Acta Medica Okayama

Volume 25, *Issue* 1 1971 *Article* 6

FEBRUARY 1971

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Eiji Chikata*

*Okayama University,

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Abstract

For the purpose of elucidating more exact relationship between the process of carcinogenesis and aggregate-forming ability, we performed rotation cultures of a series of five liver cell lines derived from rats fed DAB for various period of d:l ys. As a result we found a tendency of the cells obtained from rats fed DAB for a longer period to form larger aggregates. The differences of the aggregate.forming ability among these cell lines were demonstrated well within one day, and more prominently after three days in rotaion culture. Histologically, the aggregates of all cell lines were composed of cuboidal epithelial cells, especially in some cell lines showing gland-like structures.

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*PMID: 4258665 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **25**, 57—64 (1971)

AGGREGATE-FORMING ABILITY OF LIVER CELL LINES DERIVED FROM DAB-FED RATS IN ROTATION CULTURE

Eiji CHIKATA

Division of Pathology, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. J. Sato)

Received for publication, December 14, 1970

Cytological studies of a series of liver cell lines from DAB-fed rats have been made as one of methods for clarifying DAB-carcinogenesis (1, 2). The colony analysis of the cell lines revealed that these cell lines showed accelerated growth potentials and morphological changes in proportion to the duration of DAB-feeding (2). Other methods might demonstrate the differences in the character of the cell lines especially concerning the process of carcinogenesis.

KURODA reported that malignant cells form larger aggregates than normal cells in rotation culture (3, 6). In the present experiment the aggregate-forming ability (aggregability) of the cell lines derived from DAB-fed rats were studied.

MATERIALS AND METHODS

Cells: A series of liver cell lines (dRLN-53, dRLN-60, dRLN-61, dRLa-74 and dRLh-84) obtained from the rats fed DAB for various periods of days served for the study (1, 2). These cell lines were cultured in LD medium with 20 per cent bovine serum. They were subcultured using 0.2 per cent trypsin in phosphate buffered saline. These cell lines in monolayer culture are shown in Photos 8a to 12a.

Rotation Culture: Following the principles of the procedures described by MOSCONA (4) and KURODA (3), the cells in monolayer culture were dispersed by 0.2 per cent trypsin solution and filtrated through twice folded lens paper. After the centrifugation at 800 rpm for 5 min., the cells were resuspended in Eagle's MEM with 20 per cent heat inactivated bovine serum, and 3×10^5 cells/3ml. were inoculated into 25-ml Erlenmayer flasks. In order to prevent the attachment of the cells to the glass, flasks were coated with silicone. The flasks were placed on a gyrotory shaker (New Brunswick Scientific Company, New Jersey, U. S. A.) rotated at 70 rpm at 37°C for three days and in some experiments eight days. The aggregability was compared by the mean diameter of 20 aggregates, which were measured on microphotographs.

Histological Studies on Aggregates: The aggregates were centrifuged at 1000 or 1500 rpm for 10 min. The supernatant being decanted, the aggregates were fixed

with Carnoy's fixative for at least 30 min. Then the samples were dehydrated with ethanol, packed with celloidin, and embedded into paraffin. The sections thus prepared were stained with hematoxylin and eosin.

RESULTS

1) *Comparison of the Aggregability*: The size of aggregates of each cell line was compared in Fig. 1. Fig. 2 shows the growth of aggregates of each cell line during the rotation culture for three days. It was revealed that the cell lines from rats fed DAB for a longer period of days produced larger aggregates with an exception of the cell line dRLa-74.

Though the differences in the aggregability of these cell lines were well demonstrable after 24 hours in rotation culture, distinct differences

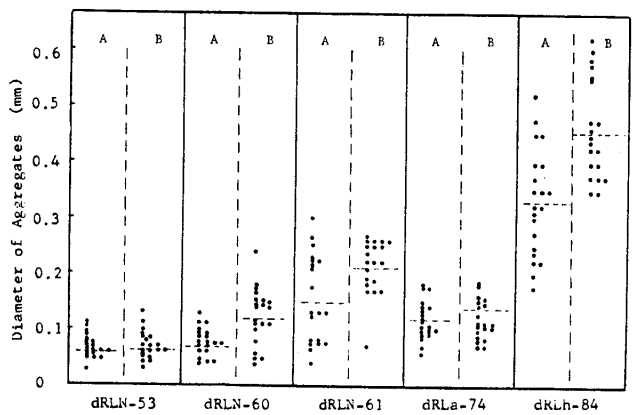
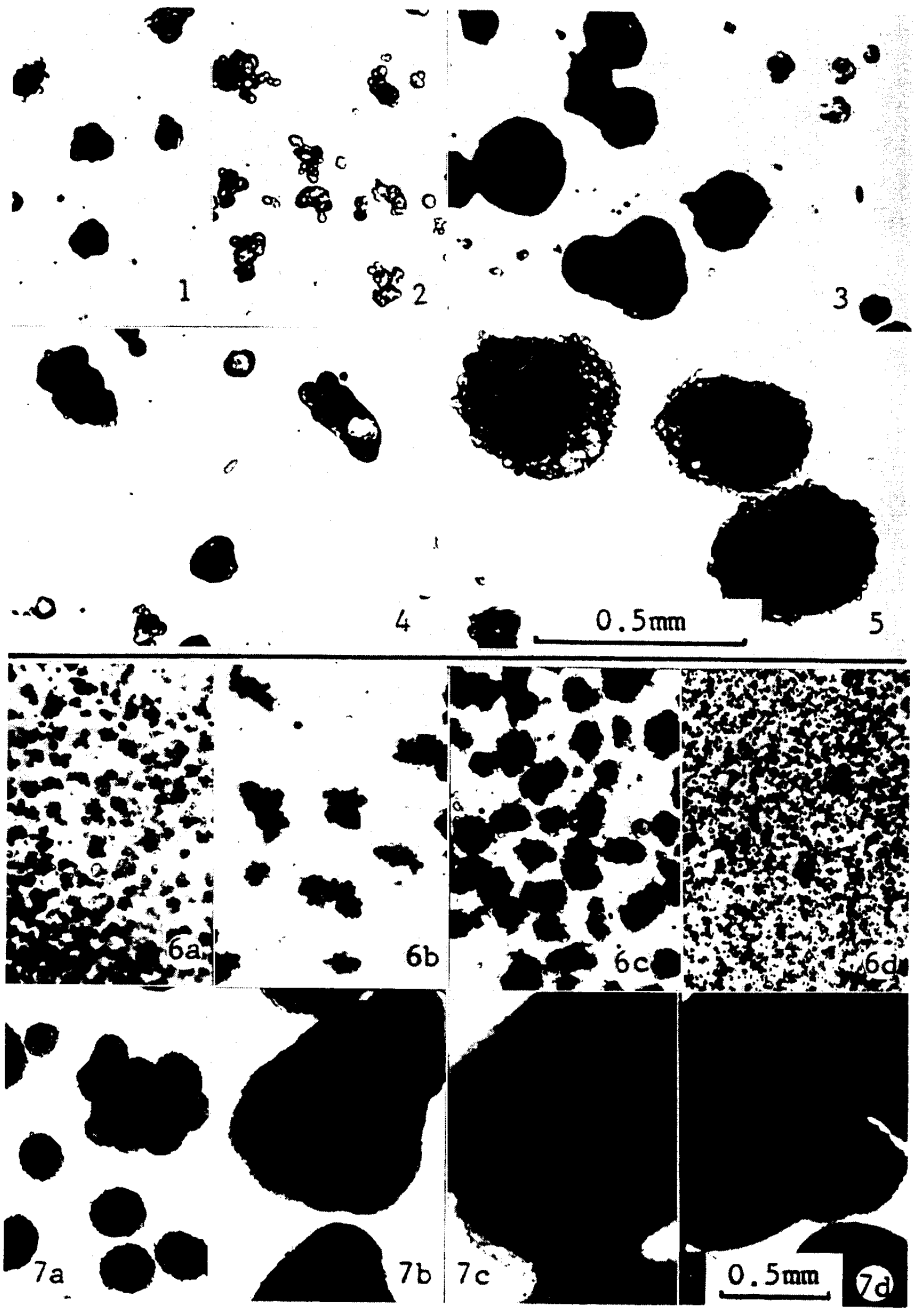


Fig. 1 Comparison of the aggragability of liver cell lines from DAB-fed rats in rotation culture. Cells dispersed with 0.2 per cent trypsin were inoculated into 25-ml Erlenmayer flasks (3×10^6 cells/3 ml Eagle's MEM+20% BS). They were placed on a gyratory shaker rotated at 37°C for 3 days. Twenty aggregates of each sample were measured for their diameter on microphotographs. Each point represents the diameter of one aggregate. Horizontal dotted lines show the mean diameter of 20 aggregates. A: 24 hrs., B: 72 hrs.

Photo 1 Aggregates of the cell line dRLN-53 after 24 hrs in rotation culture. ×40.
Photo 2 Aggregates of the cell line dRLN-60 after 24 hrs in rotation culture. ×40.
Photo 3 Aggregates of the cell line dRLN-61 after 24 hrs in rotation culture. ×40.
Photo 4 Aggregates of the cell line dRLa-74 after 24 hrs in rotation culture. ×40.
Photo 5 Aggregates of the cell line dRLh-84 after 24 hrs in rotation culture. ×40.
Photos 6a-d Aggregates of the cell line dRLN-60 after (6a) one, (6b) 3, (6c) 5, and (6d) 8 days in rotation culture. ×40.
Photos 7a-d Aggregates of the cell line dRLh-84 after (7a) one, (7b) 3, (7c) 5, and (7d) 8 days in rotation culture. ×40.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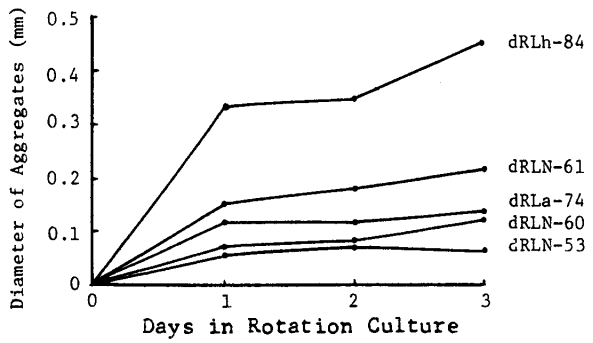

Fig. 2 Growth of the aggregates of liver cells from DAB-fed rate in rotation culture for 3 days. Culture conditions were the same as explained in Fig. 1. The size of aggragates of each sample is the mean diameter of 20 aggregates.

were elicited on the third day of the culture, since the growth rate of the aggregates was higher in the liver cell lines from rats fed DAB for a longer period. The size and shape of the aggregates of each cell line are shown in Photos 1 to 5.

2) *Histological Findings of Aggregates*: The aggregates of each cell line obtained on the third day of rotation culture were subjected to the microscopic observation after hematoxylin-eosin staining (Photos 8b to 12b). In general the aggregates of the cell lines used consisted of cuboid epithelial cells.

In the cell lines dRLN-53 and dRLN-60, several cells associated loosely to form small gland-like structure (Photos 8b and 9b).

The aggregates of the cell line dRLN-61 were composed of densely packed cells with no necrotic centers (Photo 10b).

In the cell line dRLa-74, some aggregates showed characteristic gland-like appearance (Photo 11b).

In the cell line dRLh-84, the fingings of aggregated were similar to those of the cell line dRLN-61 with some exceptions that the former

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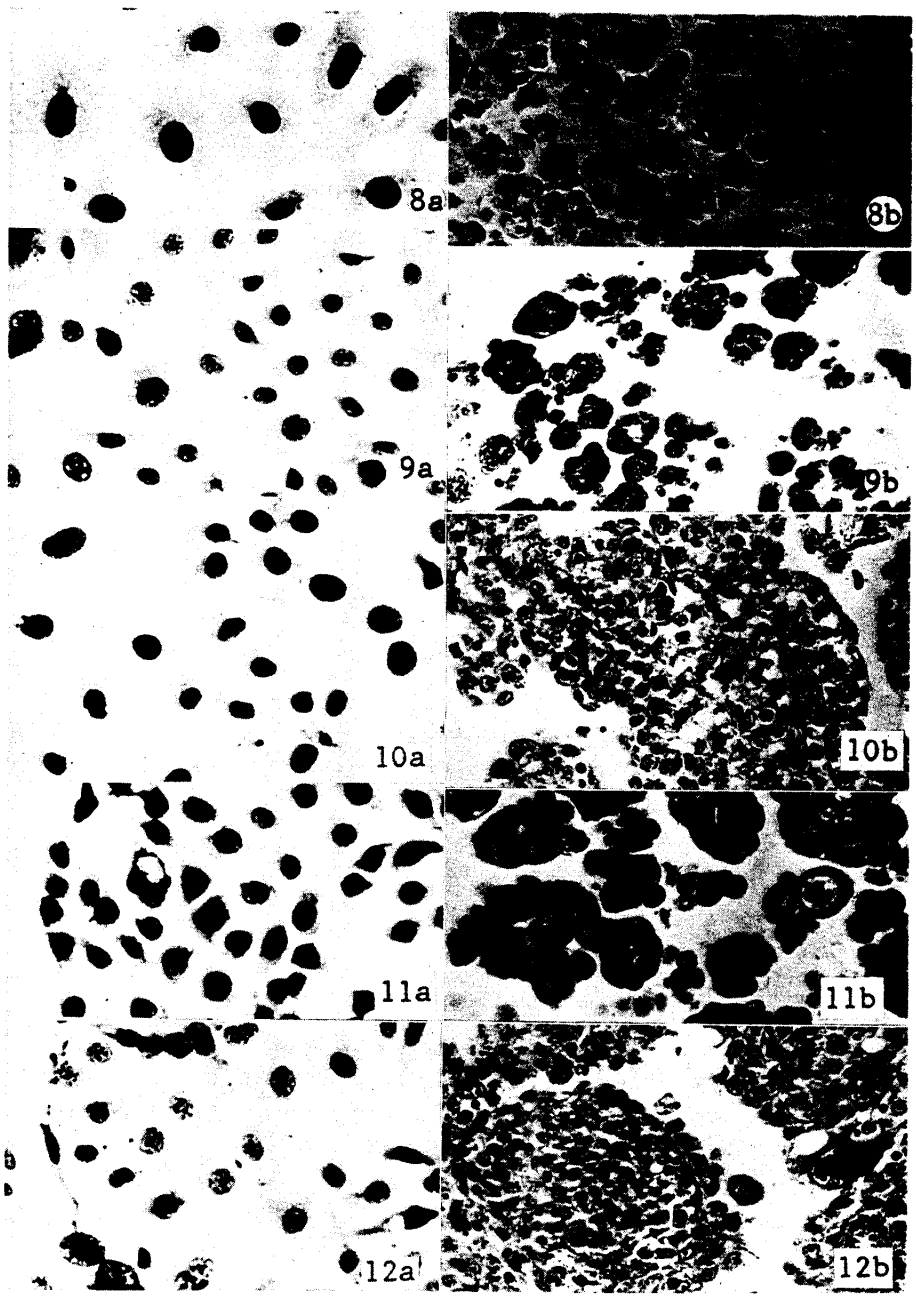
Photo 8a, b The cell line dRLN-53; 8a, Cells in monolayer culture. Giemsa staining. ×100: 8b, A section through aggregates. Hematoxylin and eosin staining. ×200.

Photo 9a, b The cell line dRLN-60; 9a, Cells in monolayer culture. Giemsa staining. ×100; 9b, A section through aggregates. Hematoxylin and eosin staining. ×200.

Photo 10a, b The cell line dRLN-61; 10a Cells in monolayer culture. Giemsa staining. ×100; 10b, A section through aggregates. Hematoxylin and eosin staining. ×200.

Photo 11a, b The cell line dRLa-74: 11a, Cells in monolayer culture. Giemsa staining. ×100; 11b, A section through aggregates. Hematoxylin and eosin staining. ×200.

Photo 12a, b The cell line dRLh-84: 12a, Cells in monolayer culture. Giemsa staining. ×100; 12b, A section through aggregates. Hematoxylin and eosin staining. ×200.


8a
8b
9a
9b
10a
10b
11a
11b
12a
12b

aggregates had necrotic centers and some giant cells (Photo 12b). It was rare to detect mitotic figures in all the cell lines.

3) *Changes of the Aggregates in 8-day Rotation Culture*: Among the five cell lines used, the cell lines, dRLN-60 and dRLh-84, were cultured in gyratory shaker as long as 8 days, medium being renewed 3 and 6 days after the start of culture. In the both cell lines, further growth of aggregates was observed on the fifth day. On the eighth day of rotation culture, the aggregates of dRLN-60 disrupted into clusters consisted of several cells, and those of the cell line dRLh-84 occasionally into 2 to 3 smaller aggregates (Photos 6a-7d).

Microscopic findings of the sections of these aggregates demonstrated that picnotic cells increase as the culture period increases till 8 days in dRLN-60. In the cell line dRLh-84, area of central necrosis increases as the size of aggregates increases.

DISCUSSION

KURODA reported that spontaneously developed mouse mammary gland tumor cells and virally *in vitro* transformed chick embryo cells showed higher aggregability compared with their respective controls (3). NAMBA *et al* compared the aggregability of 4NQO-transformed rat liver cells in culture, and their recultured hepatoma cells, with that of untreated controls. It was shown that the aggregability increased in the ascending order of control, 4NQO-transformed, and hepatoma cells (5). KURODA also compared the aggregability of some of the cell lines used in our experiment (6). However, there seem to be no exact reports concerning the aggregability of the cells in the process of DAB-carcinogenesis. Our findings disclosed that approximately in proportion to the duration of DAB-feeding, liver cells showed larger aggregates. These data apparently suggest that in dRLN-53, dRLN-60, and dRLN-61 cells, which have no tumorigenecity yet, the process of carcinogenesis proceeds stepwise.

The cell line dRLa-74 formed exceptionally smaller aggregates than dRLN-61 cells. This might have been due to excessive treatment of the cells with trypsin on dispersion, because of tight cohesiveness of the cell line.

PESSAC and ALLIOT suggested the role of cell surface materials in the mechanism of aggregation of malignant cells, using cultured mouse tumor cell lines (7). The problems of mechanisms which played a role in forming aggregates of different size among the rat liver cell lines used in the present experiment are under investigation (8).

Histological examination of the aggregates of each cell line showed characteristic association of cuboidal epithelial cells. The cell line dRLa-74 demonstrated gland-like (adenocarcinomatous) appearance, while the cell line dRLp-84 with higher malignancy than the former (dRLa-74) showed the feature of undifferentiated liver cell carcinoma.

Thus histological findings of cell aggregates formed in rotation culture appear to represent the structure of tissues *in vivo* where cultured cells originated. Thus, it is suggested that the present culture method might be applied to histopathological studies of cells *in vitro*, which differ strikingly from cells *in vivo* in many respects.

MOSKOWITZ described that cultured mammalian cell lines, *i. e.*, HEp 2, WISH, and a clone of L, formed aggregates, which grew and reproduced themselves by breaking up into fragments periodically after several weeks in rotaion culture without showing prominent central necrosis (9).

However, in the present experiment, the cell lines dRLN-60 and dRLh-84 exhibited different patterns of growth of aggregates during eight days of rotation culture ; the disruption of aggregates and death of cells in the former, and prominent central necrosis in the latter. These observations might reflect the adaptability of each cell line to the rotation culture, rather than aggregability.

It is concluded that for the elucidation of differences of aggregability among the cell lines, one day of rotation culture is susficient, and three days of the culture will demonstrate more accurate differences.

SUMMARY

For the purpose of elucidating more exact relationship between the process of carcinogenesis and aggregate-forming ability, we performed rotation cultures of a series of five liver cell lines derived from rats fed DAB for various period of days.

As a result we found a tendency of the cells obtained from rats fed DAB for a longer period to form larger aggregates. The differences of the aggregate-forming ability among these cell lines were demonstrated well within one day, and more prominently after three days in rotaion culture. Histologically, the aggregates of all cell lines were composed of cuboidal epithelial cells, especially in some cell lines showing gland-like structures.

ACKNOWLEDGENTS

The author wishes to express sincere thanks to Prof. JIRO SATO for many useful suggestions and to Dr. YUKIAKI KURODA, of the Nat. Inst. Genet., Mishima, for helpful discussions.

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