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Evaluation of Tumor Cell Response to Hyperthermia with Bioluminescent Imaging

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Abstract

Background: Hyperthermia is used in combination with radiotherapy and/or chemotherapy in the treatment of various types of cancer. Currently, the tumor cell response to hyperthermia is determined largely based on the size reduction of tumor mass, which is insensitive.

Methods: We tested the feasibility of bioluminescent imaging (BLI) in evaluation of the tumor cell response to hyperthermia by exposing luciferase-expressing MDA-MB-231-luc human breast cancer cells to high temperature (43 ^{o}C) for 10 minutes to 2 hours. The tumor cells were the imaged and the light signal generated by the tumor cells was quantified with BLI. To validate its usefulness, the light signal intensity was comparatively analyzed with the tumor cell clonogenicity and cell viability, which were measured with classic clonogenic and MTT assays.

Results: The light signal intensity determined by BLI was closely correlated with the absolute number of viable cells as well as the cell viability measured with the traditional MTT assay under normal culture condition. Relative to the clonogenicity of tumor cells after exposure to hyperthermia, however, BLI underestimated, while MTT assay overestimated the cell viability. Difference in the interpretation of tumor cell clonogenic ability following hyperthermia with BLI, MTT dye, and clonogenic assay may be due to the different mechanisms of the three measurements as well as the fact that hyperthermia can induce cell damage at levels of both transient and permanent.

Conclusions: BLI is sensitive, convenient, and potentially valuable in the evaluation and monitoring of tumor cell response to treatments including hyperthermia.

Keywords: Hyperthermia, tumor cell response, bioluminescent imaging, breast cancer

Introduction

Hyperthermia, also called thermal therapy or thermotherapy, is used in the treatment of various types of cancer in combination with other therapies (1,2). Hyperthermia has been shown to increase the sensitivity of some cancer cells to radiation and enhance the efficacy of anticancer drugs (3). To date, the anti-tumor efficacy of hyperthermia *in vivo* is largely based on the reduction of tumor size, which is insensitive, especially at the early stage of treatment. In *in vitro* studies, the tumor cell response to hyperthermia is mainly evaluated with cell viability and clonogenic assays, which differ widely in their mechanisms and applications (4). Some assays are based on the cellular uptake of vital dyes and/or intracellular enzyme activity such as neutral red, alamar blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium (5-7). Some other assays depend on the uptake of DNA precursors such as radiolabeled thymidine (8). In general, dye uptake-based assays such as MTT are suitable for estimating cell proliferation and evaluating the rapid cellular response to treatments (9). Considering that tumor growth and recurrence depend on the surviving clonogens, clonogenic measurements are more useful than dye-based cell viability assays when investigating therapeutic efficacy (10-12). Irrespective of the mechanisms of these assays, their application is limited to *in vitro* evaluation of the cellular viability or clonogenicity. A rapid, sensitive and noninvasive technique is desired for determination of the tumor response to therapy both *in vitro* and in living subjects.

Luciferase gene is widely used as a reporter in biochemical assays and gene expression analysis (13, 14). Luciferase catalyzes the conversion of its substrate D-luciferin to oxyluciferin in the presence of oxygen, Mg^{2+}, and ATP, which is accompanied by release of photons. Luciferase-based bioluminescent imaging (BLI) is becoming more and more attractive because of its capability for rapid and noninvasive monitoring of subtle changes in tumors (15-18). Because the light intensity depends on the level and activity of both luciferase and ATP, we hypothesized that the light intensity could reflex the cell viability and cell response to treatment. In the present study, we tested the hypothesis using BLI to evaluate the cytotoxicity of hyperthermia (43 ^{o}C) to human breast cancer MDA-MB-231-luc cells. MDA-MB-231-luc cells are stably transfected with the firefly luciferase gene, express high level of luciferase, and produce strong light when they are incubated with the luciferase substrate D-luciferin. The results showed that the light intensity measured with BLI was closely correlated with the absolute cell number as well as the cell viability measured with the traditional MTT assays under normal culture conditions, indicating the usefulness of BLI in cell viability evaluation. After exposure of the cells to hyperthermia, the light intensity decreased rapidly and significantly. Relative to the clonogenicity determined with colony formation assay, BLI tended to underestimate and MTT assay tended to overestimate the cell viability.

Materials and Methods

Cell line and hyperthermia treatment

MDA-MB-231-luc human breast cancer cell line was used in this study (Caliper, Alameda, CA). Cells were routinely maintained as monolayers in RPMI1640 medium and kept at 37 °C in humidified atmosphere containing 5% CO_2. The medium was

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supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (50 units/ml), and streptomycin (50 µg/ml) (Invitrogen, Carlsbad, CA). To test the relationship between light signal intensity and viable cell number, cells were seeded in sterile 96-well plates at various cell densities (25 to 5 x 10^3 cells/well) and allowed to grow overnight before measurements. For hyperthermia treatment, cells (1 x 10^4 cells/well) were seeded in sterile 96-well plates, grow overnight, and was then subjected to hyperthermia by sealing the plates with parafilm, enclosing in a Ziploc bag, and immersing into a water bath of 43 ± 0.1 ℃ for 10 to 120 minutes (min). Controls were similarly sealed in Ziploc bags but immersed in a 37℃ water bath. After heating, plates were ready for BLI, MTT assay, and clonogenic measurement.

Bioluminescent imaging

Luciferase-based BLI was performed with a highly sensitive, cooled CCD camera, which is mounted in a light-tight specimen box (Caliper IVIS spectrum optical imaging system). After heating, the old medium was removed from each well and 100 µl of D-luciferin solution (150 ug/ml in RPMI medium) (Caliper) were added. The plate was placed on the stage of specimen box, which was maintained at 37 ℃. Images were captured at 5 min after addition of D-luciferin. The imaging time was set to 1 min. The light emitted from each well was detected, integrated, digitized, and displayed with the acquisition and analysis software (Caliper). The bioluminescent signal from each well was measured and expressed as total flux (photons per second, p/s). At least 5 replicates were performed in all experiments and each experiment was repeated at least 3 times.

MTT and clonogenic assays

MTT assay was used to measure the tumor cell viability, which is based on the reduction of tetrazolium dye MTT (Sigma, St. Louis, MO) by viable cells. After exposure to 43 ℃, the old medium was replaced with MTT solution (100 µl; 0.5 mg/ml phenol red-free RPMI1640). Three hours after incubation at 37 ℃, the absorbance was determined at the wavelength of 560 nm with a multiwell spectrophotometer (BIO-RAD, Hercules, CA). For the clonogenic assay, cells were trypsinized and pipetted to single cell suspension. All cells were plated on 100-mm tissue culture dishes with fresh medium and kept at 37 ℃, 5% CO_2 incubator for 10-14 days. Colonies with more than 50 cells were counted.

Statistical analysis

All correlation analyses were performed with the statistical software Origin 7.0 (OriginLab, Northampton, MA).

Results

Correlation between imaging-based light intensity and cell viability

BLI showed that the light signal intensity increased with increased cell number (Fig. 1A). A close correlation between the bioluminescent signal intensity and absolute cell number was obtained over the range from 25 to 5 x 10^3 cells per well (R = 0.99, $P < 0.0001$). As few as 125 cells could be detected clearly (Fig. 1B). Similar correlative results were also obtained with MTT assay between the absorbance at 560 nm and cell number (R = 0.99, $P < 0.0001$) (Fig. 1C). The bioluminescent signal intensity was also closely correlated with the formazan absorbance at 560 nm obtained with MTT assay (R = 0.98, $P < 0.0001$) (Fig. 1D).

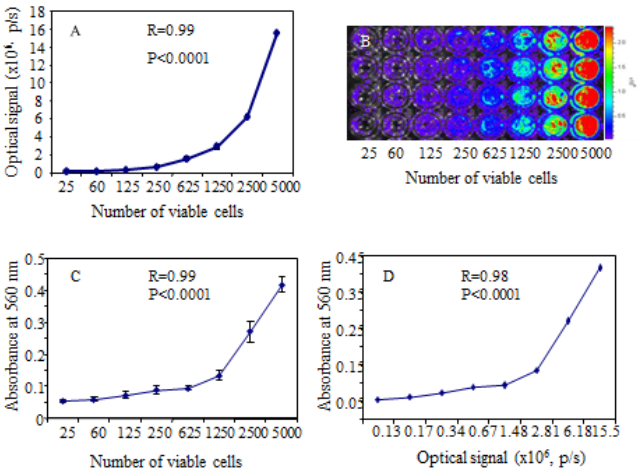

Figure 1. Comparison of MTT assay and BLI for measurement of cell viability under normal culture condition. 1A: correlation between absolute cell number and light signal intensity; 1B: a representative bioluminescent image of cells at various intensities; 1C: correlation of the absorbance at 560 nm and absolute cell number; and 1D: close correlation between MTT assay absorbance and bioluminescent signal intensity.

Evaluation of tumor cell response to hyperthermia

To test the cell response to hyperthermia, cells were subjected to hyperthermia (43 ^oC) for 10 to 120 min. At 10 min after exposure to hyperthermia, the light signal intensity from the treated cells decreased to 77% of the intensity from the control cells (untreated). A dramatic decrease of the light intensity was then detected at 20 min, showing only 22% of the control intensity (Fig. 2). With prolonged exposure to hyperthermia, the light signal intensity from the treated cells decreased further, showing only 9%, 3.6%, and 2% of the control light intensity at 30, 60, and 120 min, respectively. The corresponding cell viability measured with MTT assay was 95%, 90%, 90%, 83%, and 68% of the controls, and the colony number measured with clonogenic assay was 94%, 99%, 85%, 62%, and 7% of the controls at 10, 20, 30, 60, and 20 min, respectively, after exposure to hyperthermia (Fig. 2). These results suggested that BLI might underestimate, while MTT assay might overestimate the cell viability of tumor cells relative to the cell colony forming ability following exposure to hyperthermia.

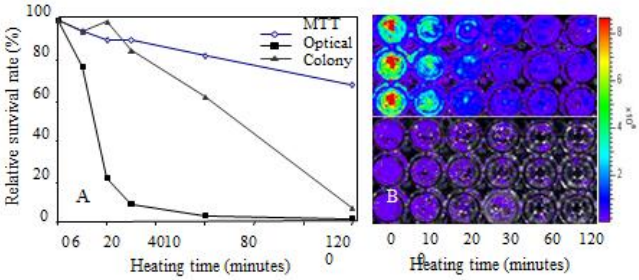

Figure 2. Evaluation of tumor cell response to hyperthermia at 43^oC for different durations from 10 to 120 min, indicating BLI may underestimate and MTT may overestimate the cell viability with respect to the clonogenic potential as estimated by clonogenic assay (2A). 2B shows the bioluminescent images of cells with (lower panel) and without (upper panel) heat treatment. The relative survival rate is reported as percentage relative to 100% survival for untreated controls.

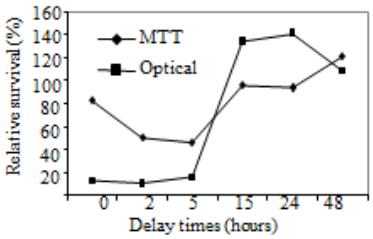

Figure 3. Delayed assessment of the cell viability following 1 hour treatment at 43 ^{o}C with BLI and MTT assay, indicating continued cell damage within the initial 5 hours followed by recovery of cells from damage.

Evaluation of the tumor damage recovery following hyperthermia

Heating can cause cell damages at different levels. Damages may be transient or permanent. Cells may lose enzyme activity as well as clonogenic potential, or may lose just one of these two properties. With this in mind, delayed assessment of the cell viability was performed at 3, 5, 15, 24, 48, and 72 hours after the cells returned to normal culture condition following 1 hour heating at 43 ^{o}C. Delayed assessment permits a recovery of the cells from transient but not from permanent damage. Immediate assessment of the cell viability after 1 hour exposure to hyperthermia showed a significantly low level of the light intensity relative to the controls not subjected to hyperthermia. The low level of signal intensity remained for more than 5 hours and a complete recovery of the light signal intensity was observed at ~15 hours (Fig. 3). Similar tendency was also observed for the cell viability measured with MTT assay, showing continued decrease of the cell viability for 5 hours followed by gradual recovery (Fig. 3). The light signal intensity at both 24 and 48 hours was higher than that of the controls, which might be due to the rapid cell proliferation following recovery from hyperthermia damage. MDA-MB-231-luc cells are known to have a cell cycle time of about 23 hours (19).

Discussion

Bioluminescent signal is a measurement of the luciferase expression level and activity, oxygen, and ATP content of the viable cells (20). Because the light signal intensity reflects the metabolic activity of viable cells, we hypothesized that BLI would be useful to evaluate tumor response to treatments including hyperthermia under the contextual influences of whole biological systems. To test this hypothesis, we first evaluated the feasibility with BLI to determine viable cell number using luciferase-expressing breast cancer cells under normal culture condition. As expected, the absolute number of viable cells was closely correlated with the bioluminescent signal intensity. Measurement with BLI was extremely sensitive. As low as 125 cells could be detected and fewer than 100 cell difference could be identified. The close correlation between the light signal intensity and the cell viability estimated with traditional MTT assay further indicate that the BLI-based light signal intensity could be used as an indicator of the cell viability.

The results that rapid and dramatic decrease of the light signal intensity after exposure to hyperthermia suggest that hyperthermia has a significant effect on the light generation. The significant loss of bioluminescent signal was less consistent with the loss of clonogenic potential of tumor cells. Immediate assessment with BLI appeared to underestimate the clonogenic potential of cells. This result may be partially explained by the shape decrease of ATP content but not clonogenic potential of the cells because hyperthermia has been shown to damage mitochondria (21). Hyperthermia may also down-regulate the luciferase expression and/or inactivate its activity. Further studies are necessary to determine the level and activity of ATP and luciferase after hyperthermia. Considering the effect of transient damage on light generation, delayed assessment after cells returned to normal temperature was designed to allow the cell recovery from transient damage. Indeed, relative to the control, continued low cell signals from BLI were observed for the initial 5 hours and the signal intensity then recovered gradually to the control levels. Similar results were also obtained with MTT assay. It appeared that transient damage to cells by hyperthermia significantly influenced the light production.

The difference in the interpretation of clonogenic ability following hyperthermia with BLI, MTT and clonogenic assay may be partially explained by the damages at different levels. Clonogenic ability is largely affected by the permanent cell damage. Whereas BLI and MTT assay reflect both transient and permanent damages. Compared to MTT assay, BLI is rapid, convenient and more sensitive [16, 17, 22]. Importantly, BLI can be repeated for the same cells at different times. Our study with cells in culture provides the insight towards noninvasive monitoring of tumor response to hyperthermia or other treatments *in vivo* with BLI.

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References

1. van der Zee J. Heating the patient: a promising approach? Ann Oncol 2002;13:1173-84.
2. Wust P, Hildebrandt B, Sreenivasa G, Rau B, Gellermann J, Riess H, Felix R, Schlag PM. Hyperthermia in combined treatment of cancer. Lancet Oncol 2002;3:487-97.
3. Dahl O, Dalene R, Schem BC, Mella O. Status of clinical hyperthermia. Acta Oncol 1999;38:863-73.
4. Bellamy WT. Prediction of response to drug therapy of cancer, a review of in vitro assays. Drugs 1992:44:690-708.
5. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000;267:5421-6.
6. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol. Annu Rev 2005;11:127-52.
7. Lechpammer S, Asea A, Mallick R, Zhong R, Sherman MY, Calderwood SK. Development of an XTT tetrazolium salt-based assay for detection of specific hyperthermia sensitizers in a high-flux screening programme. Int J Hyperthermia 2002;18:203-15.
8. Senekowitsch-Schmidtke R, Ruth F, Bernatz S. Radiolabeled thymidine: a sensitive tracer for early tumor response and recurrence after irradiation. J Nucl Med 1999;40:1702-5.
9. Hamid R, Rotshteyn Y, Rabadi L, Parikh R, Bullock P. Comparison of alamar blue and MTT assays for high through-put screening. Toxicol In Vitro 2004;18:703-10.

10. Miret S, De Groene EM, Klaffke W. Comparison of in vitro assays of cellular toxicity in the human hepatic cell line HepG2. J Biomol Screen 2006;1:184-93.
11. Kawada K, Yonei T, Ueoka H, Kiura K, Tabata M, Takigawa N, Harada M, Tanimoto M. Comparison of chemosensitivity tests: clonogenic assay versus MTT assay. Acta Med Okayama 2002;56:129-34.
12. Tomasovic SP, Barta M, Klostergaard J. Neutral red uptake and clonogenic survival assays of the hyperthermic sensitization of tumor cells to tumor necrosis factor. Radiat Res 1989;119:325-37.
13. Bloquel C, Trollet C, Pradines E, Seguin J, Scherman D, Bureau MF. Optical imaging of luminescence for in vivo quantification of gene electrotransfer in mouse muscle and knee. BMC Biotechnol 2006;6:16.
14. Shinde R, Perkins J, Contag CH. Luciferin derivatives for enhanced in vitro and in vivo bioluminescence assays. Biochem 2006;45:11103-12.
15. Massoud TF, Gambhir SS. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. Genes Develop 2003;17:545-80.
16. Greer III LF, Szalay AA. Imaging of light emission from the expression of luciferases in living cells and organisms: a review. Luminescence 2002;17:43-74.
17. Jenkins DE, Hornig YS, Oei Y, Dusich J, Purchio T. Bioluminescent human breast cancer cell lines that permit rapid and sensitive in vivo detection of mammary tumors and multiple metastases in immune deficient mice. Breast Cancer Res 2005;7:R444-54.
18. Nogawa M, Yuasa T, Kimura S, Kuroda J, Sato K, Segawa H, Yokota A, Maekawa T. Monitoring luciferase-labeled cancer cell growth and metastatsis in different in vivo models. Cancer Lett 2005;217:243-53.
19. Zhang RD, Fidler IJ, Price JE. Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and brain metastasis. Invasion Meta 1991;11:204-15.
20. Paulmurugan R, Massoud TF, Huang J, Gambhir SS. Molecular Imaging of drug-modulated protein-protein interactions in living subjects. Cancer Res 2004;64:2113-9.
21. Miyazaki N, Kurihara K, Nakano H, Shinohara K. Role of ATP in the sensitivity to heat and the induction of apoptosis in mammalian cells. Int J Hyperthermia 2002;18:316-31.
22. Shan L, Wang S, Korotcov A, Sridhar R, Wang PC: Bioluminescent animal models of human breast cancer for tumor biomass evaluation and metastasis detection. Ethn Dis 2008;18(2 Suppl 2):S2-65-9.