each experimental group were normalized to the luciferase activity measured on day 3. In the control group, tumor cells were implanted into the PALN but the LN did not receive GNRs, irradiation or controlled surface cooling; values for the luciferase activity in the control group were those we have previously published (Okuno et al., 2013) [15]. The relation between normalized luciferase activity and time (days) after inoculation is shown in Fig. 5B. The luciferase activity in the cooling + irradiation group increased over time and at each time point did not differ significantly from values in the control group. However, the luciferase activity in the cooling + irradiation + GNRs group was significantly reduced on day 5 (\( P < 0.05 \), control vs cooling + irradiation + GNRs), although subsequently it increased. Thus, laser treatment with cooling and GNRs resulted in a temporary reduction in the tumor burden of the PALN.

Changes in the size of the PALN, measured using a high-frequency ultrasound imaging system, are shown in Fig. 6. Representative images for the cooling + irradiation and cooling + irradiation + GNRs groups are presented in Fig. 6A. Figure 6B shows the changes in PALN size during the 9 days after inoculation. PALN size was normalized to that on day 0 and the values for luciferase activity in the control group were those we have previously reported (Okuno et al., 2013) [15]. The PALN size increased with time in the control group, consistent
Figure 6. In vivo anti-tumor effects measured using a high-frequency ultrasound system. The tumor in the PALN was irradiated with NIR laser light on days 3 and 4. A. Two-dimensional high-frequency ultrasound images, showing the temporal changes in LN size in the cooling + irradiation (n = 5) and cooling + irradiation + GNRs (n = 5) groups. The dashed line indicates the PALN region. B. Changes in the size of the PALN with time in the control (Okuno et al. [15], n = 6), cooling + irradiation (n = 5) and cooling + irradiation + GNRs (n = 5) groups, assessed using three-dimensional high-frequency ultrasound. The values are normalized to those on day 0. There were no significant differences between groups in PALN size at any of the time points.

with an increase in tumor volume. There appeared to be a trend for the PALN size to increase more slowly in the cooling + irradiation and cooling + irradiation + GNRs groups, although significant differences were not detected between the three experimental groups at any time points. We also investigated the tissue damage induced by irradiation of the PALN in the presence and absence of GNRs (Fig. 7). As shown in Fig. 7A, macroscopic skin damage was not detected after irradiation, whether in the presence or absence of GNRs. Figures 7Ba and 7Bb show histological sections stained with H&E, taken after

Figure 7. Evaluation of tissue damage. A. Skin damage following laser irradiation of the PALN with use of the skin temperature control system. (a), (b), (c): Representative images from the cooling + irradiation group (n = 5). (d), (e), (f): Representative images from the cooling + irradiation + GNRs group (n = 5). Macroscopic damage to the skin over the PALN was not observed following irradiation with NIR light on days 3, 4 and 9 after inoculation with tumor cells, irrespective of whether GNRs were present. The tumor in the PALN was irradiated on days 3 and 4. B. Histological analysis of tissue damage in the PALN on day 9. (a), (b): Representative images from the cooling + irradiation group (n = 5). (c), (d): Representative images from the cooling + irradiation + GNRs group (n = 5). (a), (c): Scale bar, 500 μm; arrows, direction of laser beam. (b) Magnified view of the square in (a). Scale bar, 100 μm. The tumor (T) under the capsule is viable. (d) Magnified view of the square in (c). Scale bar, 100 μm. The capsule (Cap) is intact. The tumor (T) under the capsule is viable or shows partial necrosis (N).
irradiation with laser light: the tumor (T) observed under the skin was viable. Figures 7Bc and 7Bd show H&E-stained sections prepared after irradiation in the presence of GNRs: the capsule (Cap) was intact, while the tumor under the capsule was viable or exhibited signs of necrosis (N).

Discussion

We have demonstrated the potential of PTT with NIR laser light, GNRs and a skin temperature control system for treating LN metastasis without causing significant skin damage. The skin temperature control system maintained the skin temperature at 45°C by varying the laser intensity. The irradiation time was estimated numerically and controlled to ensure that the damage function \( \Omega_{md} \) was < 3. These protocols resulted in antitumor effects in tumor-bearing LNs, but avoided macroscopic skin damage (Fig. 7A) or disruption of the capsule (Fig. 7Bd). Our novel findings extend previous observations, and provide proof of concept that a combination of PTT (using NIR laser light and GNRs) with controlled skin surface cooling can be developed into a novel clinical therapy for LN metastasis that avoids damage to the skin.

In the present study, the light transmittances of the skin temperature control system and skin were 75% and 65%, respectively. The actual laser intensity that reached the LN was estimated to be 49% of the applied radiation.

We numerically estimated the temperature and damage fields in the LN when the skin temperature control system was used, based on the assumption that the LN was an isotropic bio-simulated material. The temperature field produced was oval in shape, and the maximal temperature was generated at a depth of about 1 mm below the skin surface. The maximal temperature was estimated to be 55°C after 10 sec of irradiation, and about 70°C after 100 sec of irradiation (Figs. 3, 4). The damage was localized to an area about 3 mm in depth, but did not occur in the region near the skin surface where the damage function \( \Omega_{md} \) was < 3. The temperature decreased exponentially to 37°C in the depth and radial directions, indicating that heat was being transferred out by the skin temperature control system (Fig. 4) [19]. More accurate predictions of the temperature distribution will require accurate values for the optical and thermal properties of LNs. In particular, the temperature dependence of the thermophysical properties will also need to be taken into account.

In macroscopic assessments, we confirmed that the skin and LN capsule were intact after irradiation in the presence of GNRs. Although there was evidence of tumor necrosis, some tumor remained viable, suggesting that the GNRs may not have distributed uniformly in the tumor. It has been reported that the penetration depth of laser light and subsequent temperature distribution were decreased by gold nanoparticles [20, 21]. It has also been estimated that 5,000 gold nanoparticles per cell are required to cause cellular damage [22]. Thus, further studies are required to optimize the concentration of GNRs so as to cause damage within a limited area and depth.

The energy of NIR laser light is converted into heat energy by the longitudinal plasmonic response of GNRs [23] without physicochemical hybridization [24]. However, since GNRs are synthesized in aqueous solution containing hexadecyltrimethylammonium bromide (CTAB), the clinical application of GNRs has been limited by the toxicity of CTAB. In order to reduce this toxicity, a new synthetic process to remove CTAB has been designed [25], and human albumin nanoparticles containing GNRs have been developed [26].

In the present study, a model of PALN metastasis was developed by the injection of tumor cells, and a combination of GNR injection, irradiation with NIR laser light and skin temperature control was shown to be a potentially effective anticancer therapy that did not induce macroscopic skin damage. The present method could potentially be modified for use in the clinic as follows: GNRs are injected into the sentinel LN and delivered to downstream LNs via the lymphatic network; then, the sentinel LN and downstream LNs are irradiated by NIR light. Lymphatic delivery of GNRs would
allow for a lower dose to be used, compared to systemic delivery of GNRs, thereby reducing the accumulation of GNRs in organs such as the liver and spleen [27]. Further studies, based on the use of a lymphatic delivery system, will be required to optimize the laser intensity, nanoparticle concentration and exposure time through estimation of thermal damage. In addition, a hybridized cooling, laser irradiation and GNR injection system will need to be developed.

Acknowledgements

The authors thank the Biomedical Research Core of Tohoku University Graduate School of Medicine for technical support. This study was supported in part by JSPS KAKENHI Grant Numbers 26293425 (SM), 26670856 (SM) and 26242051 (TK), and in part by the JSPS through the Bilateral Programs (Japan-South Africa) (TK).

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