Application of Crystal Violet Staining Assay to Drug Sensitivity Test for Human Malignant Tumor

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We applied an assay using crystal violet to test for anticancer drug sensitivity. In brief, after short-term incubation in a microplate, surviving tumor cells were fixed with methanol and stained with crystal violet. After washing out the dead cells, the remaining cells attached to the plastic plate were dissolved in ethanol and the intensity of absorbance was measured at 570 nm by spectrophotometer.

We investigated the relationship between the number of tumor cells and the intensity of absorbance and found that the intensity appeared to be in direct proportion to the number of surviving cells.

The reliability of the crystal violet assay was confirmed by the results of a ^{3}H-thymidine uptake assays.

Our study demonstrated that the crystal violet assay is a simple, rapid and reliable method for evaluation regarding anticancer drug sensitivity.

key words: Drug sensitivity testing, Crystal violet assay, Human tumor cell lines, ^{3}H-thymidine uptake assays

Introduction

In order to perform successful chemotherapeutic treatment of malignant tumors, the most sensitive drug should be selected for each individual patient. Determination of chemosensitivity is therefore very important. Various *in vitro* assays of chemosensitivity have been reported by several investigators, including colony forming assays[1,2], ^{3}H-thymidine uptake assays[3,4] and a microcytotoxicity assay[5]. However, we consider that none of them are entirely satisfactory.

A chemosensitivity testing system should be simple and easy to use, it should provide rapid reproducible responses with reliable specific results that are easy to standardize, and it should be economical.

Therefore, we introduced the crystal violet staining procedure to a chemosensitivity assay system.

Materials and Methods

Cell Lines

Four cell lines were used. Human osteosarcoma cell lines, NY and Huo-3N1, human oral cavity carcinoma, HSC-2, and human tongue carcinoma, HSC-3, were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan).

The cell lines, NY, HSC-2, and HSC-3, were cultured in Eagle's MEM medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol (2ME), 100μg/ml kanamycin and 10% heat-inactivated fetal calf serum (FCS) (Flow Laboratories, Inc., McLean, VA). Huo-3N1 cells were cultured in RPMI1640 medium (GIBCO Laboratories, Grand Island, NY) containing 2mM glutamine, 5×10^{-5} M 2ME, 100μg/ml kanamycin and 10% FCS. The cells were cultured in monolayer fashion and harvested by trypsinization or by using 5mM EDTA in PBS.

Anticancer Drugs

Stock solutions of the anticancer drugs were prepared in sterile buffered 0.9% NaCl solution and stored at −20°C. Subsequent dilutions were also made with 0.9 % NaCl solution. Tumor cells were exposed to the anticancer drugs at the following final concentrations (μg/ml): methotrexate (MTX), 50, 500; adriamycin (ADM), 0.5, 5.0; cisplatin (CDDP), 0.5, 5.0; vincristine (VCR), 2.0, 20; nimustine (ACNU), 2.0, 20; actinomycin-D (ACT-D), 0.5, 5.0. The concentrations of each drug approximately corresponded to those used in hamster osteosarcoma tumor tissues which had been passaged in our laboratory, for which the experimental results have been previously published.[6,7]

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Crystal Violet Assay

Crystal violet powder was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Tumor cells were washed twice with Eagle's MEM or RPMI1640 medium, and were added, at a concentration of 10^4 cells in 50μl, to the individual wells of 96-well flat-bottomed microtiter plates (Falcon, Becton-Dickinson and Co., Lincoln Park, NJ). After 48h incubation at 37℃ in a 5% CO_2-enriched atmosphere, the cells were exposed to each anticancer drug for 4-6h. After 48h incubation, cells were stained with 50μl of 0.5% crystal violet/20% methanol solution for 5 min, and rinsed out. The stained cells were solubilized with 100μl of 50% ethanol containing 0.05M NaH_2PO_4, and the absorbance of each well was measured at 570nm with a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc., Helsinki, Finland). As a control, RPMI1640 or Eagle's medium containing 2ME, glutamine, kanamycin, and 10% FCS was used in place of anticancer drug.
The survival rate was calculated from the following formula : % Survival=

$$\frac{\text{Absorbance of drug treated-Spontaneous absorbance*}}{\text{Absorbance of control-Spontaneous absorbance*}}$$

*The intensity of wells incubated without cells

The percentage survival was used to evaluate the drug sensitivity of tumor cells. All samples were examined in triplicate.

^{3}H-Thymidine Uptake Assay

Culture of tumor cells in microtiter plates and exposure to each anticancer drug were performed in the same manner as for the crystal violet assay. After 24h incubation, 20μl of 25μCi/ml ^{3}H-thymidine (New England Nuclear Co., Boston, MA) was added to the medium in each well. After again incubated for 24h, the plates were stored at −20°C prior to assay. After thawing, cultured cells were harvested onto glass filter strips, to which 0.5% DPO/toluene was added. ^{3}H-thymidine incorporation was measured by liquid scintillation counter. Drug sensitivity was assessed from the results of ^{3}H-thymidine incorporation into DNA, and it was expressed as the percentage survival, calculated in the same way as for the crystal violet assay.

Results

Linearity of Crystal Violet Assay

In order to examine the relationship between the number of tumor cells and the intensity of absorbance at 570nm, the cells were plated at different concentrations (2.5×10^3 to 4×10^4 cells/well) and measured after 24h incubation. As shown in Fig. 1, the intensity of absorbance increased linearly with increase in number of cells. At the same time, we ascertained the increase of the number of cells under the microscope (before staining). This result suggests a close relationship between the intensity of absorbance and the number of surviving cells.

Testing of Drug Sensitivity

The crystal violet assay and the ^{3}H-thymidine uptake assay both indicated that the cytocidal effect of each drug was dose-dependent (Fig. 2).

In addition, when the percent suvival was classified into four groups (<30%, 30—50%, 50—70%, and>70%), the results from both assays performed on the same tumor cells showed good correspondence ; 8/12 for NY, 7/12 for Huo-3N1, 10/12 for HSC-2, and 10/12 for HSC-3. The total correspondence rate was 35/48.

Furthermore, drug sensitivity for tumor cells detected by the crystal violet assay was correlated ($p<0.01$) with that detected by ^{3}H-thymidine uptake assay (Fig. 3).

Discussion

In vitro chemosensitivity assays are associated with many problems, for example, the environment of the cells *In vitro* is different from that *in vivo* and may alter their chemosensitivity. In addition, optimum drug concentrations and exposure times for treatment still remain undetermined. However, until now we have had to depend on *in vitro* assays to predict clinical responses to anticancer drugs.

The crystal violet staining procedure for drug sensitivity testing was introduced in an attempt to overcome some of the many disadvantages of *in vitro* assays. Crystal violet belongs to the group of basic triphenylmethane dyes, and is conventionally used for Gram staining in bacteriology. In cell biology, Ruff et al.[8] have utilized staining with crystal violet to measure the cytotoxicity of tumor necrosis factor. Prochaska[9] reported that in murine hepatoma cells, the absorbance of crystal violet was linearly correlated with the cell number.

In our crystal violet assay, tumor cells were fixed with methanol and stained with crystal violet after drug treatment and short-term incubation. After the dead cells were washed out, the staining absorbance was quantified by optical density recorder. This method utilizing the crystal violet assay system has several advantages : 1) it is very simple to perform, 2) it allows rapid mass sample evalution, and 3) this

system dose not require the use of radioisotopes.

It has been pointed out that one disadvantage of crystal violet staining is that it simply reflects the quantity of protein and cannot distinguish viable from dead cells. However, we doubt that this is a disadvantage, because viable cells can attach to the plastic plate even after washing, whereas dead cells damaged by anticancer drugs can not attach. So only viable cells are subjected to measurement in our assay using plastic-adherent cells. Further the result of the crystal violet assay was compared with that of the ^{3}H-tymidine uptake assay, one of the methods to estimate the number of surviving cells. The comparative results of two methods showed a good correlation.

Our results indicate that an anticancer drug sensitivity testing system using the crystal violet assay would be very useful in the selection of anticancer drugs for patients with malignant tumor.

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Footnotes

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