The Modified High-Density Survival Assay is the Useful Tool to Predict the Effectiveness of Fractionated Radiation Exposure

Yoshikazu KUWAHARA1, Miyuki MORI1, Toshiyuki OIKAWA1, Tsutomu SHIMURA1, Yosuke OHTAKE2, Shiro MORI3, Yasuhito OHKUBO2 and Manabu FUKUMOTO1*

Ionizing radiation/Modified high-density survival assay/Clonogenic assay/MTT assay/Clinically relevant radioresistance.

The high-density survival (HDS) assay was originally elaborated to assess cancer cell responses to therapeutic agents under the influence of intercellular communication. Here, we simplified the original HDS assay and studied its applicability for the detection of cellular radioresistance. We have recently defined clinically relevant radioresistant (CRR) cells, which continue to proliferate with daily exposure to 2 gray (Gy) of X-rays for more than 30 days in vitro. We established human CRR cell lines, HepG2-8960-R from HepG2, and SAS-R1 and -R2 from SAS, respectively. In an attempt to apply the HDS assay to detect radioresistance with clinical relevance, we simplified the original HDS assay by scoring the total number of surviving cells after exposure to X-rays. The modified HDS assay successfully detected radioresistance with clinical relevance. The modified HDS assay detected CRR phenotype, which is not always detectable by clonogenic assay. Therefore, we believe that the modified HDS assay presented in this study is a powerful tool to predict the effectiveness of fractionated radiotherapy against malignant tumors.

INTRODUCTION

Radiotherapy is one of the major therapeutic modalities for eradicating malignant tumors and is especially excellent in preservation of tissue function. The objective of radiotherapy is to achieve local control by killing tumor cells. In response to radiation, cell death occurs with various characteristics or through different pathways: rapid phase death soon after irradiation, delayed interphase death following arrest in G2 phase and mitotic death after cell division.1) Clonogenic assay is the gold standard for measuring in vitro radiosensitivity and evaluating the overall consequences of these early and late responses to radiation. In this assay, the number of cells seeded per dish is adjusted so that a relatively constant number of surviving colonies in each culture dish is obtained.2) However, for several cell lines plating efficiency of both control and irradiated cells is dependent upon the density of cells seeded for colony formation, that is, cell inoculum levels result in a non-linear relationship with colony formation.3) Moreover, plating efficiency usually decreases after irradiation.4,5) These indicate that the outcome of clonogenic assay might sometimes be biased due to cell-contact effects or plating efficiency.

Exposure to ionizing radiation or anti-cancer agents induces early apoptotic and necrotic cell death,6,7) but also triggers accelerated senescence and mitotic catastrophe, which are manifested several days after the induction of DNA damage.8–11) Biological assays to measure cell survival have been achieved by several assays such as the ³H-thymidine incorporation assay and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The ³H-thymidine incorporation assay primarily detects the proliferative activity of cells, and MTT assay metabolic activity of cells.12) Therefore, these assays cannot detect cell survival as a consequence of early and late cell death.

*Corresponding author: Phone: +81-22-717-8507, Fax: +81-22-717-8512, E-mail: fukumoto@idac.tohoku.ac.jp
1Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan; 2Department of Radiopharmacology, Tohoku Pharmaceutical University, Sendai 981-8558, Japan; 3Division of Oral and Maxillofacial Surgery, Department of Oro-Maxillofacial Surgical Science, Tohoku University Graduate School of Dentistry, Sendai 980-8575, Japan.
I declare that all of the authors have no conflicts of interest, nor are we involved in anything that may be considered to be a perceived conflict of interest.
doi:10.1269/jrr.09094
Recently, the “high density survival” (HDS) assay was developed by Mirzayans et al.\textsuperscript{13} The HDS assay can be applied to cells with extremely low cloning efficiencies by facilitating cell-to-cell interaction before and after radiation exposure. As is the case for clonogenic assay, the HDS assay involves incubation of cells for prolonged periods after irradiation, and therefore provides an integrated measure of all cytotoxic responses, encompassing early and late cell death. However, the original HDS assay requires radiolabeled thymidine, which necessitates specialized facilities and making the assay difficult to perform routinely. In this report, we have developed a modified HDS assay, which eliminates the use of radionucleosides. Our modified HDS assay is easy to perform because the outcome of the effects of genotoxic stresses is obtained by simple cell counting without the need for specialized materials or devices.

The standard fractionated exposure protocol of radiotherapy for tumor treatment consists of about 2 Gy a day, 5 days a week for over a period of 5 to 8 weeks.\textsuperscript{2} Therefore, we have defined “clinically relevant radioresistant (CRR) cells” which continue to grow even after exposure to 2 Gy/day of X-rays for more than 30 days in vitro (> 60 Gy in total dose).\textsuperscript{14} We recently established CRR cell lines, HepG2-8960-R, SAS-R1 and SAS-R2, independently. In this study, we confirmed the modified HDS assay as a suitable method to evaluate CRR phenotype. We also examined direct applicability of the modified HDS assay to non-adherent cells.

**Materials and Methods**

**Cell lines**

Human tumor cell lines, HepG2 (liver carcinoma), SAS (oral squamous cell carcinoma), MOLT-17 (T-cell leukemia), U937 (histiocytic lymphoma) and K562 (erythroleukemia), were obtained from the Cell Resources Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. We established CRR cell lines HepG2-8960-R from HepG2, and SAS-R1 and SAS-R2 independently from SAS as described previously.\textsuperscript{14,15} CRR cells were exposed to 2 Gy of X-rays every 24 hours for more than 1 year. All the cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich Inc., St Louis, MO, USA) supplemented with 5% fetal bovine serum (Gibco Invitrogen Corp., Carlsbad, CA, USA) in a humidified atmosphere at 37°C with 5% CO\textsubscript{2} in air. During this study, we paid special attention to maintain culture conditions constant. For cell counting, adherent cells were harvested with 0.25% trypsin in phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and single cell suspension was prepared. The number of surviving cells was counted by dye exclusion test using 0.4% trypan blue (DOJINDO LABORATORIES, Kumamoto, Japan) in physiological saline and a hemacytometer. Pipetting without trypsin treatment was performed for the preparation of single cell suspension of non-adherent cells (i.e., MOLT-17, U937 and K562). X-ray exposure experiments performed in this study were conducted 48 hours after the last maintenance radiation.

**Irradiation**

Irradiation of X-rays was performed in a 150-KVp X-ray generator (Model MBR-1520R, Hitachi, Tokyo, Japan) with a total filtration of 0.5 mm aluminum plus 0.1 mm copper filter. The dose rate measured by a thimble ionization chamber (IC 17A, Far West Technology, Goleta, CA, USA) was 1.01 Gy/minute.

**Assay of radioresistance with clinical relevance**

Exponentially growing cells (1 × 10\textsuperscript{5}) were seeded in 25 cm\textsuperscript{2} flasks (Nalge Nunc International, Rochester, NY, USA) 48 hours before the first experimental irradiation. Those cells were exposed to 2 Gy of X-rays every 24 hours for 40 consecutive days. On day 3 after the first irradiation, the number of surviving cells was counted by the dye exclusion test and 1/10 of cell suspension was transferred to a new flask. The same procedure was further carried out on day 5, 7, 10, 14, 18, 21, 25, 30, 35, 39, and 42.

**Fig. 1.** Schematic illustration of the modified high-density survival assay. Exponentially growing cells were seeded in a 25 cm\textsuperscript{2} flask at 5 × 10\textsuperscript{5}/flask and incubated for 48 hours. The cells were then exposed to various doses of X-rays and incubated for another 72 hours. Subsequently, 1/10 of the cells of each flask were seeded into a new 25 cm\textsuperscript{2} flask and incubated for further 72 hours. Then total number of cells in each flask was counted by trypan blue dye exclusion test.
10, 20 and 30. Cell number was counted on day 40. If the total cell number in a flask was less than \(1 \times 10^5\), all cells were transferred to a new flask.

**Clonogenic assay**

Clonogenic assay was performed as previously described. Briefly, \(10^3\) cells were exposed to a single dose of X-rays at 2, 5 and 10 Gy, and were seeded into a gelatin-coated 60-mm tissue culture dish. Colonies consisting of 50 cells or more were counted after incubation at 37°C for 10 days.

**The modified high-density survival assay**

The modified HDS assay was elaborated from the original HDS assay in order to develop an assay without the use of radiolabeled nucleosides and with easy handling. Exponentially growing cells were seeded in 25 cm\(^2\) flasks at \(5 \times 10^5/\text{flask}\) and incubated for 48 hours. The cells were then exposed to various doses of X-rays and incubated for another 72 hours. Subsequently, 1/10 of the cells of each flask were seeded into a 25 cm\(^2\) flask and incubated for further 72 hours. Then total number of cells in each flask was counted by trypan blue dye exclusion test. Schematic illustration of this assay is presented in Fig. 1.

**Evaluation of plating efficiency, and the average number of cells produced from single cells within 5 days**

Cells were plated at the density of \(10^2, 10^3\) and \(10^4\) cells per 60 mm tissue culture dish (Becton, Dickinson and Company, Franklin Lakes, NJ USA) and were incubated for 5 days at 37°C. After fixing with 99.5% methanol and staining with Giemsa solution (Merek & Co., Inc., Whitehouse Station, NJ, USA), plating efficiencies and the average number of cells produced from single cells within 5 days were counted under a light microscope.

**Statistic Analysis**

All experiments were performed in triplicates, and the mean and standard deviation at each data point were calculated and statistical significance was determined by the Student’s \(t\)-test.

**RESULTS**

**Establishment of clinically relevant radioresistant cell lines from SAS**

In order to confirm whether SAS-R1 and SAS-R2 acquired the CRR phenotype or not, SAS, SAS-R1 and SAS-R2 cells were exposed daily to 2 Gy of X-rays for 40 consecutive days, and total number of cells at the appropriate time point was scored. The growth rate of parental SAS was plotted against the fractionated radiation dose. (Fig. 2A) The survival fraction of SAS-R1 and SAS-R2 after exposure to more than 5 Gy of X-rays was significantly lower than those of parental SAS. (Mean ± SD; SAS vs SAS-R1 and SAS vs SAS-R2, \(*p < 0.01, **p < 0.001\))

**Table 1. Plating Efficiency in Relation to Seeded Cell Number**

| Cell line     | Plating efficiency |
|--------------|-------------------|
|               | 100 (cells/dish)  | 1000  | 10000 |
| HepG2         | 4.3 ± 1.5         | 53.3 ± 4.9* | ND    |
| HepG2-8960-R  | 3.3 ± 1.5         | 2.9 ± 0.7  | 17.1 ± 4.7 |
| SAS           | 74.3 ± 11.9*      | 83.9 ± 6.0* | ND    |
| SAS-R1        | 6.3 ± 4.9         | 5.4 ± 0.9  | 9.5 ± 0.8 |
| SAS-R2        | 1.7 ± 1.5         | 2.8 ± 0.6  | 8.5 ± 0.8 |

Data are expressed as mean ± SD of 3 independent experiments.

ND: Not determined. Because of too many colonies, we failed to determine total number of colonies accurately.  
*; HepG2 vs HepG2-8960-R, SAS vs SAS-R1 and SAS vs SAS-R2; \(p < 0.001\)
was the highest among 3 cell lines until day 6 (12 Gy in total) but thereafter, SAS stopped proliferating and died on day 40 (80 Gy in total) (Fig. 2A). On the other hand, SAS-R1 and SAS-R2 continued to grow with daily exposure to 2 Gy of X-rays and the total number on day 40 was $1.7 \times 10^6 \pm 8.4 \times 10^5$ and $5.9 \times 10^6 \pm 6.3 \times 10^5$, respectively. These indicate that both SAS-R1 and SAS-R2 were CRR cells. However, clonogenic assay failed to detect cellular radiosistance of SAS-R1 and SAS-R2 compared with parental SAS (Fig. 2B). The surviving fractions at 2 Gy of 3 cell lines were almost the same. Furthermore, surviving fractions of SAS-R1 and SAS-R2 were lower compared with parental SAS after exposure to 5 and 10 Gy of X-rays. SAS-R2 failed to form a colony at all after exposure to 10 Gy of X-rays.

### Table 2. Total number of cells produced from single cells within 5 days

| Cell line      | Total number of cells produced from single cells (100 cells/dish) | Total number of cells produced from single cells (1000 cells/dish) | Total number of cells produced from single cells (10000 cells/dish) |
|----------------|------------------------------------------------------------------|------------------------------------------------------------------|------------------------------------------------------------------|
| HepG2          | 7.6 ± 2.8                                                        | 11.3 ± 4.3                                                        | 18.3 ± 0.8                                                        |
| HepG2-8960-R   | 6.4 ± 3.3                                                        | 9.6 ± 1.8                                                         | 16.7 ± 8.7                                                        |
| SAS            | 54.1 ± 31.2*                                                   | 68.6 ± 33.0*                                                    | ND                                                               |
| SAS-R1         | 11.1 ± 4.9                                                      | 15.1 ± 8.2                                                        | 26.3 ± 15.4                                                      |
| SAS-R2         | 5.8 ± 2.5                                                       | 6.5 ± 2.7                                                        | 21.4 ± 9.8                                                      |

Data are expressed as mean ± SD of 3 independent experiments. ND; Not determined. Because of too many colonies, we failed to determine total number of cells accurately.

*; SAS vs SAS-R1 and SAS vs SAS-R2; $p < 0.001$

Plating efficiency and the number of cells produced from single cells within 5 days

Plating efficiency of CRR cells was lower than that of parental cells (Table 1). Albeit not always but the more number of cells were seeded, the more plating efficiency was observed. The number of cells produced from a single cell within 5 days tended to increase according to the increase of the number of cells seeded in the dish. This tendency was observed in both parental and radioresistant cells (Table 2).

![Fig. 3](image1.png) **(A)** The modified high-density survival (HDS) assay of parental HepG2 and radioresistant HepG2-8960-R cells. (Mean ± SD; HepG2 vs HepG2-8960-R, *p < 0.05, **p < 0.01) **(B)** The modified HDS assay of parental SAS, radioresistant SAS-R1 and SAS-R2 cells. (Mean ± SD; SAS vs SAS-R1 and SAS vs SAS-R2, *p < 0.05, **p < 0.01).

![Fig. 4](image2.png) **(A)** Cell growth curve of non-adherent cells under exposure to fractionated X-rays. Exponentially growing cells ($1 \times 10^5$) were seeded 48 hours before the first experimental irradiation. Cells were exposed to 2 Gy of X-rays every 24 hours for 30 consecutive days. **(B)** Radiosensitivity of MOLT-17, U937 and K562 determined by the modified HDS assay. (Mean ± SD).
The total number of cells produced from single cells was significantly lower in both SAS-R1 ($p < 0.001$) and SAS-R2 ($p < 0.001$) compared with their parental cells, respectively.

**Evaluation of radiation sensitivity by the modified high-density survival assay**

Procedure of the modified high-density survival assay is presented in Fig. 1, and the result of the modified HDS assays is presented in Fig. 3. The survival rate of all the CRR cells was significantly higher than their corresponding parental cells at all the exposure doses examined.

**Effect of fractionated X-ray exposure on survival of non-adherent cells**

The effect of daily exposure to 2 Gy of X-rays on survival of non-adherent hematopoietic cells is shown in Fig. 4A. By day 5 (10 Gy in cumulative dose), almost all MOLT-17 cells died, in contrast, U937 and K562 continued to grow until day 10. Thereafter, the number of surviving cells of K562 and U937 decreased and died out by day 60. These results suggested that K562 was the most radioresistant and MOLT-17 was the most radiosensitive among three cell lines examined.

**Radiosensitivity of non-adherent cells determined by the modified high density survival assay**

We determined whether the modified HDS assay developed in this study was applicable to non-adherent cell lines or not. MOLT-17 was the most radiosensitive and K562 was the most radioresistant among three cell lines (Fig. 4B).

**DISCUSSION**

Since cells treated with toxic agents do not die immediately after treatments, short-term assays which measure viability by dye-based staining underestimate overall cell death rates. Therefore, clonogenic assay has been used almost exclusively over a number of years for determining radiosensitivity in vitro. However, as discussed by Mirzayans et al., clonogenic assay is limited to cells with the ability of colony formation and is labor intensive.

Recently, we established CRR cell lines, which continue to grow with daily exposure to 2 Gy of X-rays for more than 30 days. However, the CRR phenotype was not detectable by the MTB assay or other colorimetric assays. Even adopting gelatin-coated plates or radiation-inactivated feeder layers, radioresistance of CRR cells was not detectable by clonogenic assay (data not shown). As is reported, plating efficiency and cell growth in colony assay were cell density dependent. Although the clonogenicity was not always improved in all the cell lines examined, both the number of colonies and the number of cells per colony increased according to the increase of cell density at seeding in the present study. In clonogenic assay cells are plated out at very low densities, obviating the effects of cell-to-cell communications. Because of high cell density, we thought that the HDS assay could overcome the problem of retardation of cell growth associated with colony assay and might reflect the CRR phenotype. Moreover, the modified HDS assay can measure the late effect of the irradiation and is easier to perform compared with the clonogenic assay. Compared to corresponding parental cell lines, the modified HDS assay detected radioresistance of all 3 CRR cell lines.

Determination of cellular radiosensitivity of non-adherent cells can be done using 96 well dishes, where cells are seeded at limiting dilution and colony growth can be observed over time, or can be done by monitoring growth in soft agar. However, the procedure is laborious. In this study, we determined whether the modified HDS assay can easily detect cellular radiosensitivity of non-adherent cells, and can predict the effectiveness of fractionated radiation exposure. For these purpose, we first examined the effect of exposure to fractionated 2 Gy of X-rays on the survival of three non-adherent cell lines. By day 20 of serial exposure to 2 Gy/day of X-rays, K562 cells were the most radioresistant and MOLT-17 cells were the most radiosensitive. This tendency was consistent with the results of the modified HDS assay.

Our preliminary study revealed that transplanted SAS-R1 tumors into athymic nude mice subcutaneously are more resistant against fractionated X-ray radiation compared with tumors of parental SAS cells. Therefore, we consider that the HDS assay presented in this study is a convenient and standard assay to predict the effectiveness of conventional fractionated radiotherapy.

**ACKNOWLEDGEMENTS**

This study was supported in part by the Grants-in Aid from the Ministry of Education, Science, Sports and Culture and the Ministry of Health, Labour and Welfare of Japan.

**REFERENCES**

1. Aldridge DR and Radford IR (1998) Explaining differences in sensitivity to killing by ionizing radiation between human lymphoid cell lines. Cancer Res 58: 2817–2824.
2. Hall EJ and Giaccia AJ (2006) Radiobiology for the radiologist. Philadelphia: Lippincott Williams & Wilkins.
3. Pomp J, et al (1996) Cell density dependent plating efficiency affects outcome and interpretation of colony forming assays. Radiother Oncol 40: 121–125.
4. Fitzek M and Trott KR (1993) Clonal heterogeneity in delayed decrease of plating efficiency of irradiated HeLa cells. Radiat Environ Biophys 32: 33–39.
5. Liu ZZ, et al (2005) Cell cycle and radiosensitivity of progeny of irradiated primary cultured human hepatocarcinoma cells. World J Gastroenterol 11: 7033–7035.
6. Lichter AS and Lawrence TS (1995) Recent advances in radiation oncology. N Engl J Med 332: 371–379.
7. Gudkov AV and Komarova EA (2003) The role of p53 in...
determining sensitivity to radiotherapy. Nat Rev Cancer 3: 117–129.
8. Roninson IB, Broude EV and Chang BD (2001) If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resist Updat 4: 303–313.
9. Roninson IB (2003) Tumor cell senescence in cancer treatment. Cancer Res 63: 2705–2715.
10. Castedo M, et al (2004) Cell death by mitotic catastrophe: a molecular definition. Oncogene 23: 2825–2837.
11. Shay JW and Roninson IB (2004) Hallmarks of senescence in carcinogenesis and cancer therapy. Oncogene 23: 2919–2933.
12. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63.
13. Mirzayans R, et al (2007) A sensitive assay for the evaluation of cytotoxicity and its pharmacologic modulation in human solid tumor-derived cell lines exposed to cancer-therapeutic agents. J Pharm Pharm Sci 10: 298s–311s.
14. Kuwahara Y, et al (2009) Clinically relevant radioresistant cells efficiently repair DNA double-strand breaks induced by X-rays. Cancer Sci 100: 747–752.
15. Wang L, et al (2007) Analysis of Common Deletion (CD) and a novel deletion of mitochondrial DNA induced by ionizing radiation. Int J Radiat Biol 83: 433–442.
16. Brown JM and Wouters BG (1999) Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res 59: 1391–1399.
17. Uchida H, et al (2004) Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth in vitro and in vivo. Mol Ther 10: 162–171.
18. Hulspas R, et al (1997) In vitro cell density-dependent clonal growth of EGF-responsive murine neural progenitor cells under serum-free conditions. Exp Neurol 148: 147–156.
19. West CM, et al (1998) The intrinsic radiosensitivity of normal and tumour cells. International journal of radiation biology 73: 409–413.

Received on August 8, 2009
Revision received on December 29, 2009
Accepted on February 2, 2010
J-STAGE Advance Publication Date: April 12, 2010