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Chemosensitivity testing of primary human renal cell carcinoma by a tetrazolium based microculture assay (MTT)

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Summary. MTT staining procedures have been used in chemosensitivity testing of established cell lines of human and other sources as well as of human leukaemias, but only limited information on its application in primary solid human tumors is presently available. We have evaluated MTT staining in primary human Renal Cell Carcinomas (RCCs), studied various factors interfering with the optimal use, and finally applied it in subsequent chemosensitivity testing. The method depends on the conversion of a water-soluble tetrazolium salt (MTT) to a purple colored formazan precipitate, a reaction effected by enzymes active only in living cells. Single cell suspensions of RCCs were obtained either by enzymatic dispersion or by mechanical dissagregation, filtered through gauze, and purified by Ficoll density centrifugation. Tests were carried out in 96-well microculture plates. 10^4 viable tumor cells per well at 4 h incubation time with 20 µg MTT/100 µl total medium volume yielded best results. Formazan crystals were dissolved with DMSO, and the plates were immediately measured on a microculture plate reader at 540 nm. Under these criteria, linearity of the system could be demonstrated. For chemosensitivity testing, cells were continuously exposed to a number of drugs prior to the MTT staining procedure. Reproducibility of results was assessed and confirmed by culturing RCCs in flasks additionally, resubmitting them after 1, 2, and 4 weeks to the MTT assay. We conclude that the semiautomated MTT assay offers a valid, rapid, reliable and simple method to determine the degree of chemoresistance in primary human RCCs.

Key words: Primary human renal cell carcinoma – MTT staining assay – Chemosensitivity testing

Chemosensitivity testing by clonogenic assays [6, 10] is suitable in established tumor cell lines [11] but exhibits limited value in primary human carcinomas [18] due to low plating efficiencies, clumping artefacts, and prolonged duration.

In an attempt to circumvent the technical pitfalls of stem cell tests, several short-term assays have been developed, including dye exclusion techniques [27], tritiated thymidine uptake [22, 24], radiolabeled glucose utilisation [25], and automated imaging analysis of crystal violet-stained cells [9]. Provided that these methods are accompanied by appropriate quality control measures they may show comparability with standard soft agar systems [21, 22, 25–27], but they did not allow processing of large numbers of samples. This prompted the introduction of automated colorimetric bioassays. They made use of tetrazolium compounds to determine cytotoxic effects of drugs [2, 5, 17].

Tetrazolium salts measure the activity of dehydrogenase enzymes only in living cells, which cleave the tetrazolium ring [19]. Thus, the conversion of a soluble yellow 1-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide into a purple colored MTT-formazan precipitate (Fig. 1) reflects cell viability [13]. MTT may also serve to improve detection of drug cytotocity in colony formation assays [1], and has the advantage over many other stains [8] of being nonlethal [12], allowing cells to be recovered if desired. The original procedure [16] underwent considerable modifications [3, 7] and is providing same information as other methods to determine drug responsiveness in tumor cell lines [2, 3, 5]. Sofar, it has not been used in primary solid human tumors, and therefore factors involved in its optimal application under these circumstances are not well known. The present study describes the adaption of the MTT test to measure the effect of antineoplastic agents on primary human renal cell carcinomas (RCCs).

Materials and methods

Cell suspension

Fresh tumor specimens from primary human RCCs were placed in chilled (4°C) Hank's balanced salt solution (HBSS), and processed within one hour after nephrectomy. Representative samples of

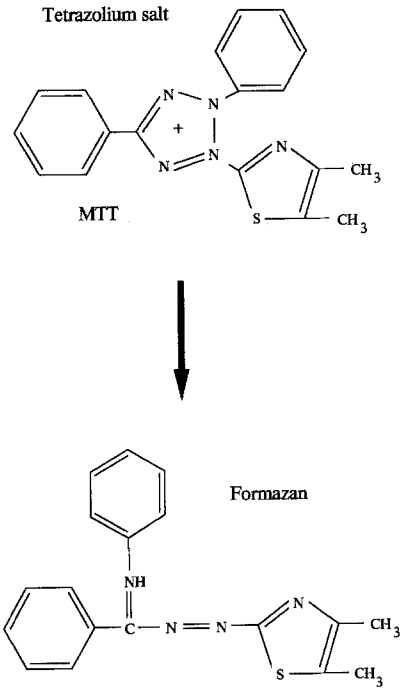

Fig. 1. Conversion of MTT to MTT formazan

different parts of the probes were freed from fat and necrotic areas, and HE stained smears were used to rule out major admixture of non tumorous material. Single cell suspensions were prepared either by enzymatic dispersion (Dispase/Collagenase 0,6 U/ml, 2 h; Boehringer Mannheim) or by mechanical disaggregation (steel mesh), followed by filtration through gauze (pore size 50–100 µm) under sterile conditions. The enzymatic method yielded only slightly higher quantities of metabolic active cells, but proved to be more time consuming. Erythrocytes and other cellular contaminants were separated by Ficoll density centrifugation (density 400 g, 10 min, 4° C; Seromed). The tumor cell pellet was washed twice in chilled (4°C) HBSS and resuspended in RPM 1640 culture medium without phenylred, supplemented with 20% serum. Experiments in triplicate were carried out to evaluate the influence of the serum contents: New born calf serum (NBS), fetal calf serum (FKS), and synthetic NU serum (Flow Laboratories).

These procedures resulted in greater than 80% viable cells, as assessed by trypan dye exclusion test; more than 90% of them were classified cytologically (HE stain) as tumor cells.

Table 1. Effect of serum supplement to optical density in a MTT staining assay. Conditions as described in materials and methods, 16 h incubation period

Serum	Background	Blank[b]	SEM[c]
NBS	0.016	0.091	± 0.002
	0.017	0.094	± 0.002
	0.014	0.089	± 0.002
FKS	0.0018	0.042	± 0.001
	0.018	0.043	± 0.001
	0.015	0.039	± 0.001
NU	0.016	0.024	± 0.001
	0.017	0.022	± 0.001
	0.017	0.021	± 0.001

[a] RPM 1640, without phenylred, without cells, MTT (200 µg/ml), 16 h incubation, then addition of DMSO (1.5:1 per total volume), read at 540 nm immediately
[b] background, supplemented with 20% serum
[c] 4 replicate wells

Microculture tetrazolium assay

Cell suspensions were adjusted to the cell number desired in a Neugebauer counting chamber, dispensed by multichannel pipettes into 96-well flat bottom microculture plates (Flow), and incubated for 2 h (37°C, 5%CO_2, 100% relative humidity) to allow cells to recover from mechanical agitation. MTT (Sigma) was dissolved in phosphate buffered saline (PBS) at 5 mg/ml, and stored at 4°C protected from light for 4 weeks maximum. After sterile filtration (Millipore) to remove a small amount of insoluble residue present in some batches, aliquots were freshly diluted (1:5) in prewarmed culture medium, and added to each well, resulting in 20 µg MTT/100 µl total medium volume. Optimal cell density and incubation time were established by experiments in quadruplicate, the mean of 4 replicate wells determined each point.

Measurements

150 µl organic solvents were pipetted to each well. According to previous suggestions, isopropanol [7], acidificied (0,04 N HCl) isopropanol [5, 16], or dimethyl sulfoxide (DMSO; Merck) [2, 3] were examined in quadruplicate. After vibration on a mechanical plate shaker, color intensity was measured on a microculture plate reader (Titertek Multiscan Plus MK II). To demonstrate the absorbance spectrum of MTT formazan solutions generated by RCCs, filters from 405 to 590 nm were assessed in triplicate.

Chemosensitivity testing

Drugs, already available in solution, were stored in original bottles at 4°C for 2 weeks maximum. Others were prepared according to manufacturers instructions, portioned into multiple aliquots, and kept at −70°C. Just prior to use, vials were removed from refrigerator or thawed, respectively, and serially diluted in culture medium.

RCCs were exposed to antineoplastic agents from 16 h minimum to 7 days maximum, followed by the MTT staining assay. In case of drugs interfering with color absorbance (e.g., anthracyclines), cells were isolated by centrifugation and resuspended in fresh medium before addition of MTT.

Peripheral wells of the microculture plates served as blanks (culture medium without cells containing cytostatics and MTT). Values were expressed as:

$$\% \text{ viable cells} = \frac{\text{Absorbance of Test} - \text{Absorbance of Blank}}{\text{Absorbance of Control} - \text{Absorbance of Blank}} \times 100$$

The mean of 4 replicate wells determined each point in our experiments.

To explore reproducibility of results, RCCs were additionally cultured in monolayer flasks, harvested after 1, 2 and 4 weeks, and resubmitted to the MTT staining procedure as described ($n = 14$).

Results

Precipitation of serum in organic solvents may influence the optical density [7]. In fact, medium containing 20% NBS showed much higher background absorbance than

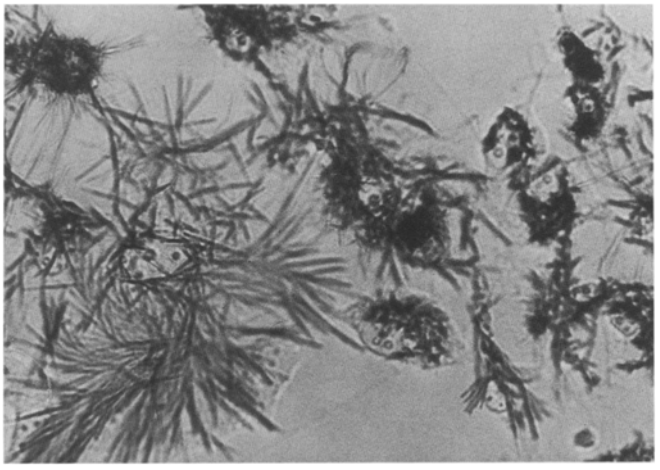

Fig. 2. MTT formazan crystals generated by RCCs. Conditions as described in materials and methods except for cell growth in Petri dish. 4 h exposure time of 10^4 cells to 200 µg MTT/ml culture medium

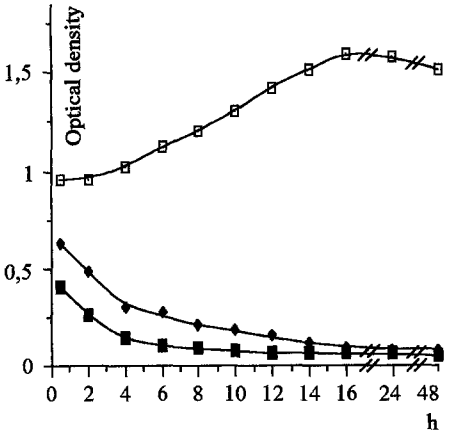

Fig. 4. Stability of MTT formazan color reaction after addition of solvents. Standard MTT assay as described in materials and methods, read at 540 nm. Plates stored at room temperature, 4 replicate wells determining each point. ⊡ = DMSO; ◆ = isopropanol; ■ = 0.04 N HCl/isopropanol

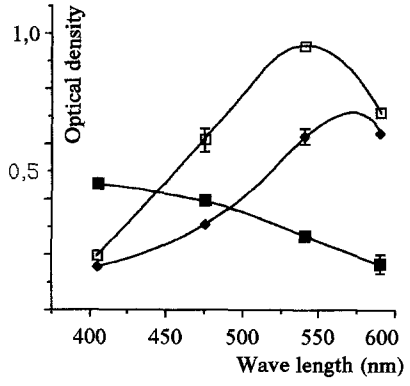

Fig. 3. Absorbance spectrum of MTT formazans generated by RCCs in various organic solvents. Standard MTT assay as described in materials and methods, 4 replicate wells determining each point ± SEM (no errors bars shown when within size of symbol). Filters from 405–590 nm. ⊡ = DMSO; ◆ = isopropanol; ■ = 0.04 N HCl/isopropanol

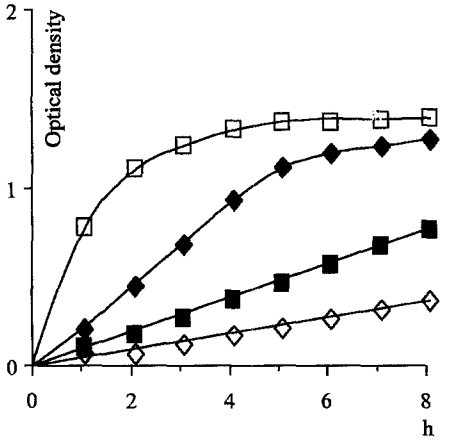

Fig. 5. Linearity of MTT formazan formation by RCCs depending on cell number and time. Cell number per well, 20 µg MTT/100 µl total medium volume. 4 replicate wells determining each point ± SEM (always within size of symbol). □ = 20,000 cells; ◆ = 10,000 cells; ■ = 5,000 cells; ◇ = 2,000 cells

medium supplemented with 20% FKS or synthetic NU (Table 1).

In this test, reduction activities of cells lead to MTT formazan production. These crystals (Fig. 2) may be difficult to dissolve [3], the organic solvents used may have a major impact on the absorbance spectrum [2, 7], and stability of the final color reaction remained to be established.

We shared the preference of previous investigators [2, 3] to DMSO for routine application, as it dissolved the formazan crystals extremely rapidly (5 min) without excessive agitation. Moreover, it exhibited highest optical density values as compared to (acidic) isopropanol (Fig. 3), and in addition to that, stability of the absorbance measured up to two hours could only be achieved when using DMSO (Fig. 4). In consequence, plates were always read at 540 nm (absorbance peak of DMSO).

The deviation of the average absorption of 4 replicate wells was extremely small. Where no cells were involved (blanks), values within 2% of the mean were obtained (Table 1). Cell effected color reaction showed moderately increased errors but stayed in general within 5% of the mean (Fig. 5).

Variations of cell number and incubation time revealed linearity of the system to an optical density of about 1,0 only when 10^4 viable cells/well were plated at 4 h incubation time (Fig. 5). For the subsequent in vitro evaluation of cytotoxic agents and biological response

Table 2. Chemosensitivity testing of RCCs using a MTT staining assay. Conditions as decribed in materials and methods. Continuous drug exposure time ranging from 16 h minimum to 7 days maximum prior to MTT staining assay

Drug	Max. conc. µg/ml	*n*	Resistant	Sensitive[a]
Vinblastine	1	35	27	8
Cisplatinum	3	34	29[b]	5
Carboplatinum	7.5	34	29[b]	5
Bleomycine	1	32	30	2
TNF	0.01	32	30	0
a-2-Interferon	0.01	30	29[c]	1[c]
Doxorubicin	3	30	27[d]	3
Epirubicin	3	30	27[d]	3
Daunomycine	3	15	15	–
5-DU	10	12	12	–
Methotrexate	1	12	12	–
MMC	1	10	10	–

[a] defined as less than 65% remaining activity of untreated controls
[b] cross resistance
[c] one case representing exactly 65% remaining activity of untreated controls
[d] cross resistance

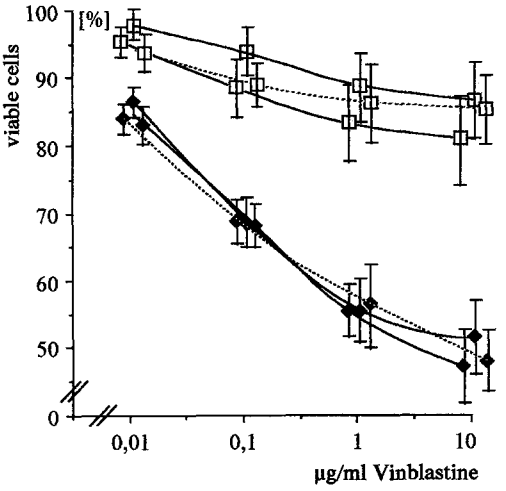

Fig. 7. Reproducibility of chemosensitivity testing results using a MTT staining assay. RCCs maintained in monolayer cultures containing RPM 1640 medium supplemented with 20% NU serum, and harvested for subsequent chemosensitivity testing. 16 h vinblastine exposure time, followed by standard MTT assay as described in materials and methods, 4 replicate wells determining each point in all experiments, $n = 14$, average in each point ± SD. ▣ = resistant (n=6); ◆ = sensitive (n=8); —— = immediately; ····· = 2 weeks in culture; —— = 4 weeks in culture

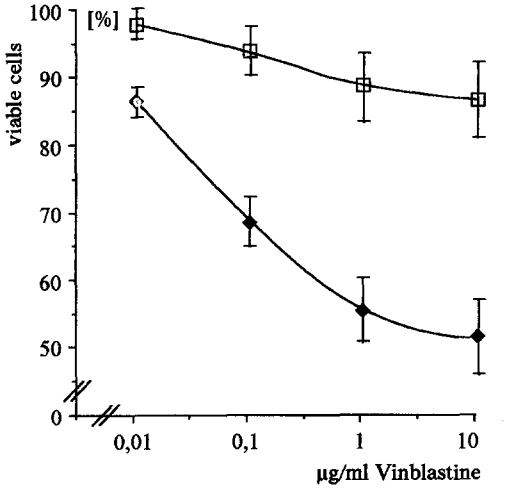

Fig. 6. Detection of vinblastine resistance in RCC using a MTT staining assay. 16 h exposure time to vinblastine, followed by standard MTT assay as described in materials and methods. 4 replicate wells determining each point in all experiments, average in each point ± SD. ▣ = resistant (n=27); ◆ = sensitive (n=8)

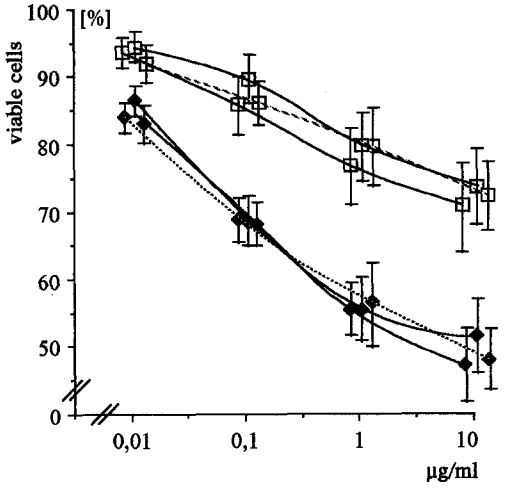

Fig. 8. Drug response of monolayer cultures of human RCCs using a MTT staining assay. RCCs maintained in monolayer cultures containing RPM 1640 medium supplemented with 20% NU serum, and harvested for subsequent chemosensitivity testing. 16 h drug exposure time, followed by standard MTT assay as described in materials and methods, 4 replicate wells determining each point in all experiments, $n = 8$ (group of vinblastine sensitive tumors), average in each point ± SD. ▣ = Cisplatinium ($n = 8$); ◆ = vinblastine ($n = 8$); —— = immediately; ····· = 2 weeks in culture; —— = 4 weeks in culture

modifyers in RCCs, standard tests then included 10^4 viable cells at 4 h incubation followed by solubilisation of formazan crystals with DMSO.

Chemosensitivity testing of 35 human RCCs confirmed a high degree of chemoresistance to a panel of antineoplastic agents (Table 2). In clinical trials [20] vinblastine has so far proved to be the most potent single agent in metastatic RCC with response rates of maximally 20%. Assessing vinblastine in the MTT short-term assay (Fig. 6) 8 of 35 tumors could be classified as sensitive to this drug when a threshold below 65% of untreated controls at 16 h exposure time was used as discrimination between resistant and sensitive. None of the other substances tested revealed a similar effect (Table 2).

Primary monolayer cultures of the same tumor may serve to ascertain reproducibility of chemosensitivity

testing results [9]. in 14 RCCs, which underwent the MTT staining method as described after culturing up to 4 weeks response to vinblastine did not change (Fig. 7). In general, the relative degree of resistance observed for each drug was maintained (Fig. 8).

Discussion

There is no doubt that the MTT staining assay has great potential as a rapid method of survey for drug responsiveness in cell lines [2–4]. However, to assess primary human solid tumors such as RCCs, several shortcomings of the original procedure have to be circumvented.

It is obvious that in contrast to established cell lines, in primary tumors purification of the specimens may have impact on the color reaction, as non-tumorous cell contaminants may also reduce the dye or interfere with other mechanisms designated to determine cell viability. This specific problem is persistant in all short-term assays. Hence, much attention has to be paid to the preparation of cell suspensions involving a number of separation steps, as described. We are aware of the fact that Ficoll gradients may not guarantee homogenity of tumor cell pellets and that stained cytological specimens may not distinguish to 100% between normal and neoplastic cells. However, we think that the small but varying amount of other cells than RCCs obtained in our suspensions would, if of any importance, distinctively enhance the variation of values (Figs. 3, 5, 6) due to inpredictable differences in metabolic activities. Further support for the view that this systematic error may be neglegible, derives from the observation that monolayer cultures, a necessary part of the purification procedures prior to cloning of cell lines, ascertained reproducibility of our results (Figs. 7, 8).

Removal of supernatants prior to solubilisation of MTT formazan crystals as suggested by other authors [7] to prevent false readings of optical density caused by serum precipitates in organic solvents, can easily be achieved in adherent cell lines. But this is not possible in the case of floating cells such as RCCs in microculture plates, and even centrifugation of the plates [23] can not ensure that loss of cells or variations in residual volume [17] are avoided. This problem could be solved by employing synthetic NU serum, which did not increase blank absorbance (Table 1).

Evaluation of 106 different cell lines in a MTT staining assay [2] demonstrated significant cell related differences, thus indicating that assessment of optimal plating density and incubation time for each cell type is mandatory. If these optimal conditions are met, it is even possible to determine retrospectively the number of cells per assay, provided that the absorption curve is linear [5]. In our tests, 10^4 viable cells per well at 4 h incubation time fulfill these conditions (Fig. 5).

The majority of modifications in MTT staining assays concerned changes in the application of organic solvents [3, 7]. In primary RCCs mineral oil [3] had to be excluded, since it functions only in adherent cells. DMSO [3, 4] exhibited most satisfying solution properties, clearly advantageous to (acidic [16]) isopropanol [7]. Moreover, highest values of optical density were obtained with it (Fig. 3), and stability of the color reaction was found up to two hours (Fig. 4). We have started to compare our MTT staining method to extablished test systems like the clonogenic assay [14] or the in vitro short-term test [15]. Preliminarily, we can report on 4 of 5 tumors showing growth in soft agar. These 4 RCCs were resistant in all 3 assays to several drugs tested. Hence, support for the view that the end point of the tetrazolium based systems closely approximate that of the other in vitro methods [2] may be assumed.

Regarding the precautions as described, the semiautomated MTT assay offers a valid, rapid and simple method to assess the degree of chemoresistance in human RCCs.

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