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Effect of 3'-methyl-N,N-dimethyl-4-aminoazobenzene in the presence or absence of liver microsomes on the liver cells having low tumor-producing capacity in culture.

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Abstract

A cell strain having low tumor-producing capacity was exposed in culture to 3'-methyl-N,N-dimethyl-4-aminoazobenzene (3'-Me-DAB) in the presence or absence of liver microsomes, and whether or not the cells will progress to those having high tumor-producing capacity was examined. When transplanted into rats, the cells treated with 3'-Me-DAB four (Exp-I) or thirteen times (Exp-II) formed larger tumors than untreated control cells, the latter treatment being more efficient in this regard. Furthermore, the tumors formed by the cells treated with 3'-Me-DAB in the presence of liver microsomes were considerably larger than those formed by the cells treated with 3'-Me-DAB alone. The subcutaneous tumors produced by the cells treated with 3'-Me-DAB with S-15 Mix showed poorly differentiated histology compared with those produced by control and 3'-Me-DAB-treated cells. The frequency of lung metastasis tended to increase by 3'-Me-DAB with S-15 Mix. The cells treated with 3'-Me-DAB in the presence or absence of liver microsomes differed from untreated control cells in vitro in some properties, including the size of aggregates in rotation culture, plating efficiency in liquid medium and morphology. These observations suggest that cell malignancy was promoted by 3'-Me-DAB alone as well as by 3'-Me-DAB in the presence of liver microsomes.

KEYWORDS: liver cells, low tumor-producing capacity, 3'-Me-DAB, microsomes, in vitro carcinogenesis

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EFFECT OF 3'-METHYL-N,N-DIMETHYL-4-AMINOAZOBENZENE IN THE PRESENCE OR ABSENCE OF LIVER MICROSOMES ON THE LIVER CELLS HAVING LOW TUMOR-PRODUCING CAPACITY IN CULTURE

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Abstract. A cell strain having low tumor-producing capacity was exposed in culture to 3'-methyl-N,N-dimethyl-4-aminoazobenzene (3'-Me-DAB) in the presence or absence of liver microsomes, and whether or not the cells will progress to those having high tumor-producing capacity was examined. When transplanted into rats, the cells treated with 3'-Me-DAB four (Exp-I) or thirteen times (Exp-II) formed larger tumors than untreated control cells, the latter treatment being more efficient in this regard. Furthermore, the tumors formed by the cells treated with 3'-Me-DAB in the presence of liver microsomes were considerably larger than those formed by the cells treated with 3'-Me-DAB alone. The subcutaneous tumors produced by the cells treated with 3'-Me-DAB with S-15 Mix showed poorly differentiated histology compared with those produced by control and 3'-Me-DAB-treated cells. The frequency of lung metastasis tended to increase by 3'-Me-DAB with S-15 Mix. The cells treated with 3'-Me-DAB in the presence or absence of liver microsomes differed from untreated control cells *in vitro* in some properties, including the size of aggregates in rotation culture, plating efficiency in liquid medium and morphology. These observations suggest that cell malignancy was promoted by 3'-Me-DAB alone as well as by 3'-Me-DAB in the presence of liver microsomes.

Key words : liver cells, low tumor-producing capacity, 3'-Me-DAB, microsomes, *in vitro* carcinogenesis.

A large number of studies have been made on the transformation of normal rat liver cells in culture by N,N-dimethyl-4-aminoazobenzene (DAB) and 3'-methyl-N,N-dimethyl-4-aminoazobenzene (3'-Me-DAB) (1-8). Sato and coworkers suggested that malignancy of the cultured liver cells was promoted by DAB and 3'-Me-DAB in culture (3, 5, 9). In these experiments, a long-term exposure of the cells to DAB and 3'-Me-DAB was required before obtaining the final results. It was then suggested that the cells used could not carry out the metabolic action necessary for the activation of aminoazo dyes.

In the present experiment, 1) a cell strain having low tumor-producing capacity was employed and promotion of cell malignancy by 3'-Me-DAB was attempted, and 2) the cells were briefly exposed to 3'-Me-DAB in the presence

or absence of liver microsomes, and whether or not prolonged treatment of the cells with 3'-Me-DAB was necessary for a promotion of cell malignancy was examined.

MATERIALS AND METHODS

Cell culture. CL20 cells were obtained from a single cell cloning of dRLa-74 (9, 10), which was derived from the liver of a DAB-fed rat. The CL20 as well as dRLa-74 cells had epithelial-like morphology *in vitro* and low tumor-producing capacity. The transplantability of CL20 cells was stable throughout the serial cultivations. Culture medium was Eagle's minimal essential medium supplemented with 20% heat-inactivated bovine serum. These cells were subcultured once a week with 0.2% trypsin containing 1.3 mM EDTA at a concentration of 1×10^5 cells/ml. Culture was grown in a Falcon plastic dish containing 3 ml medium and incubated at 37 °C in a water-saturated atmosphere of 5% CO_2.

Chemicals. 3'-methyl-N,N-dimethyl-4-aminoazobenzene (3'-Me-DAB; Tokyo Kasei Co., Tokyo) was dissolved in dimethylsulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO) and diluted with culture medium before use. All other products were of the purest grades.

Preparation of microsome fractions. S-15 Mix was prepared as described by Kuroki and others with a slight modification (11-13). Briefly, adult male Donryu rats were given 1 mg/ml of sodium phenobarbital (Maruko Chemical Co., Nagoya) in drinking water for seven days, and were injected with 40 mg/kg of 3-methyl-cholanthrene (Wako Chemical Co., Tokyo) intraperitoneally 48 h prior to sacrifice. Liver homogenate was then successively centrifuged at 9,000 g for 10 min and 15,000 g for 20 min at 0-4 °C. The resulting supernatant was tentatively designated as "S-15 fraction". S-15 Mix consisted of 0.4 ml S-15 fraction and 1.6 ml Earle's balanced salt solution containing 16 μmol $MgCl_2$, 2 or 4 μmol NADPH and 2 μmol ATP. The effectiveness of S-15 Mix was confirmed with a chromosome aberration test using normal rat liver cells, *i.e.*, Ac2F cells (14). 3'-Me-DAB alone with 15.8 and 47.7 μg/ml induced only 1% chromosomal aberration, whereas 3'-Me-DAB with S-15 Mix 9-14% chromosomal aberration.

Carcinogen treatment. Concerning the treatment of CL20 cells with 3'-Me-DAB in the presence or absence of S-15 Mix, the cells were treated four times, once a week, in Exp-I and thirteen times, also once a week, in Exp-II; the time required for each treatment was 5 h. The cells treated with 1.5 and 34.9 μg/ml of 3'-Me-DAB were designated as CL20-DI and CL20-DII, respectively, and those treated with 1.5 and 34.9 μg/ml of 3'-Me-DAB in the presence of S-15 Mix as CL20-DIS and CL20-DIIS, respectively. Control cells treated with 1% DMSO and S-15 Mix were labeled as CL20-C and CL20-S, respectively.

Tumorigenicity test. Harvested cells were suspended in growth medium and inoculated subcutaneously into syngeneic rats less than 24 h after birth at 1×10^6 cells/rat. The tumor size in each rat was measured at the 60th day after cell inoculation. At the 90th day after inoculation, rats were autopsied and their organs were examined grossly and histologically.

Rotation culture. Rotation culture was performed according to Moscona (15) with a slight modification. Cells in monolayer culture were dispersed with 0.2% trypsin containing 1.3 mM EDTA and filtrated through a piece of double-fold lens paper. Cell suspensions containing 3×10^5 cells in 1.5 ml culture medium were inoculated into a 20 ml Erlenmyer flask and rotated on a gyratory shaker (New Brunswick Scientific Co., NJ) at 70 rpm. After 24 h of rotation culture, aggregability was compared using microphotographs according to

the mean diameter of aggregates.

Growth property test. For the estimation of cell proliferation, inocula of 1×10^5 cells in one ml medium were seeded and placed in a 35 mm plastic dish. Population doubling time was calculated from the cells in the logarithmic growth phase. Saturation density was determined from the maximum of the growth curves. After seeding 200 cells in triplicate plates, plating efficiency was determined 10 days later by fixing, staining and counting the resulting colonies.

RESULTS

Cytotoxicity. Cytotoxicity and optimal concentration of 3'-Me-DAB with or without S-15 Mix were examined on CL20 cells (Fig. 1). No difference, however, was observed between the cytotoxicity induced by 3'-Me-DAB alone and that by 3'-Me-DAB with S-15 Mix. Subsequently, two different concentrations, *i.e.*, 1.5 and 34.9 μg/ml, of 3'-Me-DAB were employed.

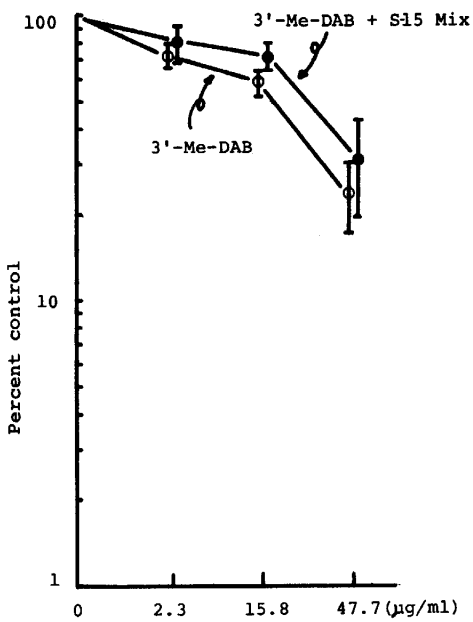

Fig. 1. The effect of 3'-Me-DAB and 3'-Me-DAB plus S-15 Mix on the proliferation of CL20 cells. Bar: mean ± SD. Cells were exposed to the chemicals for 5 h.

Back-transplantation test. In Exp-I, the cells were inoculated subcutaneously into rats at the 11th day after the last treatment. As shown in Fig. 2, the tumor sizes produced in rats at the 60th day after inoculation were compared between control and 3'-Me-DAB-treated cells. Larger tumors were obtained from the CL20-DII and CL20-DIIS cells than from the cells of CL20-C and CL20-DI, or CL20-S and CL20-DIS. In this experiment, however, no difference was observed between the cells treated with 3'-Me-DAB alone and those treated with 3'-Me-DAB in the presence of S-15 Mix.

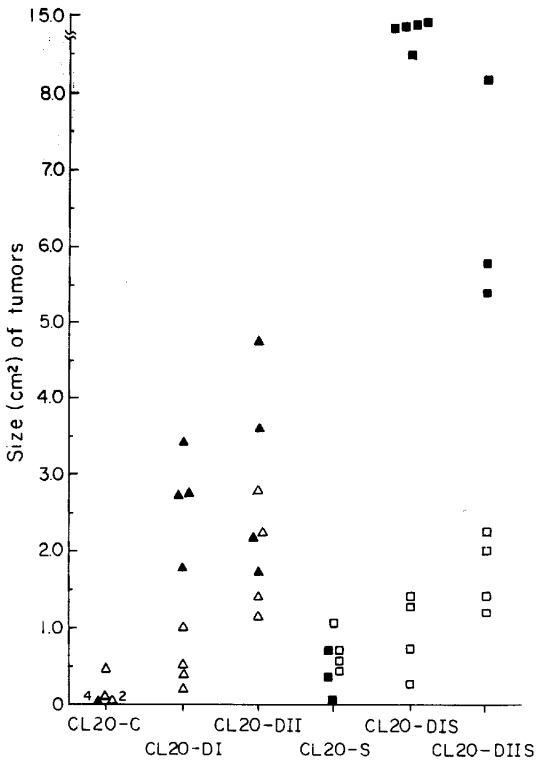

Fig. 2. The tumor size in rats at the 60th day after a back-transplantation of CL20 cells treated with or without 3'-Me-DAB in the presence or absence of liver microsomes. △, □ represent Exp-I, and ▲, ■ Exp-II. △ and ▲: in the absence of liver microsomes, whereas □ and ■: in the presence of the liver microsomes. Numerals, (2) and (4), in CL20-C indicate the number of rats examined in Exp-I and Exp-II, respectively.

In Exp-II, the cells were inoculated subcutaneously into rats at the 11th day after the last treatment and the tumor sizes were compared. 3'-Me-DAB-treated cells, *i.e.*, CL20-DI and CL20-DII, formed larger tumors than control, *i.e.*, CL20-C. Tumors in CL20-DII cells were slightly larger than those in CL20-DI cells. On the other hand, the cells treated with 3'-Me-DAB in the presence of S-15 Mix, *i.e.*, CL20-DIS and CL20-DIIS, formed much larger tumors than CL20-DI, CL20-DII and CL20-S as a control (Fig. 2). Among these cells, CL20-DIS cells formed the largest tumors.

All the subcutaneous tumors originally produced showed epithelial differentiation of varying degrees. Control and 3'-Me-DAB-treated cells produced a moderately to poorly differentiated carcinoma, while the cells treated with 3'-Me-DAB in the presence of S-15 Mix produced tumors with more advanced

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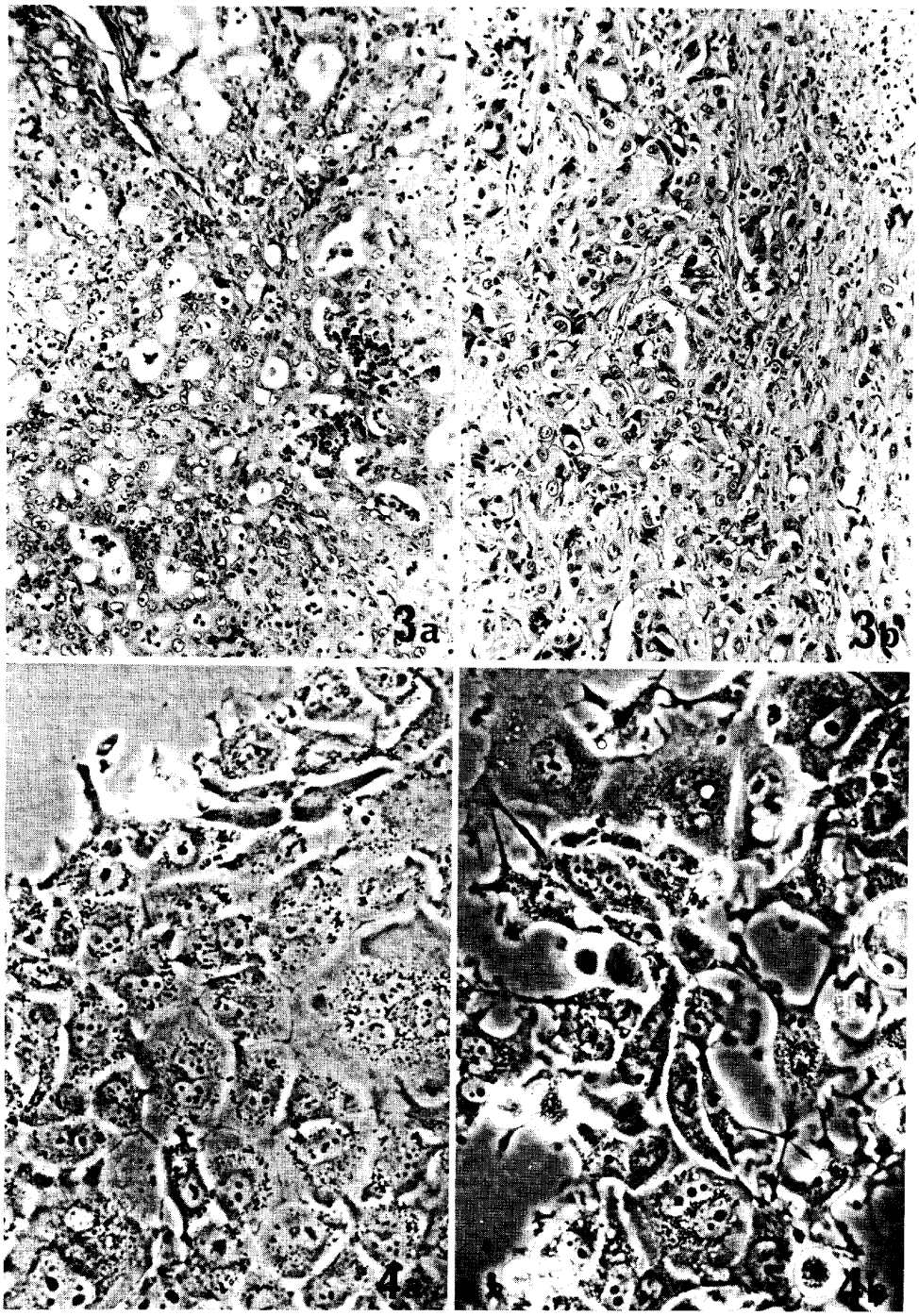
Fig. 3a. Histology of tumors produced by untreated control cells (CL20-C). Hematoxylin-eosin, × 100. 3b. Histology of tumors produced by the cells treated with 3'-Me-DAB in the presence of liver microsomes (CL20-DIIS). Hematoxylin-eosin, × 100.

Fig. 4a. Untreated control cells (CL20-C). Phase contrast microscopy, × 200. 4b. The cells treated with 3'-Me-DAB in the presence of liver microsomes (CL20-DIS). Phase contrast microscopy, × 200.


3a
3b
4a
4b

anaplasia (Figs. 3a and 3b).

Results concerning the incidence of metastatic growth of CL20 cells treated with and without 3'-Me-DAB are summarized in Table 1. Metastasis was found exclusively in the lung among various organs examined. The frequency of metastasis tended to increase by the cells treated with 3'-Me-DAB in the presence of S-15 Mix in Exp-II.

TABLE 1. INCIDENCE OF LUNG METASTASIS FOLLOWING THE INJECTION OF CL20 CELLS TREATED WITH OR WITHOUT 3'-ME-DAB IN THE PRESENCE OR ABSENCE OF LIVER MICROSOMES

Cells	No. of rats tested	No. of rats with metastasis			
		Macronodule[a]	Micronodule[b]	Total	
Exp-I					
CL20-C	3	1	0	1	1/3
CL20-DI	4	0	0	0	2/8*
CL20-DII	4	1	1	2	
CL20-S	4	0	0	0	0/4
CL20-DIS	4	0	3	3	3/8*
CL20-DIIS	4	0	0	0	
Exp-II					
CL20-C	4	0	0	0	0/4
CL20-DI	4	0	1	1	2/8*
CL20-DII	4	1	0	1	
CL20-S	3	1	0	1	1/3
CL20-DIS	1	-	1	1	4/4
CL20-DIIS	3	1	2	3	

a More than 2 mm in diameter; *b* Less than 2 mm in diameter.

* Significantly different from the group, consisting of CL-20DIS and CL20-DIIS, in Exp-II ($p < 0.01$).

TABLE 2. THE SIZE OF AGGREGATES OF CL20 CELLS TREATED WITH OR WITHOUT 3'-ME-DAB IN THE PRESENCE OR ABSENCE OF LIVER MICROSOMES

Cells	Diameter (mm) (Mean ± SD)	
	Exp-I	Exp-II
CL20-C	0.15 ± 0.03 (23)	0.25 ± 0.03 (6)
CL20-DI	0.28 ± 0.06 (7)*	0.35 ± 0.10 (6)*
CL20-DII	0.33 ± 0.03 (6)*	0.44 ± 0.08 (5)*
CL20-S	0.19 ± 0.03 (15)	0.43 ± 0.03 (6)
CL20-DIS	0.28 ± 0.04 (7)*	0.56 ± 0.03 (6)*
CL20-DIIS	0.35 ± 0.02 (6)*	0.60 ± 0.07 (6)*

* Significantly different from CL20-C or CL20-S ($p < 0.05$).

No. in parentheses: No. of aggregates examined.

Size of aggregates in rotation culture. We have suggested that aggregability of rat liver epithelial-like cells in rotation culture was suitable for determining malignant transformation of cells. As shown in Exp-I and Exp-II of Table 2, 3'-Me-DAB-treated cells with or without S-15 Mix formed larger aggregates than control, *i.e.*, CL20-C and CL20-S. No significant difference, however, was observed between CL20-DI and CL20-DII, between CL20-DIS and CL20-DIIS, between the cells treated with 3'-Me-DAB and those treated with 3'-Me-DAB in the presence of S-15 Mix, and between Exp-I and Exp-II.

Some other properties. The change in some other properties after carcinogen treatment (Exp-II) was examined (Table 3). Except for in plating efficiency,

TABLE 3. GROWTH PROPERTIES OF CL20 CELLS TREATED WITH OR WITHOUT 3'-Me-DAB IN THE PRESENCE OR ABSENCE OF LIVER MICROSOMES (EXP-II)

Cells	Population doubling time (h)	Plating efficiency (%) (Mean ± SD)	Saturation density ($\times 10^4/cm^2$) (Mean ± SD)
CL20-C	17.4	12.2 ± 1.6	26.1 ± 3.9
CL20-DI	22.2	Not examined	39.4 ± 6.7
CL20-DII	21.6	55.5 ± 4.5*	31.1 ± 3.2
CL20-S	25.4	8.2 ± 1.0	28.6 ± 3.7
CL20-DIS	18.5	25.0 ± 1.7*	32.8 ± 3.1
CL20-DIIS	23.3	34.5 ± 5.3*	24.2 ± 3.1

* Significantly different from CL20-C or CL20-S ($p < 0.05$).

no significant difference in population doubling time and saturation density was found between control and the cells which were treated with 3'-Me-DAB with or without S-15 Mix. On the other hand, the cells treated with 3'-Me-DAB in the presence of S-15 Mix were morphologically different from the control. Especially, increased pleomorphism and atypism of nucleus and cytoplasm were observed in CL20-DIS (Figs. 4a and 4b).

DISCUSSION

According to a back-transplantation test, increasing trend of tumor size, altered histology from moderately differentiated to anaplastic type, and enhanced metastatic tendency were found in the treated cells compared with untreated control. The results have confirmed that cell malignancy was more promoted by 3'-Me-DAB in the presence of S-15 Mix than by 3'-Me-DAB alone.

Sato (5) described an increase in malignancy by the continuous treatment of rat liver cells with 3'-Me-DAB for more than 100 days in culture. Tokiwa

and Sato (9) arrived at a similar conclusion to the above experiment using dRLa-74. In the present experiment using an *in vivo* back-transplantation test, the increase in malignancy was possible by brief treatments of the cells, *i.e.*, pulse administration of 3'-Me-DAB four or thirteen times for 5 h each time, instead of 100 consecutive days. Such an effect of the brief treatments on the cells was also found in a few markers *in vitro* as discussed below. These results suggest, among several possibilities, a possibility that CL20 cells have a certain amount of aminoazo dye-activating enzymes. If we accept the above possibility, the time for exposure of the cells to 3'-Me-DAB for obtaining an increase in cell malignancy can be much shortened whenever any cells having a potent procarcinogen-activating enzyme *in vitro* are employed. Biochemical study concerning this aspect is under progress using the cultured cells.

Sixty days after back-transplantation of CL20 cells (Fig. 2), four pulse treatments (Exp-I) showed a dose response to 3'-Me-DAB concentration, irrespective of S-15 Mix, whereas the tumor size did not change significantly. In contrast, thirteen pulse treatments (Exp-II) demonstrated larger tumors in the group with the presence of S-15 Mix than those without S-15 Mix. The incidence of lung metastasis following injection of only the CL20 cells with the presence of S-15 Mix in Exp-II (Table 1) was significantly higher than other groups. Based on the above facts, the frequency of pulse-treatments with 3'-Me-DAB to CL20 cells may accelerate the potency of cell malignancy, and the presence of S-15 Mix in culture medium may further enhance the acceleration of malignant potential of the cells.

In order to determine useful parameters for malignant transformation of epithelial cells *in vitro*, various attempts have been made but none of them has been successful. A few investigators (16-21), however, support the possible presence of the markers for malignant transformation of cultured cells including aggregate-forming capacity. In the present experiments, three markers, namely, aggregate-forming capacity, plating efficiency in liquid medium and cell morphology, were well correlated with the results of *in vivo* experiments. These markers will be useful in obtaining the information regarding the promotion of cell malignancy.

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