Indocyanine Green-lactosome and Near-infrared Light-based
Intraoperative Imaging and Photodynamic Therapy for
Metastatic Bone Tumors

Toshinori Tsukanishi1, Toru Funayama2, Eiichi Ozeki3, Isao Hara3, Tetsuya Abe1, Shinzo Onishi1, Masashi Yamazaki1 and Masataka Sakane1

1 Department of Orthopaedic Surgery, University of Tsukuba,
1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575 Japan
2 Department of Orthopaedic Surgery, Kempoku Medical Center Takahagi Kyodo Hospital,
1006-9 Ageho-cho, Kamitetsuna, Takahagi, Ibaraki 318-0004, Japan
3 Technology Research Laboratory, Shimadzu Corporation,
3-9-4 hikaridai Setkacho, Sarakugun, Kyoto 619-0237 Japan
sakane-m@tsukuba-seikei.jp

The reduction of local recurrence of metastatic spinal tumors after intratumoral excision improves the quality of life. We have developed a novel photodynamic therapy (PDT) comprising an indocyanine green (ICG)-labeled nanocarrier (ICG-lactosome) and near-infrared light for use as an intraoperative adjuvant therapy. ICG-lactosomes accumulated in bone metastases from breast cancer because of enhanced permeability and retention effect. The cytotoxic effects of this novel PDT on human breast cancer cells were confirmed. In conclusion, ICG-lactosomes enable selective tumor imaging and represent a potential new PDT photosensitizer for adjuvant therapy during surgery for bone metastases from human breast cancer.

Keywords: Photodynamic therapy, Indocyanine green, Nanocarrier, Near-infrared fluorescence imaging

1. Introduction
As the efficacy of primary tumor treatment methods increase, cancer recurrences complicated by bone-related events, which significantly reduce the quality of life, have become problematic. Early diagnosis and minimally invasive treatment are key to preventing bone-related events.

Indocyanine green (ICG) shows maximum absorption in the near-infrared light spectrum (approximately 810 nm). It reaches a depth of approximately 20 – 40 mm for near-infrared irradiation in humans and therefore light shielding is not required after administration. Currently, ICG has been clinically applied for the identification of tumor-feeding blood vessels and sentinel lymph nodes. It is administered intravenously, accumulates quickly in the liver, and is excreted rapidly. Our previous study proved the photodynamic effects of ICG in a rat model of bone metastasis from breast cancer [1]; however, no selective accumulation was observed in the tumor sites. To overcome the shortcomings of ICG-photodynamic therapy, Kimura et al. developed nanoparticles that encapsulated ICG (ICG-lactosomes) [2] that could be integrated into inflammation or tumor sites in accordance with the enhanced permeability and retention (EPR) effect[3].

Our hypothesis for bone metastasis was as follows: it would be possible to confirm the extent of residual disease in the vertebral body margins using imaging with a molecular probe that localizes in the tumor and is selective for ICG. Additionally, residual disease could be evaluated using fluorescence imaging during tumor resection after microscopic examination and near-infrared light irradiation could be performed, resulting in high chances of radical resection and fewer residual tumor cells, thereby preventing relapse after surgery.
To investigate our hypothesis, we conducted in-vivo imaging and in-vitro studies using human breast cancer cells.

2. Materials and Methods

**In vivo imaging**

The MDA-MB-231 human breast cancer cell line (purchased from ATCC, Manassas, VA, USA) was cultured in Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich, Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (ATCC) and 2% penicillin-streptomycin solution (SIGMA-ALDRICH) in a humidified atmosphere with 5% CO₂. All cultures used for intracardiac injections were subconfluent and were resuspended in fresh medium 24 h before inoculation into nude mice. Cells (1 \times 10^5) were suspended in 0.1 ml of phosphate-buffered saline (PBS) and then injected into the left cardiac ventricle of female BALB/c-m mice (5 weeks old; Charles River Laboratories, Inc., Wilmington, MA, USA) using a 27-gauge needle according to a modification of the method described by Arguello et al [4] and Sasaki et al [5].

Sixty-eight days after injection, 0.5 mg of ICG-lactosomes per body was administered intravenously via the tail. After 24 h, whole-body radiography (SOFTEX-CSM-2; Softex Japan Co., Ltd., Yokohama, Japan) in the spine position and fluorescence imaging (Xenogen IVIS Spectrum; PerkinElmer Inc., Waltham, MA, USA) were performed. The experiments, an autopsy was conducted to examine bone metastasis. Osteolytic sites observed on radiogram and the liver were dissected, fixed in a 10% formalin solution, decalcified, and processed for hematoxylin and eosin staining. Coronal cross-sections were obtained to evaluate cancer cells invasion.

**In vitro studies**

MDA-MB-231 human breast cancer cells were seeded (2 \times 10^5 cells per well) into 96-well plates. The plates were divided (16 wells/treatment) into the following groups: control/untreated, ICG-lactosome administration only (ICG-lactosome), laser irradiation only (laser), and ICG-lactosome administration plus laser irradiation (photodynamic therapy: PDT; Fig. 1). Cells in the control, laser, and PDT groups were incubated in 100 μl of medium for 24 h. Cells in the ICG-lactosome group were incubated in 100 μl of medium containing 1 mg of ICG-lactosomes for 24 h. The following day, the laser group samples, treated with 100 μl of PBS and the PDT group samples, treated with PBS containing 0.5 mg of ICG-lactosomes, were treated with laser irradiation using a near-infrared medical diode laser (UDL-15; Olympus Co., Tokyo, Japan) ($\lambda = 810 \pm 20$ nm).

The irradiation conditions were set at an intensity of 298 mW and an irradiation time of 60 s. The combined total energy density was 17.9 J/cm². The medium in the irradiated wells was replaced with fresh medium every 24 h post-irradiation. The control and ICG-lactosome group wells also received fresh medium every 24 h. The cells in all groups were incubated for 96 h post-treatment. The cells were examined microscopically, and cell viability was measured using a WST-1 assay every 24 h after treatment for 96 h. The mean absorbance in the WST-1 assay (an indicator of cell viability) was analyzed using the Tukey-Kramer test for comparisons of multiple groups.

3. Results

**In vivo imaging**

On the radiogram, osteolytic changes were observed in the right mandible and in the proximal sites of the left humerus and the right tibia (Fig. 2, 3, left). IVIS showed that the fluorescence intensity was enhanced in the osteolytic lesions and in the liver (Fig. 3, right). On histological examination, the cancer cells were found to proliferate in the bone marrow near the osteolytic changes. Tumor cells were not present in the liver (Fig. 4).

![Figure 1: Experimental protocol.](image-url)
PDT group than in the other 3 groups at each time point. Cell viability in the ICG-lactosome group did not differ significantly from that in the control group (Fig. 6).

| ICG lactosome | PDT |
|---------------|-----|
| 0             |     |
| 24            |     |
| 48            |     |
| 72            |     |
| 96            |     |

Figure 5: Microscopic examination (400× magnification). Tumor cells in the PDT group (right column) abolished at 24 h after treatment.

In vitro studies

Microscopic examination revealed that the irradiation-treated cells had almost completely disappeared at 24 h post-irradiation (Fig. 5). Cell viability was significantly lower in the PDT group than in the other 3 groups at each time point. Cell viability in the ICG-lactosome group did not differ significantly from that in the control group (Fig. 6).

Figure 6: Mean absorbance values in the WST-1 assay. The absorbance value indicates cell viability. Cell viability was significantly lower in the laser group (red) than in the control and ICG-lactosome groups. Cell viability in the PDT group (yellow) decreased significantly relative to that in the laser group.
4. Discussion

With regard to tumor imaging, ICG-lactosomes remain in the blood for a long time because the reticuloendothelial system might not recognize it as foreign, thus allowing accumulation in cancer tissues via the EPR effect. Our previous results showed that spinal metastases appeared as enhanced lesions on the other fluorescence imaging device (Clairvivo OPT plus; Shimadzu Co., Kyoto, Japan) in cases of directly injected rat breast cancer cells. [6] The current results of the present study confirmed that ICG-lactosomes were integrated in the bone metastases that showed osteolytic changes; this result was highly reproducible, and the fluorescence intensity in these lesions was significantly enhanced relative to that in non-metastatic lesions.

With regard to therapeutic applications, ICG-lactosome PDT with near-infrared light significantly reduced the number of human breast cancer cells in vitro. Because ICG absorbs light at longer wavelengths than does Photofrin (the most widely used photosensitizer in the clinical setting), ICG-lactosome PDT could penetrate more deeply into tissues than could Photofrin PDT. This would therefore expand the range of options to treat various cancer stages.

Ongoing studies in animal models are in progress to confirm the effectiveness and underlying mechanisms of ICG-lactosome PDT.

5. Conclusion

ICG-lactosomes, with a potential therapeutic application in PDT, are also useful for selective tumor imaging during surgery for bone metastases from human breast cancer.

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