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Comparison of colorimetric MTT and clonogenic assays for irradiation and cisplatin treatment on murine fibrosarcoma SA-1 cells

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Background. *The aim of our study was to determine the relationship between cell survival of SA-1 tumor cells measured by clonogenic assay and MTT assay after irradiation and cisplatin treatment.*

Materials and methods. *Survival of SA-1 cells was measured after irradiation (2-8 Gy) and cisplatin treatment (0.05-0.5 μg/ml) by clonogenic assay performed 7 days after treatment, and by MTT assay performed on day 3, 4, 5, and 7 after the treatment.*

Results. *The results showed good correlation between MTT assay and clonogenic assay for irradiation doses below 4 Gy. For higher doses good correlation between MTT and clonogenic assay was determined only when MTT assay was performed on day 5 and 7 after the treatment. In the case of cisplatin treatment, similar pattern was observed, good correlation between IC_{50} values for MTT and clonogenic assay was found when MTT assay was performed on day 5 and 7 after the treatment.*

Conclusion. *Results of our study confirmed the results of previous studies addressing this topic and further support the use of MTT test as an alternative test for clonogenic test as a predictive assay of tumour response to the radio or chemotherapy.*

Key words: sarcoma, experimental-radiotherapy-drug therapy; colony forming units assay; cisplatin; colorimetry-methods; triazoles

Introduction

The use of predictive assays in radio and chemotherapy is getting more and more attention in the last years, especially, because some clinical studies demonstrated good correlation between a predictive assays and clinical response to therapy.[1-8] Numerous different approaches, such as measurement of either survival or growth of cells, tumour cell kinetics, determination of chromosomal or DNA damage following gene expression and mea-

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surement of tumour hypoxia, were tested in order to predict tumour or normal tissue response of particular patient to radio or chemotherapy.[9-11] The rationale of predictive assays is to identify patients before the commencement of therapy, in whom the dose could be either increased or decreased according to the sensitivity of tumour or normal tissue that is determined by predictive assay.[9-11]

Clonogenic assay, which measures directly the ability of tumour cells to proliferate, is the predictive assay which has been most often tested in connection with tumour response to radiotherapy. Tumour cell sensitivity showed to be a good predictor of tumour response in some tumour types, however in others this relationship was not determined.[1-8] There are several drawbacks associated with this test, *i.e.* the non-ability of some tumour cell to form colonies and also quite big costs. Clonogenic assay can also be time consuming in the cases of cells with low plating efficiencies and long doubling times.[9-11]

Therefore, several alternative non-clonogenic tests were developed, which measure either growth of the cells or DNA damage. Growth assays estimate survival by comparing the number of viable cells in treated and control groups. Several different methods are currently used for measuring growth of the cells *i.e.* measurements of cells number, dye exclusion, isotope precursor uptake, and measurement of cell metabolism.[11-15]

One of the latter methods is the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay.[12] The MTT assay quantifies the ability of viable cells to reduce a yellow tetrazolium salt to purple formazan crystal using the mitochondrial enzyme succinate dehydrogenase. The MTT assay is rapid and semi-automated and was shown in some studies to be a good replacement for clonogenic assay.[12-17]

The aim of this study was to determine the relationship between cell survival of SA-1 tumour cells measured by clonogenic assay and MTT assay after irradiation and cisplatin treatment.

Materials and methods

Cell line

Fibrosarcoma SA-1 cells (Jackson Laboratory, Bar Harbor, ME) were used in experiments. Cells were grown in Eagle's minimum essential medium (EMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated foetal calf serum (FCS; Sigma). Cells were routinely subcultured twice per week and were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Doubling time of the SA-1 cells was 18 hours.

Determination of relationship between cell number and optical density

To determine the relationship between cell number and optical density exponentially-growing cells were trypsinized and suspended in EMEM supplemented with 10% FCS. The cells were diluted to final concentrations in EMEM and plated in round-bottomed 96-well microtiter plates (Costar, Badhoevedorp, The Netherlands). The cell densities used were $1x10^3$, $3x10^3$, $5x10^3$, $1x10^4$, $1.2x10^4$, $1.5x10^4$, $3x10^4$ $5x10^4$, $7.5x10^4$ and $1x10^5$ cells per well. MTT solution (5 mg/ml; 25 µl) was added to each well after 3 h, which was the time needed for cells to seed. The microtiter plates were then incubated for another 3 hours at 37°C in an incubator containing humidified atmosphere and 5% CO_2. At the end of incubation period the MTT test was performed (see below).

Irradiation

Cells from the exponential growth phase were trypsinized and suspended in EMEM supplemented with 10% FCS, counted and diluted to final concentrations. For clono-

genic assay cells were plated in 60 mm Petri dishes and for MTT assay cells were placed in 100 mm Petri dishes (Costar, Beovendorf, The Netherlands). Cells were irradiated using a 220 kV X-ray Machine (Darpac, Gulmay Medical Ltd, UK) filtered with 0.6 mm Cu and 3 mm Al at a dose rate of 2 Gy/min. After irradiation, 100 µl of cell in EMEM were plated in round-bottomed 96-well microtiter plates (Costar). Final cell densities at particular radiation doses for MTT assay were as follows: 100 cells per well at 0 Gy (control); 100 cells per well at 2.0 Gy; 250 and 300 cells per well at 4.0 Gy; 1000 cells per well at 6.0 Gy; and 1500 cells per well at 8.0 Gy. One column of eight wells was utilised for each irradiation dose. Cell densities at particular radiation doses for clonogenic assay were as follows: 200 cells per Petri dish at 0 and 2.0 Gy; 400 cells at 4.0 Gy; 1500 cells at 6.0 Gy; and 6000 cells per well at 8.0 Gy. The microtiter plates as well as 60 mm Petri dishes were incubated at 37°C in an incubator containing humidified atmosphere and 5% CO_2 for 3, 4, 5 and 7 days. On day 3 50 µl of EMEM and on day 5 25 µl of EMEM were added to each well to provide nutrition for the cells and to compensate media loss due to the evaporation.

MTT test was performed 3, 4, 5 and 7 days after irradiation. Clonogenic assay was performed on day 7. Experiments were repeated four times in duplicates for MTT assay and in triplicates for clonogenic assay.

Treatment with cisplatin

Exponentially growing cells were trypsinized and suspended in EMEM supplemented with 10% FCS at a concentration of $2.2x10^3$ cells/ml for MTT assay. Ninety µl of cell suspension was seeded in well and 10 µl of medium containing different cisplatin concentration was added to each well. For clonogenic assay 300 cells were seeded in Petri dish containing 10 ml of medium with specific cisplatin concentration. The cisplatin concentrations used were 0.05, 0.1, 0.2, 0.3 0.4, 0.5 µg/ml. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3, 4, 5 and 7 days. At the end of incubation period MTT test or clonogenic assay were performed as described below.

MTT assay

Survival of SA-1 cells after irradiation and treatment with cisplatin was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. At the end of incubation time (3, 4, 5, or 7 days) MTT solution (25 µl of 5 mg/ml solution) was added to each well and the microtiter plates were further incubated for 3 hours at 37°C. The microtiter plates were then centrifuged at 2000 rpm to collect the formed formazan crystals at the bottom of the rounded shaped wells. EMEM containing MTT solution was carefully removed with a pipette and then the formazan crystals were dissolved in 100 µl of dimethyl sulfoxide (Sigma). The microtiter plates were shaken for 99 seconds to ensure adequate solubilization and the absorbance of the resulting solution was measured at 540 nm using an Anthos microplate reader (Anthos, Austria).

Using the calibration curve, absorbance at different doses was converted to cell number. Platting efficiency (PE) was calculated by dividing the number of cell obtained with the number of cells seeded. Surviving fraction was calculated by dividing the PE of treated cells with PE of the control.

Clonogenic assay

Clonogenic assay was performed 7 days after irradiation or treatment with cisplatin. Colonies were fixed and stained with crystal violet (Sigma). Colonies of less than 50 cells were not counted. Plating efficiency and surviving fraction were calculated for each treatment group.

Statistical analysis

Linear regression was used to fit the experimental data of cell number with corresponding optical densities to obtain the calibration curve and Pearson correlation coefficient was calculated. One-way analysis of variance and Turkey-Keuls test were used to compare the data between clonogenic and MTT assay at different doses.

Results

Calibration

To calculate the surviving fraction after irradiation and cisplatin treatment using the MTT assay the calibration curve was constructed determining the correlation between cell number and optical densities. There was a good reproducibility between replicates and between the experiments with standard errors of the arithmetic mean below 10%. We found a linear relationship between cell number and optical densities (Figure 1). The correlation coefficient between the cell number and optical densities was 0.992. The equation, which described best the experimental data, was

$$y = 2.6 \cdot 10^{-5} x + 0.07.$$

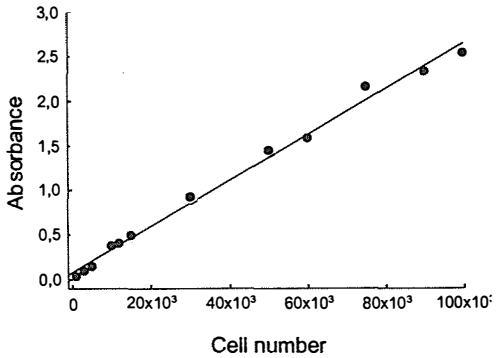

Figure 1. The calibration curve for absorbance against cell number of SA-1 tumour cells. Data represents AM±SE. Experiments were performed four times in duplicates.

This equation was therefore used in further irradiation and cisplatin treatment experiments to convert the optical density measured into cell number, which allowed us to calculate the surviving fraction of cells when MTT test was employed.

Comparison of assays after irradiation

To determine the correlation between MTT and clonogenic assay, MTT test was performed on day 3, 4, 5 and 7 after the irradiation (Figure 2). Up to 4 Gy dose of irradiation the survival curves were almost identical for all tested groups. However, when MTT test was performed on day 3 the cell kill was not detected for higher doses. Nevertheless, when MTT test was performed on day 5 or 7 very good correlation with clonogenic test was obtained (r=0.984 and 0.997, respectively).

Comparison of assays after cisplatin treatment

To determine the correlation between MTT and clonogenic assay, MTT test was per-

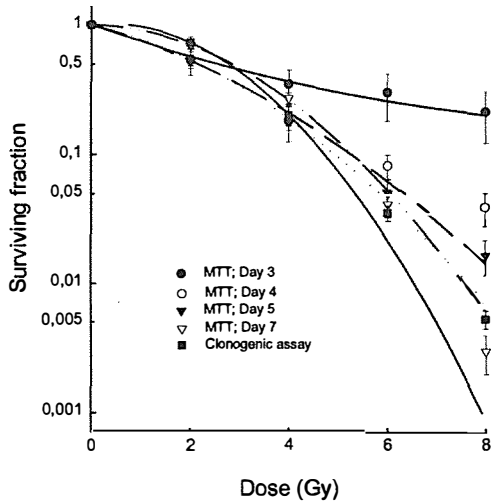

Figure 2. Comparison of cell survival after irradiation measured by clonogenic assay and MTT assay. Data represents AM±SE. Experiments were performed four times in duplicates.

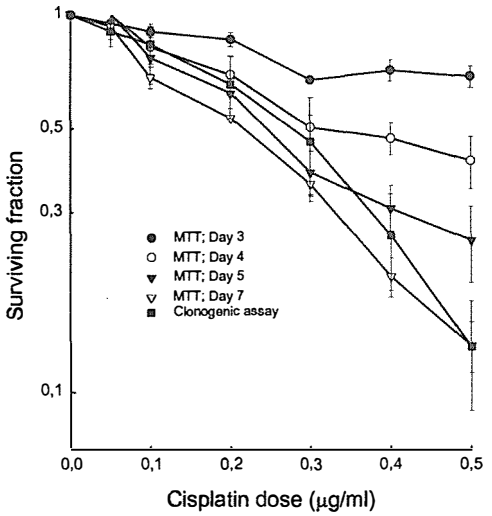

Figure 3. Comparison of cell survival after treatment with cisplatin measured by clonogenic assay and MTT assay. Data represents AMISE. Experiments were performed four times in duplicates.

formed on day 3, 4, 5 and 7 after the cisplatin treatment. MTT assay gave higher IC_{50} value when performed on day 3 or on day 4 (IC_{50} > 0.5 μg/ml and 0.4 μg/ml, respectively), but good correlation was found between IC_{50} values of MTT assay performed on days 5, and 7 and clonogenic assay (IC_{50}= 0.25, 0.22, and 0.28 μg/ml, respectively).

Discussion

This study shows good correlation between MTT assay and clonogenic assay for irradiation for doses below 4 Gy. For higher irradiation doses the good correlation between MTT and clonogenic assay was determined only when MTT assay was performed on day 5 and 7 after the treatment. In the case of cisplatin treatment, similar pattern was observed, good correlation between IC_{50} values between MTT and clonogenic assay was determined, when MTT assay was performed on day 5 and 7 after the treatment.

The use of MTT assay to measure the number of viable cells depends on the assumption that the production of formazan crystals and the resultant absorbance is proportional to the number of cells. Linear relationship between absorbance and cell number was previously reported for low cell number up to $1x10^5$.[13-15,17] For higher cell inoculum the relationship was not linear and therefore surviving fraction could not be calculated by comparing the absorbance with cell number.[18] In our study, we also found a linear relationship between absorbance and cell number. We used low cell inoculum in both, irradiation and chemotherapy experiments therefore, cell number at the end of incubation period did not exceed linearity of the calibration curve. This experimental protocol enabled us to calculate the surviving fraction and thus direct comparison of MTT test with clonogenic assay.

In our experiments on SA-1 tumour cells we found a very good correlation of MTT assay and clonogenic assay when cell were irradiated at 2 Gy. For higher doses, results between both assays correlated well only for longer incubation times in MTT assay. Similar results were published previously testing correlation between MTT and clonogenic assay on different cell lines.[12,13,18,19] In these studies, some of the tested cell lines did not show good correlation at 2 Gy, but at higher doses, indicating that use of MTT assay can sometimes be restricted by radiation dose ranges.

Use of MTT assay in determining chemosensitivity of tumours was already established.[5-7] In a study of Carmichel *et al.* four chemotherapeutic drugs were tested in three cell lines to determine the correlation between MTT and clonogenic assay.[17] They found excellent agreement between the IC_{50} values for melphalan, adriamycin and cisplatin, whereas for vinblastine, MTT assay appeared more sensitive.[17] In this study MTT assay was performed 4 days after the treatment. In our study, good agreement between MTT and clonogenic assay was obtained only when at least 5 days incubation was used in MTT assay, demonstrating that for reliable results incubation time for MTT

assay should be adjusted with regard to the doubling time of tested cells. It should be long enough to allow chemotherapeutic drugs to exert their cytotoxic potential.

In conclusion, results of our study confirmed the results of previous studies addressing this topic and further support the use of MTT test as an alternative test to clonogenic test as a predictive assay of tumour response to the radio or chemotherapy.

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