

AN *IN VITRO* SCREENING METHOD FOR ANTITUMOR AND/OR
ANTITUMORIGENIC SUBSTANCES INVOLVING THE
TRANSFORMATION OF CHICK EMBRYO
FIBROBLASTS INFECTED WITH
ROUS SARCOMA VIRUS

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An efficient *in vitro* screening method for antitumor and/or antitumorigenic substances was established. The method is based on determining inhibitory effect of a compound on an RSV-induced tumorigenic process and the growth of the established tumor cells in a single assay system in the presence of normal cells. These effects were determined by inhibition of focus formation in a culture of chick embryo fibroblasts, while the nonspecific cytotoxic effect was determined by inhibition of the protein content of the same culture. The efficiency of this method in screening drugs was confirmed by testing various clinical and preclinical compounds.

Virus induced Rous sarcoma in chicken has been proposed as a model for experimental therapy of tumors¹⁾. The inhibitory effects of some antitumor agents on the oncogenic transformation *in vitro* of chick embryo fibroblasts (CEF) infected with Rous sarcoma virus (RSV)^{2,3)} have also been studied. In this connection, recent reports on some inhibitors of *src*-gene expression and on some regulators of tumor growth are interesting for their possible application in the above model systems in search of new antitumor agents^{4,5)}.

The purpose of the present report is to introduce our new screening method for determining the inhibitory effect of a compound on a tumorigenic process and/or on the growth of established tumor cells in a single assay system where appropriate normal cells are simultaneously present. This method can readily discriminate specific inhibitors from nonspecific, toxic substances by determining the inhibitory effect of a test compound on (1) focus formation on a plate containing CEF layer infected with RSV and on (2) total cell proteins of the same plate. The result (1) represents the effect on tumorigenic process as well as on tumor cell growth, and (2) the nonspecific toxic effect on normal cells because the population of virus-induced tumor cells is relatively small (see below). Although protein synthesis of transformed cells in foci must be faster than that of normal cells, the increase in the amount of protein due to these tumor cells was found to be negligible considering the overwhelming number of normal cells under our assay conditions. The usefulness of this assay system was tested by determining the effect of 13 clinically useful antitumor drugs. With each compound, the above described effects (1) and (2) were determined and an index (1)/(2), a kind of chemotherapeutic index (CTI) was calculated. Our experiment showed that there were big differences of CTI between the compounds tested. Correlation between our CTI and the clinical effectiveness of these antitumor drugs is discussed.

Materials and Methods

Virus and Chick Embryos

Rous sarcoma virus, Schmidt-Ruppin strain type A (SR-ASV-A), was kindly supplied by Dr. S. KAWAI, Institute of Medical Science, Tokyo University. Eggs of White Leghorn line S (leukemia, Mareck disease free) were obtained from Nippon Institute of Biological Science, Tokyo, and 9- to 10-day-old chick embryos were used.

Cells, Media and Culture Conditions

Eagle minimum essential medium (MEM) supplemented with 10% Tryptose phosphate broth (Difco), 5% calf serum, 0.5% chick serum and 60 mg/liter kanamycin was used for cell culture. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. Procedures for preparation of CEF and viral infection generally followed those of RUBIN^{6,7)} with the modification that the medium was renewed within 20 hours before cell harvest for secondary cultures. The secondary cultures of CEF were submitted to assay as follows. For an assay, 1 × 10⁸ CEF suspended in 4-ml culture medium were infected with 200 focus forming units of SR-ASV-A (no viral infection in control) and placed into a 60-mm Petri dish (Falcon). After 20 hours incubation, the culture fluid was removed and 5 ml of medium containing 0.75% (w/v) Bacto agar (Difco) was added. Thus, a cell sheet with an overlayer of gelled medium was formed. A test sample, dissolved in 2.0 ml of the medium was added on top of the gelled layer within 24 hours after the cell seeding and culture was reinitiated. A control run received 2.0 ml of the medium, otherwise the same as a test run.

Determination of Biological and Biochemical Effects

An assay unit consisted of 3 dishes. The number of foci and the amount of protein in a dish were determined (see below) at day 8 of cultivation and the results of 3 dishes were averaged. In order to determine the number of foci and the amount of cell protein in a dish, the cell sheet was first fixed by the method of SIMINOFF and REED⁸⁾ with minor modification; the top liquid medium was removed and the remainder including the cell sheet, together with gelled layer was fixed with 2 ml of 95% ethyl alcohol-glacial acetic acid (3:1) for 20~30 minutes. The fixative and the agar were then carefully removed; the fixed cell sheet on the dish was gently washed with sufficient amount of tap water and was dried at 37°C in an incubator. The number of foci was counted on a colony counter (Digital Colony Counter EKDS DC-3). Then, the same fixed cell sheet was submitted to determination of the amount of protein by the method of LOWRY⁹⁾ and OYAMA¹⁰⁾ with crystalline bovine serum albumin (BSA) as standard. Inhibitory effect of a test-substance on cell-protein synthesis is expressed by the following equation.

$$\left(1 - \frac{\text{the amount of protein of a test run}}{\text{the amount of protein of control run}}\right) \times 100$$

Results and Discussion

Increase in the Amount of Proteins of CEF, with or without Virus Infection, on Cultivation

Culture of a set of 18 dishes, each dish being seeded with 1 × 10⁸ CEF infected with 200 FFU of SR-ASV-A, was initiated (day 0). For comparison, a control run with uninfected CEF was performed in parallel. At days 1 to 9 of the culture, 2 dishes of each run were terminated; their protein contents were determined and averaged. With the virus-infected culture, foci became visible from day 4 on and increased in numbers as well as in size day by day: the number of foci per dish reached 215 on day 9. The protein content of these virus-infected cultures was not significantly different from that of uninfected cultures as illustrated in Fig. 1; both increased almost in parallel with cultivation time. Although a focus consists of densely populated transformed cells, the amount of protein due to about 200 foci did not make any significant difference in total amount of protein in a culture dish.

Inhibition of Focus Formation and Cell Growth by Various Agents

Several anticancer drugs in clinical use and some compounds whose biological activity have been reported were tested in our assay system. With each drug, the concentration required to reduce the number of foci to 50% of the no-drug control (effective dose 50) and the amount required to reduce the protein content to 50% of the no-drug control (toxic dose 50) were determined. Toxic dose 50/effective dose 50 (TD_{50}/ED_{50}) is referred to as chemotherapeutic index (CTI). The results are shown in Table 1. Vinblastin showed the highest and largest CTI among those tested. Among anthracycline antibiotics, 4'-*O*-tetrahydropyranlyadriamycin (THP-adriamycin) showed the largest CTI (15.9). Possible usefulness of the compound as indicated here is consistent with promising data from recent phase 2 studies. The large ED_{50} and TD_{50} values of cyclophosphamide suggest this compound has to be activated *in vivo* for its clinical activity. An active substance was found in the culture broth of *Bacillus laterosporus* MG-162aF2 by this assay and purified. The structure was determined and it was named spergualin¹¹⁾. Our assay method can be applied to crude materials, such as culture filtrates of microorganisms. Another merit of our assay system is that antitumor agents with high specificity to tumor cells can be distinguished from nonspecific toxic agents by a single test. A third merit is possible application to determine inhibitory effects of a test compound on virus infection and/or on viral tumorigenic process by adding the compound at an earlier stage of the assay. A fourth merit is its ease of manipulation,

Fig. 1. Growth rate of normal CEF and SR-ASV-A infected CEF.

The protein content of CEF, washed free from medium proteins, was determined by LOWRY'S method. Protein content of 10^8 CEF, counted under a microscope, was found to be equal to 0.958 mg of BSA.

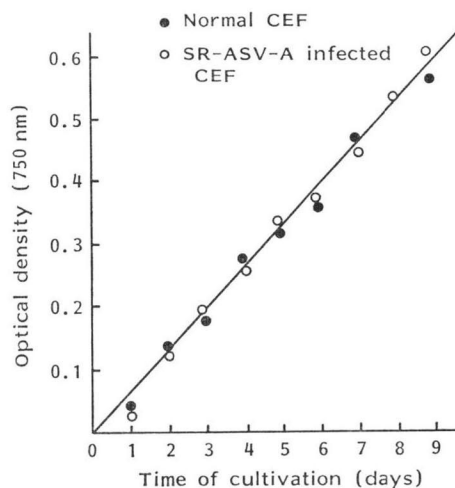


Table 1. Effect of anticancer agents on CEF-cell growth and transformation with SR-RSV-A.

Agent	Inhibition of focus formation ED_{50} ($\mu\text{g/ml}$)	Toxicity of CEF-cells TD_{50} ($\mu\text{g/ml}$)	CTI ^{a)} TD_{50}/ED_{50}
Nitrogen mustard <i>N</i> -oxide	2.9	10.2	3.5
Cyclophosphamide	550.0	1150.0	2.1
Triethylene thiophosphoramidate	2.4	9.0	3.8
Methotrexate	82.0	145.0	1.8
6-Mercaptopurine	16.0	34.0	2.1
5-Fluorouracil	1.3	2.2	1.7
Actinomycin D	0.0037	0.011	3.0
Mitomycin C	0.06	0.2	3.3
Daunorubicin	0.014	0.08	5.7
Adriamycin	0.0072	0.1	13.8
THP-Adriamycin	0.0066	0.105	15.9
Bleomycin	0.02	0.1	5.0
Vinblastin	0.001	0.066	66.0

a) CTI stands for chemotherapeutic index.

especially in determining protein content; since cells are adhering to the bottom of a dish, they can be washed free from proteins of the medium with buffer solution by decantation.

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