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The Optimal Condition of Performing MTT Assay for the Determination of Radiation Sensitivity

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Purpose : The measurement of radiation survival using a clonogenic assay, the established standard, can be difficult and time consuming. In this study, We have used the MTT assay, based on the reduction of a tetrazolium salt to a purple formazan precipitate by living cells, as a substitution for clonogenic assay and have examined the optimal condition for performing this assay in determination of radiation sensitivity.
Materials and Methods : Four human cancer cell lines - PCI-1, SNU-1066, NCI-H630 and RKO cells have been used. For each cell line, a clonogenic assay and a MTT assay using Premix WST-1 solution, which is one of the tetrazolium salts and does not require washing or solubilization of the precipitate were carried out after irradiation of 0, 2, 4, 6, 8, 10 Gy. For clonogenic assay, cells in 25 cm^2 flasks were irradiated after overnight incubation and the resultant colonies containing more than 50 cells were scored after culturing the cells for 10~14 days. For MTT assay, the relationship between absorbance and cell number, optimal seeding cell number, and optimal timing of assay was determined. Then, MTT assay was performed when the irradiated cells had regained exponential growth or when the non-irradiated cells had undergone four or more doubling times.
Results : There was minimal variation in the values gained from these two methods with the standard deviation generally less than 5%, and there were no statistically significant differences between two methods according to t-test in low radiation dose (below 6 Gy). The regression analyses showed high linear correlation with the R^2 value of 0.975~0.992 between data from the two different methods. The optimal cell numbers for MTT assay were found to be dependent on plating efficiency of used cell line. Less than 300 cells/well were appropriate for cells with high plating efficiency (more than 30%). For cells with low plating efficiency (less than 30%), 500 cells/well or more were appropriate for assay. The optimal time for MTT assay was after 6 doubling times for the results compatible with those of clonogenic assay, at least after 4 doubling times was required for valid results. In consideration of practical limits of assay (12 days, in this study) cells with doubling time more than 3 days were inappropriate for application.
Conclusion : In conclusion, it is found that MTT assay can successfully replace clonogenic assay of tested cancer cell lines after irradiation only if MTT assay was undertaken with optimal assay conditions that included plating efficiency of each cell line and doubling time at least.

Key Words : Clonogenic assay, MTT assay, Radiation sensitivity

INTRODUCTION

The radiation survival of cells has traditionally been measured using clonogenic assay and this has remained the investigational tool of choice following the original description of the technique in 1955.[1, 2] However, such assays require reliable cell growth in a culture media, and can be difficult and time consuming to perform in cell lines with low plating efficiencies.[3] The search for alternative methods to measure radiation survival has resulted in several evaluations. Many nonclonogenic assays quantitate the cell number based on certain metabolic functions, such as tritiated thymi-

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dine incorporation,[4, 5] glucose utilization via pentose phosphate cycle,[6] reduction of various drugs.[7] The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay is one of these. The MTT assay is a method of quantifying metabolically viable cells through their ability to reduce a yellow tetrazolium salt to a purple formazan crystal using the mitochondrial enzyme, succinate dehydrogenase.[8] The optical density of the solution resulting from the solubilisation of the formazan precipitate with an organic solvent can be detected using a multiwell spectrophotometer.[7] The main advantages of this assay are its rapidity and precision, as well as lack of any radioisotope. The MTT assay has been used successfully to test the chemosensitivity of established cell lines.[9~11]

Although short and intermediate term nonclonogenic assays have been tested in the assessment of drug sensitivity,[5, 12, 13] many of these are considered inadequate to measure radiation sensitivity. This is mainly related to the short duration of the assays. Following a dose of radiation, cells destined to die undergo one or more cell divisions.[14, 15] Therefore, a considerable period of time may elapse before these sterilized cells express their damage via a mitotic linked death and are subsequently lost from the population.[16~18] During this time interval, lethally irradiated cells appear to function normally with respect to metabolism and maintenance of intact cell structure.[15] If these assays are used for radiation survival assessment, it is important that adequate time elapse following the radiation exposure, so that sterilized cells express their damage and are lost from the population prior to performing the assay.[10]

This study has examined the use of the MTT assay as an alternative to the clonogenic assay and the optimal condition for performing this assay for the determination of radiation sensitivity.

MATERIALS AND METHODS

1. Cell lines and culture conditions

Four human tumor cell lines have been used. PCI-1 was derived from a recurrent laryngeal squamous cell carcinoma; SNU-1066, was derived from a laryngeal squamous cell carcinoma; NCI-H630 was derived from a metastatic tumor of liver. RKO was derived from a human colon cancer. SNU-1066 and NCI-H630 were received from the Korean Cell Line Bank. All four cell lines grew as monolayers in culture medium.

Table 1. The Characteristics of the Cell Lines

Name	Tumor site	Pathology
PCI-1	Larynx	squamous cell carcinoma
SNU-1066	Larynx	squamous cell carcinoma
NCI-H630	Liver	metastatic adenocarcinoma
RKO	Colon	adenocarcinoma

The culture medium for PCI-1 and RKO consisted of DMEM (Dulbecco's Modified Eagle Media, high glucose, with L-glutamine, Gibco, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 μg/ml gentamycin. For NCI-H630 and SNU-1066, RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 μg/ml gentamycin was used. Exponentially growing cultures were maintained at 37℃ in a humidified atmosphere of 95% air/5% CO_2 for all cell lines. The characteristics of the cell lines are shown in Table 1.

2. Clonogenic assay

Single cell suspensions of exponentially-growing cells were obtained by trypsinization of monolayer cultures, with cell counts performed using a Bright-Line hemocytometer (Sigma Chemical Co., St. Louis, MO, USA). Based on the results of preliminary studies, and depending on the radiation dose applied, an appropriate number of cells were plated in a 25 cm^2 polystrene culture flask (Nunc EasY Flask, Nunc, Rosklide, Denmark). PCI-1 and RKO cells were inoculated, using 300 cells for the control plates, with higher cell numbers for the treated plates. For NCI-H630, 400 cells were inoculated for the control plates, and higher cell numbers for treated plates. The control SNU-1066 plates were inoculated with 500 cells, with higher cell numbers for the irradiated plates. After an overnight incubation in a 5% CO_2 incubator at 37℃, irradiation was carried out at room temperature over a dose range of 0, 2, 4, 6, 8 Gy in PCI-1 cells and 0, 2, 4, 6, 10 Gy in NCI-H630, RKO and SNU-1066 cells using a Mark I Cs-137 irradiator (J.L. Shepherd & ass. Glendale, CA, USA). After culturing the cells for 10~14 days in a 5% CO_2 incubator at 37℃, the resultant colonies were fixed with 100% methanol and stained with 0.5% (Wt/vol) crystal violet in methanol. Colonies containing more than 50 cells were scored as the endpoint of survival, and the surviving

fractions were calculated as below.

$$S.F. = \frac{\text{Colonies formed}}{\text{Cells plated} \times \text{Plating efficiency}}$$

All experiments were done in triplicate.

3. MTT assay

Cells were harvested from exponential growth phase cultures with trypsinization. Single cell suspensions were obtained, cells counted using a Bright-Line hemocytometer (Sigma Chemical Co., St. Louis, MO, USA) and then dispersed within a Nunclon MicroWell plate (12×8, Nunc, Rosklide, Denmark) to a total volume of 100 μl/well. After overnight incubation, cells were irradiated at room temperature over a dose range of 0, 2, 4, 6, 8 Gy in PCI-1 and 0, 2, 4, 6, 10 Gy in NCI-H630, RKO and SNU-1066 cells using a Mark I Cs-137 irradiator (J.L. Shepherd & ass. Glendale, CA, USA). Preliminary experiments to test the growth pattern of cells plated at different densities and irradiated with different dose levels were performed. The cell densities used in the final experiments were as follows; PCI-1 - 1000, 500, 300 cells/well, SNU-1066 - 1250, 625 cells/well, NCI-H630 - 1000, 500 cells/well and RKO - 500, 300, 200 cells/well

Surviving fractions were measured both when the irradiated cells had regained exponential growth and when the non-irradiated cells had undergone four or more doubling times. Where possible, the assays were run for six doubling times; times for each cell line were 6 days for PCI-1, 12 days for SNU-1066, 9 days for RKO, and 12 days for NCI-H630 (four doubling times). Optimally, an incubation time of 18 days would have been appropriate for NCI-H630 cells. However refeeding of cells can lead to problems of contamination. Due to this practical consideration, incubation was limited to 12 days in this cell line. Following the appropriate incubation period, 10 μl of Premix WST-1 solution (Takara Shuzo Co. Shiga, Japan) was added to each well. WST-1 is one of the tetrazolium salts and is cleaved to the formazan dye by succinate-tetrazolium reductase (EC 1.3.99.1), which exists in the mitochondrial respiratory chain and is active only in viable cells.[8] This ready-to-use solution is containing WST-1 and an electron coupling reagent, diluted in phosphate buffered saline and does not require washing, harvesting or solubilization of the precipitate. The plates were assayed by a multiwell spectrophotometer (enzyme-linked immunosorbent assay reader; THERMOmax microplate reader, USA) at a wavelength of 490 nm after 4 hour incubation.

All experiments were done in triplicate.

4. Statistical analysis

For comparing the radiation sensitivity from these two different methods, t-tests and regression analyses of survival fraction from both methods in various radiation dose were done.

RESULTS

1. Relationship between cell number and optical density

For each cell line, the cells were dispensed into plates in serial dilution from 50,000 to 156 cells/well and incubated for 24 hours to allow attachment. MTT assays were performed as previously described manner (see 'Materials and Methods').

Data points were averaged from three wells of one row. Regression analysis revealed a high linear correlation; that is, R^2 of 0.976 between 390 and 50,000 cells seeded per well for PCI-1, R^2 of 0.986 between 195 and 50,000 cells seeded per well for SNU-1066, R^2 of 0.990 between 195 and 100,000 cells seeded per well for NCI-H630, R^2 of 0.996 for RKO. Within this range of cell numbers a conversion of the optical densities into cell numbers appeared to be unnecessary for further analysis. The results from these experiments are shown in Fig. 1.

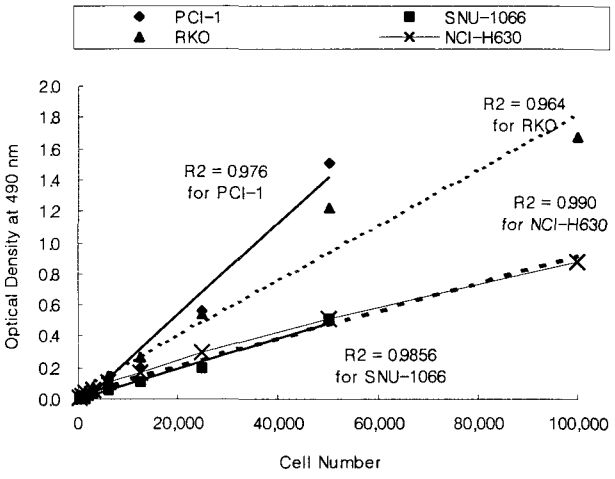

Fig. 1. Relationship between cell number and optical density in four cell lines after mixing with 10 μl of Premix WST-1 solution.

2. Survival fraction after irradiation using clonogenic and MTT assay

In the MTT assay, the survival fraction was considered to be the ratio of the optical densities of a well defined number of irradiated and control cells.

For PCI-1 cells, the plating efficiency was 64%. The cell number used for MTT assay were 1,000, 500, and 300 cells/well. Finally, the results from the plate with 300 cells/well were most appropriate for comparison with the results of the clonogenic assay. There were no significant differences between clonogenic assays and MTT assays with the survival fraction of 0.775 (±0.008)[2] for clonogenic assay and 0.711 (±0.059) at the radiation dose of 2 Gy, 0.279 (±0.009) for clonogenic assay and 0.268 (±0.057) for MTT assay at 4 Gy, 0.136 (±0.019), 0.042 (±0.037) for clonogenic assay and 0.105 (±0.028), 0.056 (±0.056) for MTT assay at 6 Gy, 10 Gy, respectively.

For SNU-1066 cells, the plating efficiency was 12%. The cell number used for MTT assay were 1250 and 625 cells/well. Finally, the results from the plate with 625 cells/well were most appropriate for comparison with the results of the clonogenic assay. There were no significant differences between clonogenic assays and MTT assays with the survival fraction of 0.315 (±0.017) for clonogenic assay and 0.338 (±0.051) at the radiation dose of 2 Gy, 0.075 (±0.004) for clonogenic assay and 0.111 (±0.029) for MTT assay at 4 Gy. Data for doses of 6 or 10 Gy showed statistical differences between two methods. The survival fractions at radiation dose of 6 Gy were 0.019 (±0.002) for clonogenic assay and 0.050 (±0.005) for MTT assay (p=0.01). At the radiation dose of 10 Gy, the survival fractions were 0.005 (±0.001) for clonogenic assay and 0.027 (±0.013) for MTT assay (p=0.04).

For NCI-H630 cells, the plating efficiency was 12%. The cell number used for MTT assay were 1000 and 500 cells/well. In this experiments, the plate with 500 cells/well were most appropriate for comparison. The survival fractions from two methods with the radiation dose of 2 Gy, 4 Gy, and 6 Gy were showed no statistical differnece except for 10 Gy. The survival fractions at radiation dose of 2 Gy were 0.622 (±0.127) for clonogenic assay and 0.688 (±0.158) for MTT assay, 0.315 (±0.077), 0.114 (±0.039) for clonogenic assay and 0.354 (±0.064), 0.188 (±0.011) for MTT assay in radiation dose of 4 Gy and 6 Gy, respectively. Survival fractions at radiation dose of 10 Gy were 0.004 (±0.003) for clonogenic assay and 0.017 (±0.013) for MTT assay (p=0.004).

For RKO cells, the plating efficiency was 31%. The cell number used for MTT assay were 500, 300 and 200 cells/well. Finally, the results from the plate with 200 cells/well were most appropriate for comparison with the results of the clonogenic assay. There were no significant differences of survival fractions in the radiation dose of 2 Gy, 4 Gy, and 6 Gy. The survival fractions at radiation dose of 2 Gy were 0.580 (±0.023) for clonogenic assay and 0.597 (±0.042) for MTT assay, 0.232 (±0.012), 0.098 (±0.008) for clonogenic assay and 0.248 (±0.011), 0.102 (±0.0081) for MTT assay in radiation dose of 4 Gy and 6 Gy, respectively. Survival fraction with the radiation dose of 10 Gy, the survival fractions were 0.013 (±0.005) for clonogenic assay and 0.043 (±0.003) for MTT assay (p=0.002). Radiation survival curves derived from two assays for four cell lines were shown in Fig. 2~5.

The α and the β value of linear-quadratic model were also calculated. There was no statistically significant differences between the two methods. But relatively small number of data points and lack of data from high dose level, These data were considered to have little meaning (data not shown).

There were good linear correlations between data from the clonogenic assay and data from the colorimetric assay by regression analyses with the R^2 values were 0.9978 for PCI-1, 0.9750 for SNU-1066, 0.9919 for NCI-H630, and

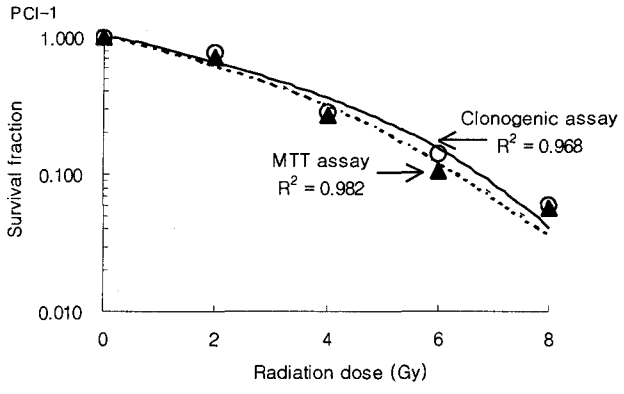

Fig. 2. Comparison of radiation survival curve from clonogenic assay (○—○) and MTT assay (144 hours - 6 doubling times, 300 cells/well, ▲···▲) for PCI-1 cells. Each points represent the mean surviving fraction of three experiments.

2) Mean (±1 standard deviation)

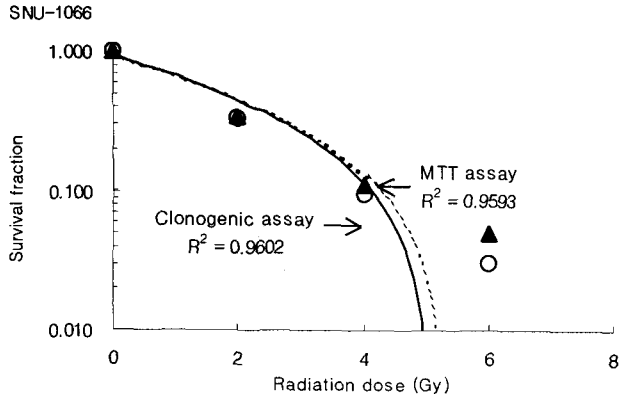

Fig. 3. Comparison of radiation survival curve from clonogenic assay (O—O) and MTT assay (192 hours - 4 doubling times, 625 cells/well, ▲···▲) for SNU-1066 cells. Each points represent the mean surviving fraction of three experiments.

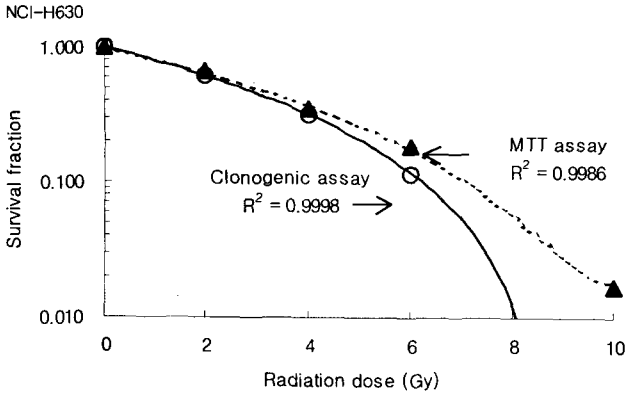

Fig. 4. Comparison of radiation survival curve from clonogenic assay (O—O) and MTT assay (240 hours - 3.3 doubling times, 500 cells/well, ▲···▲) for NCI-H630 cells. Each points represent the mean surviving fraction of three experiments.

0.9963 for RKO cells.

3. The effects of different seeding cell number and timing of measurement on survival fraction in MTT assay

Fig. 6. showed the effect of altering cell numbers and assay duration time on the radiation survival curve of NCI-H630 cells. As can be seen, the results can be potentially invalidated by seeding too many cells or by shortening the duration of the assay. At 192 and 240 hours, these potential invalidity may be due to persistent dehydrogenase activity in cells destined to die, whereas at 240 hours seeding 500 and 1,000 cells/well, confluence of control wells is a major factor.

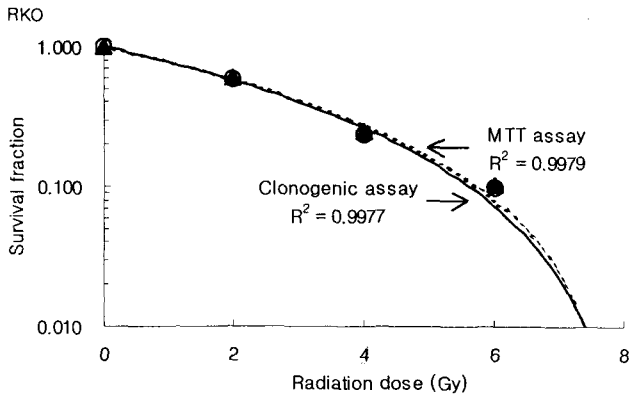

Fig. 5. Comparison of radiation survival curve from clonogenic assay (O—O) and MTT assay (192 hours - 6 doubling times , 200 cells/well, ▲···▲) for RKO cells. Each points represent the mean surviving fraction of three experiments.

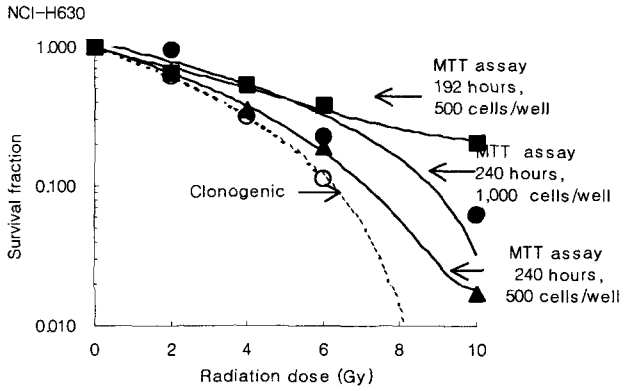

Fig. 6. Radiation survival curves (NCI-H630) for the colorimetric assay where the time of assay and number of cells/well are varied. Clonogenic assay (O···O) and MTT assay (240 hours - 3.3 doubling times, 500 cells/well, ▲—▲, 192 hours - 2.7 doubling times, 500 cells/well, ■—■, 240 hours - 3.3 doubling times, 1,000 cells/well, ●—●).

DISCUSSION

The use of MTT assay for the measurement of radiation survival has produced conflicting results. Comparable results to clonogenic assays have been obtained both when the surviving fraction of the tumor cell lines were measured following 4~7 cell doubling times and after the irradiated cells had regained exponential growth.[10, 19] Other research comparing the two assays has found that surviving fractions measured by the MTT assay were higher than those from clonogenic assays.[20, 21] However, Ramsay and colleagues con-

cluded that MTT assay increased the proportion of successful tests, and that they were of shorter duration.

The use of the MTT assay to measure the number of viable cells depends on the assumption that the production of formazan crystals and the resultant optical density is proportional to the number of cells. The relationship between cell number and optical density is not always linear and must be investigated for each cell line.[10, 19, 22)]

The MTT assay used in this study gave reproducible results with the standard deviation being generally less than 5% except NCI-H630 (about 10%). These range of standard deviation may be due to variation in growth in culture of different cell inocula as well as experimental error.

The radiation survival was slightly higher in the MTT assay than the clonogenic assay except for PCI-1. Although there were no statistical differences with the radiation dose less than 6 Gy, using a radiation dose above 6 Gy, the survival fraction measured with both assays diverged increasingly. This may due to persistent mitochondrial function in lethally-irradiated cells incapable of division.[20)] Alternatively, the surviving cells may have overgrown the dead or damaged cells to produce a higher optical density.[23)] Using 96-well microtitre plates with each well containing 0.1ml medium and having a restricted bottom surface area may lead to an early onset of plateau-phase growth. It must be remenbered that the MTT assay used in this study measures the surviving fraction of a population of clonogenic and non-clonogenic cells, whereas the clonogenic assay measures the surviving fraction of clonogenic cells only.

Therefore, at high radiation dose level, MTT assay had its limitation on application.

Preliminary experiments were necessary to establish the best number for use with each cell line. It is essential that sufficient time is allowed for cell death and loss of dehydrogenase activity and that all treatment groups, particularly the control cells, remain in exponential growth up to the point of assay. It was sometimes difficult to find a balance between plating the number of cells per well required for reliable growth of irradiated cells and plating a higher number of cells that could result in the non-irradiated cells achieving confluence. Confluence can result in overestimation of the surviving fractions.[19, 23)]

On the optimal cell number for MTT assay, although there were some limitations of data interpretation due to small number of used cell lines, less than 300 cells/well may be optimal for cells with relatively high plating efficiency above 30%. Whereas, 500 to 1,000 cells/well may be optimal for cells with low plating efficiency below 30%. More than 1000 cells/well, confluence of control wells may be a major factor of failure.

From the data of this study, the optimal timing of measurement of survival fraction in MTT assay were after 6 doubling times, at least, more than 4 doubling times were required for obtaining compatible results with the results from the clonogenic assay. In consideration of the assay condition; with the small volume of media (0.1 ml) and restricted bottom surface area of multiwell plate, assay times may be limited less than 12 days, practically. Therefore, cells with long doubling times (more than 3 days) may be inappropriate for application.

CONCLUSION

The MTT assay used in this study provided reproducible and comparable results to those from clonogenic assays used in the assessment of radiation survival. The optimal cell numbers for MTT assay were different with the plating efficiency of used cell line. From this study, cells with high plating efficiency (more than 30%), less than 300 cells/well were appropriate. For cells with low plating efficiency (less than 30%), 500 cells/well or more were appropriate for assay. The optimal time for MTT assay was after 6 doubling times, at least after 4 doubling times. In consideration of practical limits of assay (12 days, in this study) cells with doubling time more than 3 days were inappropriate for application.

This MTT assay offered a rapid, simple method for the assessment of radiation sensitivity of cell lines. However, the necessity to optimize the conditions of the colorimetric assay for each cell line may limit its usefulness in determining radiation sensitivity.

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국문 초록

방사선 감수성 측정법으로서 MTT 법 시행 시의 최적 조건에 대한 연구

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서울대학교 의과대학 암 연구소†

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목 적 : 방사선조사후 세포의 생존 분획은 세포집락 측정기법으로 확인하는 것이 표준이나 많은 비용과 시간이 소요되는 단점을 갖고 있다. 이에 생존 세포의 tetrazolium염의 자색 formazan 침전물로의 환원시키는 능력에 그 기반을 둔 MTT 기법을 사용하여 세포집락 측정기법을 대체하기위한 기법으로서의 유용성과 그 실행상의 최적조건을 규명하고자 하였다.

방 법 : PCI-1, SNU-1066, NCI-H630, RKO 등의 세포주에 0, 2, 4, 6, 8, 10 Gy의 방사선을 조사한 후 세포집락 측정기법과 MTT기법으로 세포 생존 분획을 조사하였다. 세포집락 측정기법은 25 cm^2 폴리스티렌 배양 플라스크에 방사선량에 따라 다른 수의 세포를 분주한 후 24 시간 동안 배양 후 방사선을 조사하였고 이를 10~14일 동안 배양 후 염색하여 생성된 세포집락의 수를 측정하였다. MTT기법은 침전물의 용해과정이 필요없는 Premix WST-1 시약을 이용하여 시행하였다. MTT기법은 각각의 세포주에서 세포수와 흡광도간의 선형관계와 최적 실험조건을 확인한 이후 시행하였다. 이 기법은 방사선을 조사받은 세포에서는 지수적 성장을 회복한 이후와 방사선을 조사받지 않은 세포는 4회 이상의 세포분열을 거친 후에 시행하였다. 세포집락 측정기법 및 MTT기법을 통하여 얻은 세포 생존율을 구한 후 이를 지표로 비교하였다.

결 과 : 각 기법으로 얻은 세포 생존율의 표준편차는 5% 내외였다. 2가지 방법으로 구한 세포 생존율은 t-test로 비교하였을 때 0~4 Gy에서는 통계적으로 유의한 차이가 없었으며 회귀분석 결과는 선형적 관계가 있었다(R^2= 0.975-0.992). MTT 기법의 시행에 최적인 세포수는 배양효율에 따라 다른 것으로 나타났는데, 배양효율이 30% 이상이면 300개 이하가, 30% 미만인 경우는 500~1,000개가 적당한 것으로 확인되었다. MTT기법은 6 배가시간 경과 이후에 시행하는 것이 세포집락 측정기법과 가장 근접하였으며 적어도 4 배가시간 이후에 시행하는 것이 필요할 것으로 사료되었다. 이에 따르면 배가시간이 3일 이하인 세포주가 세포 민감성 측정방법으로서 MTT 기법이 세포 집락 측정 기법을 대체하여 사용하기에 적합한 것으로 사료되었다.

결 론 : 이상에서, MTT기법을 이용하여 방사선조사 후의 세포생존을 측정하기 위해서는 예비실험을 통해 각 세포주에서의 최적의 조건을 찾는 것이 필수적이며 이 조건하에서 MTT기법을 시행해야만 방사선에 의한 세포 민감성 측정에 이용될 수 있음을 확인하였다.

핵심용어 : MTT법, 세포집락 측정기법, 방사선 감수성