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Growth Characteristics of KB and RAJI Cells in the Liquid Culture Medium in Relation to the Human Tumor Stem Cell Assay for Anti-Tumor Agents

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ABSTRACT. Recently the chemosensitivity assay with clonal human tumor stem cells in liquid medium have been developed. We have investigated the optimal conditions for culture and counting of KB cells, which derived from a human epidermoid carcinoma and grow as a sheet adhered to the bottom surface of the culture, and RAJI cells, which derived from Burkitt lymphoma and grow as a suspension in liquid medium, as the representatives of the human tumor cells. Both stock cells were maintained in Eagle's MEM medium plus 10 per cent calf serum in CO_2 incubator at 37°C. Trypsinized cell suspensions were delivered in plastic wells with different culture media and incubated for different periods. Then the cell number was counted by the Coulter Counter in ISOTON II solvent. For KB cells, the culture medium should not be changed for 72 hours, otherwise enough cell number could not be obtained. To prevent the aggregation of KB cells in ISOTON II, calf serum (10%) was effective and EDTA (0.02%) had an additive effect. RAJI cells did not aggregate in ISOTON II. Ethanol and dimethylsulfoxide inhibited the growth of RAJI cells, but almost no inhibition was noted when the initial cell number was more than 8×10^4/ml. Ethanol was more toxic. Both solvents, however, did not affect the growth of KB cells.

Key words : KB cells — RAJI cells — cancer chemotherapy — *in vitro* assay — Coulter Counter

Hamberger *et al.*[1-3] and Salmon *et al.*[4,5] have shown that *in vitro* tissue culture method for cloning human tumor cell freshly biopsied samples appears to provide a simple yet quantitative approach to study the biology and chemosensitvity of neoplastic cells. Furthermore, this assay method can predict the clinical response of neoplastic tissue to the chemotherapeutic agents[6-9] and can be used as an *in vitro* phase II screening test for anti-cancer agents.[10,11] However, this clonogenic trial with agar gel medium includes some difficulties such as very low cloning efficiency, unsuccessfulness in culturing some tumor strains, laborious counting of colonies under a microscope and insolubility of some drugs in the agar media. These problems can be solved by the application of liquid media instead of the agar gel. Henceforward, the liquid medium method should be in current use because of its several advantages.[12] Therefore, we have studied the growth characteristics of two different types of tumor cells

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concerning the practical convenience. One of them is KB cell[13] which derived from a human epidermoid carcinoma and grows as a sheet adhered to the bottom surface of the culture bottle, and the other is RAJI cell[14] from Burkitt lymphoma which grows as a free suspension in the liquid medium. Items examined were the effects of the change of the culture media, the initial cell density and the solvents for drugs on the cell growth and the counting efficiency by Coulter Counter equipment[15] in ISOTON II solvent with different additives.

MATERIALS AND METHODS

Eagle's KB cell strain of human epidermoid carcinoma, kindly supplied by Dr. K. Hosokawa, Kawasaki Medical School, was maintained in Roux bottle containing 10 ml of Eagle's MEM medium plus 10 per cent calf serum (Grand Island Biological, U.S.A.) at 37°C in 5% CO_2 : 95% air. Stock bottle contained at the start approximately 6.0×10^4 cells in 10 ml of the medium, which was renewed every 72 hours. Maximum cell density in the stock bottle was kept below 6×10^6 cells/ml medium by periodic subculturing to maintain growing cells. To inoculate in Falcon 3008 Microwell plates (Falcon, U.S.A.) for an assay, the culture medium was decanted from the stock bottle, and 3.0 ml of 0.2% trypsin solution was added into the bottle. The cell clumps were broken by repeated pipetting after five minutes settlement. Then, it was diluted by Eagle's MEM medium plus 10 per cent calf serum until the appropriate cell number was obtained. Each one ml of cell suspension was delivered in a plastic well for the assay. After 72 hours the incubated cell suspension was transferred in 8.0 ml of ISOTON II for counting the KB cell number. 0.5 ml of 0.2% tripsin- Hank's Ca- and Mg-free buffered saline solution (BSS (-)) was added on the cell sheet to be scraped from the surface. The trypsinized cell suspension and the rinsed phosphate buffered saline (PBS) solution of 0.5 ml were combined together with the ISOTON II, then the cells were counted. To prevent the aggregation, EDTA (0.02%) was added in Hank's BSS (-) - trypsin (0.05%) solution.

Lymphoblast-like RAJI cells, kindly supplied by Dr. M. Namba, Kawasaki Medical School, were grown in RPMI 1640 medium supplemented with 10% calf serum in plastic dishes (10×40 mm) in the same condition as in the case of KB cell. 1.0×10^4 cells were planted into a stock dish with 5.0 ml of the culture medium, and an aliquot of the cell suspension was subcultured within every 48 hours. One ml of the cell suspension was inoculated in the plastic well after the stock solution was diluted to appropriate cell number.

The proliferation rate of KB cells and RAJI cells were measured by counting the cell number by means of Coulter Counter equipment (model X_{BI}, Coulter Electronics, Inc., U.S.A.) in ISOTON II (a diluting solvent attached to the counter). All data are the means of two experiments done in triplicate.

RESULTS AND DISCUSSION

The influence of the culture medium change on the KB cell growth is shown in Fig. 1. In the case of A, the initial medium was not renewed for three days. In case B, however, it was entirely replaced by the fresh medium

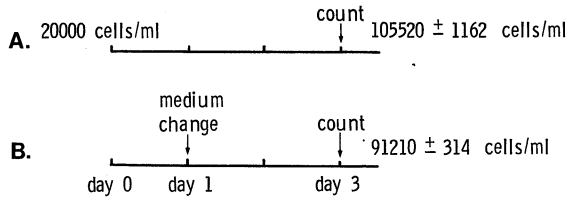

Fig. 1. Influence of the change of culture medium on KB cell growth.
KB cells were seeded in the plastic wells at the initial cell density of 2.0×10^4 cells/well in one ml of the culture medium.
A : Whole medium in the plastic well was renewed after 24 hours of seeding.
B : No medium was changed throughout the experiment.

on day 1 after seeding. The proliferation rate in the case B was 86% of that in the case A in spite of the presence of enough nutrients for the cell growth. KB cells require an incubation time before starting their growth, since the cells can grow only after adhering to the bottom surface. Therefore, to maintain KB cell growth at the constant level the condition in the culture bottle should not be perturbed by changing the culture media. It is futile to change the culture medium for the cloned stem cells of KB type within three days.

On the contrary, RAJI cells grow as the suspension in the medium. The growing cells assemble on the bottom in the well, but not adhere to its surface. Therefore, the repetitive pipetting just before the inoculation seems to be important to get the uniform cell suspension. However, the repeated pipetting retarded the cell growth when the cell number was low (Fig. 2), but did not when the cell number was over 4×10^4/ml. The growth rate of RAJI cell was rather dependent on the initial cell density in the range examined. Therefore, it seems that for the drug sensitivity test the suitable cell density of suspension-type cells is more than 4.0×10^4 cells/ml culture medium.

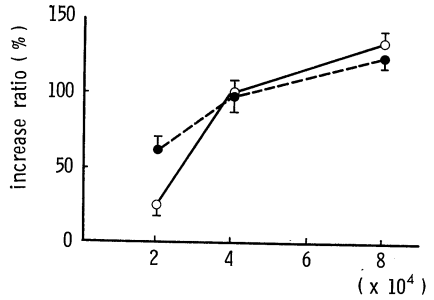

Fig. 2. The influence of the initial cell density and/or the repeated pipetting on RAJI cell growth.
RAJI cells were mixed well by the repeated pipetting (—○—) or not (—●—) and seeded in plastic wells at the densities of 2.0×10^4, 4.0×10^4 or 8.0×10^4 cells/ml in the culture media. The cells were counted after 48 hours. Vertical bars indicate S. E. of the mean.

Water-soluble test compounds were dissolved in PBS solution of which five to 100 μl was added to one ml of the cell suspension. On the other hand, water-insoluble compounds were generally dissolved in ethanol or

dimethylsulfoxide because these solvents were proved to have little effect on the cell growth of many strains.[2] However, both ethanol and dimethylsulfoxide showed the cytotoxicity not on KB cells but on RAJI cells as shown in Fig. 3. RAJI cell growth was completely inhibited by the addition of 10 μl ethanol or dimethylsulfoxide when the cell number was 2.0×10^4 cells/ml. The grade of this inhibition decreased gradually with increasing cell density. Ethanol was more toxic than dimethylsufoxide to RAJI cells. The selection of a solvent for the water-insoluble compounds is an important factor for the drug sensitivity test.

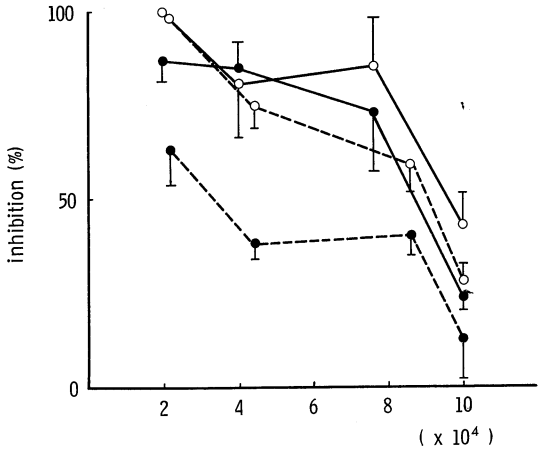

Fig. 3. Cytotoxic effect of ethanol and dimethylsulfoxide on RAJI cell growth. RAJI cells were seeded in plastic wells at the densities of 2.0×10^4, 4.0×10^4, 7.6×10^4 and 1.0×10^5 cells/well in one ml of the culture medium containing 10 μl (—○—) or 5 μl (—●—) of ethanol, and of 2.2×10^4, 4.4×10^4, 8.6×10^4 and 1.0×10^5 cells/well in one ml of medium containing 10 μl (---○---) or 5 μl (---●---) of dimethylsulfoxide. The cells were counted after 48 hours incubation.

$$\text{Inhibition} = 100\ \frac{\log\frac{D}{I}}{\log\frac{C}{I}} \times 100$$

I : Initial cell number, C : Control, D : Exposed cell number to ethanol or dimethylsulfoxide.

Although the liquid medium method is more convenient than the agar method, the measurement of the cell protein concentration[16] or counting the cell number under the microscope[17] is troublesome and time-consuming, and it is inaccurate because of the denaturation of the cell protein or an aggregation of the cells, respectively. These barriers have recently been removed by a development of Coulter Counter equipment, in which a special solvent, ISOTON II, is used as a diluent of the cell suspension. We examined the effect of ISOTON II on the aggregation which diminishes the counting accuracy. The time course study on the initial cell density and the cell count is shown in Fig. 4. By a Coulter Counter many samples can not be measured altogether at one time. The greater the sample number is, the more time it takes, and during the measurement the cells might aggregate in the counting solution. RAJI cells,

however, did not show any aggregation under the examined condition (Fig. 4, A). On the other hand, KB cells (Fig. 4, B) showed aggregation after eight hours in the calf serum-free ISOTON II solution containing 1.0 ml of 0.2% trypsin. The aggregation grade of the trypsin-treated KB cells could be reduced by adding the calf serum as much as 10% in ISOTON II. But even in such a solution, the aggregation occurred when the cell suspension of high density

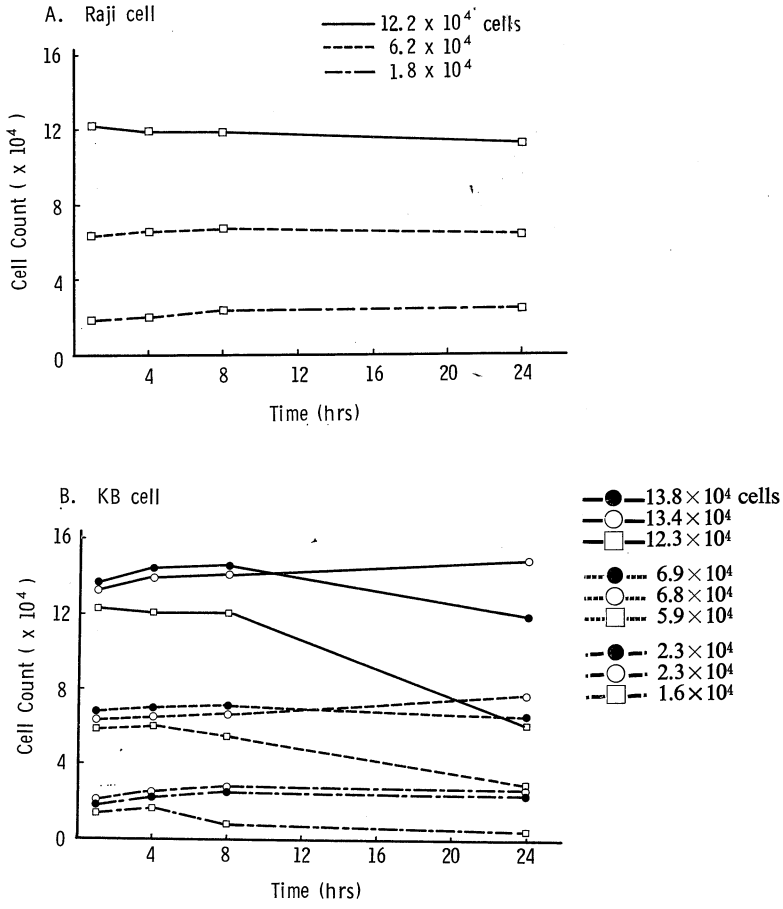

Fig. 4. The relation of the cell density and the counts of RAJI cells and KB cells in ISOTON II with or without calf serum. The decrease of the counts is attributed to the aggregation. S.E. of mean in each point was at most ten per cent. Initial cell counts are shown in the figures.

A) RAJI cells were suspended in 9.0 ml of ISOTON II and 1.0 ml of the culture medium containing 10% calf serum. There were no marked decrease in cell count during 24 hours.

B) KB cells were suspended in the following solution : ① 9.0 ml of ISOTON II and 1 ml of 0.2% trypsin solution without calf serum (□). Cell count showed marked decrease. ② 8.0 ml of ISOTON II, 0.5 ml of 0.2% trypsin solution, 0.5 ml of PBS and 1.0 ml of the culture medium containing 10% calf serum (●). Cell count showed slight decrease. ③ 8.0 ml of ISOTON II, 0.5 ml Hank's solution containing EDTA (0.02%) and 0.05 % trypsin and 1.5 ml of the culture medium containing 10% calf serum (○). Cell counts were not decreased at all.

was allowed to stand for a long period. We can obtain a constant result if KB cells are counted within eight hours at the density of $1.0-1.5 \times 10^5$ cells in 10 ml ISOTON II containing 10% of calf serum. Even after more than eight hours, 0.02% EDTA with 0.05% trypsin added in ISOTON II containing 10% calf serum could prevent the aggregation completely.

The tumor cells which grow as a free suspension such as RAJI cells can be more easily employed for an anti-cancer screening assay in the liquid medium. On the other hand, the tumor cells which grow as a sheet adhered to the bottom surface such as KB cells have more difficulties for this purpose. However, if the conditions of culure and counting are settled, such kind of the tumor cells, too, can be used for chemosensitivity assay in the liquid medium satisfactorily.

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