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Photothermal therapy of tumors in lymph nodes using gold nanorods and near-infrared laser light

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ABSTRACT

Lymph node dissection for regional nodal metastasis is a primary option, but is invasive and associated with adverse effects. The development of non-invasive therapeutic methods in preclinical experiments using mice has been restricted by the small lymph node size and the limited techniques available for non-invasive monitoring of lymph node metastasis. Here, we show that photothermal therapy (PTT) using gold nanorods (GNRs) and near-infrared (NIR) laser light shows potential as a non-invasive treatment for tumors in the proper axillary lymph nodes (proper-ALNs) of MXH10/Mo–*lpr*/*lpr* mice, which develop systemic swelling of lymph nodes (up to 13 mm in diameter, similar in size to human lymph nodes). Tumor cells were inoculated into the proper-ALNs to develop a model of metastatic lesions, and any anti-tumor effects of therapy were assessed. We found that GNRs accumulated in the tumor in the proper-ALNs 24 h after tail vein injection, and that irradiation with NIR laser light elevated tumor temperature. Furthermore, combining local or systemic delivery of GNRs with NIR irradiation suppressed tumor growth more than irradiation alone. We propose that PTT with GNRs and NIR laser light can serve as a new therapeutic method for lymph node metastasis, as an alternative to lymph node dissection.

1. Introduction

Regional lymph node dissection is considered a priority when tumor metastases are detected by imaging modalities (ultrasound, CT, MRI or PET) [1,2], provided that the lymph nodes are resectable and the patient fit for surgery [3]. However, this is a highly invasive procedure. Since the therapeutic efficacy of radiotherapy or chemotherapy is relatively low, novel, minimally-invasive treatments for lymph node metastasis are greatly needed.

Photothermal therapy (PTT) is less invasive than radiotherapy, chemotherapy and surgical management [4]. In PTT, optical energy is converted into heat by tissue absorption, causing irreversible tissue damage through thermal denaturing of proteins (and DNAs) and tissue coagulation [5]. Gold nanoparticles, which are non-toxic, non-immunogenic, stable and biocompatible [6], can facilitate PTT by absorbing light, and minimize collateral damage to normal tissue by accumulating near a tumor through bioconjugation [5]. Near-infrared (NIR) laser light has a 'therapeutic window' corresponding to a wavelength band that is minimally absorbed by the blood and soft tissues and does not excite autofluorescence [7]. Since NIR laser light can penetrate soft tissues to depths exceeding 5 cm [8], its combination with gold nanoparticles offers a novel treatment for cancer. Gold nanoparticles exist as varying structures, including nanocages [5], nanowires [9], silica-cored nanoshells and nanorods [10]. Gold nanorods (GNRs) have two surface plasmon absorption bands: a long-wavelength (800–900 nm) and weaker short-wavelength (~500 nm) band due to longitudinal and transverse oscillation of electrons, respectively [11]. The absorption maximum of the longitudinal band shifts to longer wavelengths with increasing aspect-ratio; by selecting GNRs with an aspect-ratio appropriate for the NIR wavelength, effective treatment of cancer may be possible. Several types of GNR have been developed to increase tumor selectivity and efficacy, including GNRs conjugated to the anti-epidermal growth factor receptor [11,12], polyacrylic acid-coated GNRs [13], doxorubicin-loaded GNRs [14] and GNRs conjugated

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with arginine–glycine–aspartic acid peptides [15]. The selectivity of GNRs is improved further by the enhanced permeability and retention (ERP) effect [16].

In mice, PTT and GNRs have been assessed for the treatment of solid tumors [10,15,17–20], but not lymph node metastases, in part because the induction and detection of metastases in murine lymph nodes are challenging due to their small size (a few millimeters) [21]. Previously, we have used the MRL/MpJ–*lpr*/*lpr* (MRL/lpr) mouse as an animal model of lymph node metastasis, because its lymph nodes swell to a size similar to that in humans. However, the MRL/lpr strain is also utilized as a model of autoimmune diseases, and has the major disadvantage that lethal nephritis occurs at the same age (4–5 months) as lymph node swelling [22].

Recently, we developed a recombinant MXH10/Mo–*lpr*/*lpr* (MXH10/Mo/lpr) strain (by intercrossing MRL/lpr and C3H/HeJ–*lpr*/*lpr* mice) that shows widespread lymph node swelling at only 2.5–3 months of age, with lymph nodes as large as 10 mm; both the lymph node size and the onset of swelling are consistent and predictable. Moreover, these mice do not develop severe glomerulonephritis and vasculitis [23], and have a longer lifespan than MRL/lpr mice. Here, we have induced tumor development in the proper axillary lymph nodes (proper-ALNs) of MXH10/Mo/lpr mice, and evaluated the use of PTT in combination with GNRs for the treatment of these tumors.

2. Materials and methods

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

2.1. Gold nanorods

Bare gold nanorods (bareGNRs) (aspect ratio: 6.6; surface plasmon resonance (SPR) peak: 1050 nm; 30-HAR-1064) and neutravidin polymer-conjugated gold nanorods (GNRs) (aspect ratio: 6.7; SPR peak: 1065 nm; axial diameter: 10 nm; length: 67 nm; D12-1064-PN-50) were used (Nanopartz). Fluorescent GNRs (FluoGNRs) were prepared by conjugation (30 min, room temperature) of GNRs (12.4×10^{12} particles/mL in phosphate-buffered saline [PBS]) with Atto 590-biotin (excitation: 598 nm; emission: 624 nm; Sigma-Aldrich); excess dye was removed by three centrifugation ($5000 \times g$, 5 min) and washing steps. The zeta potentials of the GNRs (6.0×10^{10} particles/mL in distilled water) were measured (ELSZ-2 analyzer; Otsuka) as: bareGNRs, 46.54 ± 0.69 mV; GNRs, -13.79 ± 1.33 mV; and FluoGNRs, -16.65 ± 2.11 mV ($n = 3$ for each). GNR absorption spectra were measured using a UV-visible near-infrared spectrophotometer (V-7200; JASCO). FluoGNR configuration (6.0×10^{10} particles/mL in distilled water) was examined with a transmission electron microscope (HT7700; Hitachi) operated at 80 kV.

2.2. Cell culture

KM-Luc/GFP cells [1], which stably express a fusion of the 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 with a *Mycoplasma* detection kit (R&D Systems).

2.3. Mice

MXH10/Mo/lpr mice, established by intercrossing MRL/lpr and C3H/HeJ–*lpr*/*lpr* (C3H/lpr) strains [23], 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). The longitudinal diameter of the proper-ALN, measured using a digital caliper, was 9.76 ± 0.35 mm ($n = 6$).

2.4. Induction of metastasis in the proper-ALN

Tumor development in mice ($n = 57$) was induced by injection into the proper-ALN of 3.3×10^5 KM-Luc/GFP cells/mL, suspended in a mixture of 10 μL PBS (Ca^{2+}- and Mg^{2+}-free) and 20 μL of 400 mg/mL Matrigel (Collaborative Biomedical Products), under the guidance of a high-frequency ultrasound imaging system (Vevo770; VisualSonics) with a 25 MHz transducer (RMV-710B). To measure the luciferase activities of tumors growing in the proper-ALNs, luciferin (150 mg/kg; Promega) was injected intraperitoneally under anesthesia (2% isoflurane in oxygen). 10 min after injection, luciferase bioluminescence was measured for 30 s using an *in vivo* luminescence imaging system (IVIS; Xenogen); this procedure was carried out on days 2, 3, 4, 5, 7 and 9 post-inoculation. The proper-ALN size was measured using an *in vivo* high-resolution three-dimensional microimaging system (VEVO770; VisualSonics) with a 25 MHz transducer (RMV-710B; axial resolution 70 μm, focal length 15 mm) set at 50% transmission power [24].

2.5. Blood biochemistry investigations

The toxicity of systemic GNR injection was evaluated using serum biochemistry tests. Four mice were injected intravenously with GNRs (12.4×10^{12} particles/mL, 100 μL), and four controls with PBS (100 μL). On day 17 after injection, blood samples from the caudal vena cava (taken under general anesthesia) were centrifuged ($2000 \times g$, 5 min) to obtain serum. Hepatic and renal injuries were evaluated from serum measurements of total bilirubin (T-BIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) (Oriental Yeast).

2.6. GNR Biodistribution

Mice were injected intravenously with GNRs (12.4×10^{11} particles/mL) in PBS, and sacrificed after 12 h, 24 h or 17 days ($n = 3$ for each). Blood was drawn from the abdominal aorta, and the organs and tissues quickly removed and freeze-dried for 20 h. Samples in nitrohydrochloric acid were heated (160 °C for 5 min, 190 °C for 45 min), dispensed into 25 mL PBS, and analyzed for metal concentration by inductively-coupled plasma mass spectrometry (ICP–MS, HP4500, Hitachi). Values for each sample were divided by the quantity of GNRs injected to obtain a percentage (%).

2.7. NIR laser light irradiation

Laser light of 1.5 W/cm^2 from a continuous Nd: YVO4 air-cooled laser (1064 nm; beam diameter: TEM_{00}, 0.6 mm; CYD-010-TUBC; Neoarc) was delivered to the target site by an optical fiber (fiber diameter: 400 μm; collimator diameter: 20 mm). The collimator head was fixed to a three-dimensional stage control system (Mark-204-MS; Sigma Koki). The temperature at the irradiated site was measured by functional thermography (1.07 mrad spatial resolution, 0.05 °C minimum temperature resolution; TVS-500; Nippon Avionics).

2.8. Treatment of tumors in the proper-ALNs with PTT

To evaluate the anti-tumor effects of laser irradiation (1.5 W/cm^2, 180 s) and local/systemic GNR delivery, luciferase activities of tumors in the proper-ALNs were measured on days 2, 3, 4, 5, 7 and 9 after tumor inoculation. The proper-ALN volume was measured on days 0, 6 and 9 (Vevo770 high-frequency ultrasound system). Based on studies of human skin burns, the possible occurrence of skin burning was evaluated through macroscopic observations of redness, blistering, ulceration, and full-thickness necrosis of the skin.