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Abstract

Morphological comparison at colonial level was made on a series of established liver cell lines derived from rats fed 4-dimethylaminoazo-benzene (DAB) for various periods of days for the purpose of elucidating more accurately the differences in morphology and growth patterns among these cell lines. Colonies of each cell line produced by the single cell plating technique were compared with regard to colony size, density and piling-up of cells, atypism and pleomorphism of cells, and the migration of cells from colonies. Plating efficiency of each cell line was also compared. The cultured rat liver cells obtained from those rats fed DAB for a longer period of days showed higher plating efficiency, and increased the incidence of large-sized, dense, and piled-up colonies, of colonies consisted of cells having nuclear atypism and pleomorphism, and of irregularly margined colonies with migrating cells. The correlation between the present results and the process of DABcacinogenesis is discussed.
MORPHOLOGY AND GROWTH PATTERNS OF COLONIES OF LIVER CELL LINES DERIVED FROM RATS FED WITH 4-DIMETHYLAMINOAZOBENZENE

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Since the early works on the liver carcinogenesis by azo dyes (1, 2), many chemical agents have been proved to be able to produce liver tumors (3, 4, 5). 4-Dimethylaminoazobenzene (DAB) and its related compounds, however, have been used in many experimental works on the liver carcinogenesis, because of their potent and relatively organ specific carcinogenic action against rat liver cells. Thus many transplantable liver carcinomas were produced by azo dyes in rats, and were transferred as solid tumors (6), or ascites tumors (7, 8, 9).

As tissue culture technique advanced, liver cells originated from normal rats (10, 11, 12), and liver carcinomas induced by azo dyes (13, 14, 15, 16) were cultivated and established as permanent cell lines in vitro. Besides these works, the comparative studies on morphology of liver cell lines of rats fed DAB were done by SATO and YABE (17), who demonstrated that the cells, both in the primary and after the long-term culture, showed the transitional changes of morphology and tumorigenicity in proportion to the duration of DAB-feeding.

In the present work, attempts were made to elucidate the differences in morphology and growth patterns of the cell lines at colonial level, especially with respect to the correlation between the process of carcinogenesis and the colonial morphology or growth patterns of the cultured cell lines.

MATERIALS AND METHODS

Cells and Culture Methods: Five liver cell lines of rats were used in the experiment. The cell lines were obtained from rats fed 4-dimethylaminoazobenzene (DAB) for the periods of 57, 107, 142, 191, and 312 days, and designated as dRLN-53, dRLN-60, dRLN-61, dRLa-74, and dRLh-84 cell line, respectively (17). The former 3 cell lines were obtained from livers which had no macroscopic cancerous lesions, and had no tumorigenicity at the time of the initiation of
culture, while the latter 2 cell lines were from nodular lesions of the liver tissues, and had tumorigenicity. The cell line dRLh-84 had the highest, and dRLa-74 had lower grade of tumorigenicity. The primary cultures were started by KATSUTA and TAKAOKA's method (10).

The culture medium consisted of 0.4 per cent lactalbumin hydrolysate with saline D (18) supplemented with 20 per cent of heat-inactivated bovine serum. The cell lines were cultivated from 208 days to 555 days before the start of plating as shown in Table 1. For the plating of cells into petri dishes, the culture medium was substituted by Eagle's minimal essential medium (19) supplemented with 20 per cent calf serum, 4 to 5 days previous to the plating.

Colony Analysis: Following the single cell cloning technique (20), 200 cells were plated into 45 mm petri dishes (Miharu Co.) containing 3 ml of the growth medium. The cultures were maintained in an incubator saturated with humidified air containing 5 per cent CO₂. The medium was renewed on the 6th day and the cultures were stopped 11 days after the plating. The cultures were fixed with Carnoy's fixative, and stained with Giemsa's solution.

The plating efficiency was calculated from average numbers of colonies in 3 plates per sample. For the comparison of colony size, numbers of cells per colony were counted, and grouped into 4 classes, i.e., very small (VS), small (S), large (L), and very large (VL) colonies. The numbers of cells were from 8 to 32 in VS, from 33 to 100 in S, from 101 to 1,000 in L, and more than 1,000 in VL colonies. In the case of piled-up and dense colonies, numbers of cells could not be counted exactly. The piled-up colonies as well as monolayered colonies of tight and close cohesiveness of cells were classified as dense colonies.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Duration of DAB-feeding</th>
<th>Transfer Generation</th>
<th>Culture Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>dRLN-53</td>
<td>57</td>
<td>19</td>
<td>420—431</td>
</tr>
<tr>
<td>dRLN-60</td>
<td>107</td>
<td>23</td>
<td>555—566</td>
</tr>
<tr>
<td>dRLN-61</td>
<td>142</td>
<td>41</td>
<td>364—375</td>
</tr>
<tr>
<td>dRLa-74</td>
<td>191</td>
<td>18</td>
<td>353—364</td>
</tr>
<tr>
<td>dRLh-84</td>
<td>312</td>
<td>10</td>
<td>208—219</td>
</tr>
</tbody>
</table>

RESULTS

1. Morphology of Liver Cell Lines in General:
   i) The cell line dRLN-53: The colonies were mostly uniform in size, and consisted of loose pavement-like sheet of cells which were also relatively uniform in size and shape. Neither piling-up nor migration of cells was observed. The nucleo-cytoplasmic ratio was small and 1 to 2 nucleoli were contained in each nucleus. In the cytoplasm, a pale stained area was observed just adjacent to the nucleus (Photos. 1 and 2).
Colonies of cultured rat liver cells

Photo 1: A typical colony of the cell line dRLN-53. This colony consists of loose pavement-like sheet of cells. Neither piling-up nor migration of cells are observed. Giemsa staining. × 40

Photo 2: The same colony as in Photo 1. × 200

Photo 3: A colony of the cell line dRLN-60. The colony is composed of polygonal cells. The piling-up of cells is not seen. The marginal line is slightly irregular. Giemsa staining. × 40

Photo 4: The same colony as in Photo 3. × 200
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ii) The cell line dRLN-60: The colony size was larger than that of the dRLN-53, though piling-up of cells in the center of colonies was rarely observed. Marginal lines of colonies became slightly irregular. Cells in the colonies were slightly polygonal in shape. The nucleo-cytoplasmic ratio was similar to that of the cell line dRLN-53. One or 2 nucleoli were contained in a nucleus (Photos. 3 and 4).

iii) The cell line dRLN-61: The colonies were large in general and consisted of polygonal cells. In some colonies, the center was dense and composed of 2 to 3 layered cells with some picnotic cells. The atypism and pleomorphism of cells were noted. The migration of cells and irregularity of marginal lines were frequently observed. The nucleo-cytoplasmic ratio increased. Several large nucleoli were contained in a nucleus (Photos. 5 and 6).

iv) The cell line dRLa-74: The colonies were generally large and showed piling-up, atypism and pleomorphism, and migration of cells accompanied by irregularity of marginal lines. At the same time, the detached areas were observed in some of the colonies. The nucleo-cytoplasmic ratio was increased in this cell line, having several large and marked nucleoli (Photos. 7 and 8).

v) The cell line dRLh-84: The colonies were generally large and were composed of slightly elongated cells, which, however, possessed round or oval nuclei, and might be classified into epithelial-like cells. In the center of colonies, cells detached themselves from the glass surface and formed clump-like arrangement, showing net-work appearance. Several large nucleated or multinucleated cells were contained in the colonies. Migration of cells and irregularity of marginal lines was prominent, accompanied by small daughter colonies around the mother colonies (Photos. 9 and 10).

2. Plating Efficiency of Rat Liver Cell Lines: As shown in Table 2, the plating efficiency of each cell line ranged from 24 per cent to 40 per cent, in proportion to the duration of DAB-feeding.

Photo. 5 A colony of the cell line dRLN-61. The colony is composed of dense and partly piled-up cells. Atypism and pleomorphism is noted. The marginal line is slightly irregular. Giemsa staining. ×40

Photo. 6 The same colony as in Photo. 5. ×200

Photo. 7 A colony of the cell line dRLa-74. The colony shows prominent piling up. Pleomorphism and irregularity of marginal line with cell migration is also noted. Giemsa staining. ×40

Photo. 8 The same colony as in Photo. 7. ×200
Colonies of Cultured Rat Liver Cells
Photo. 9 A colony of the cell line dRLh-94. The colony is composed of slightly elongated cells, which overlayed 2- to 3-fold. Marginal line is strikingly irregular with migrating cells. Atypism and pleomorphism is the most prominent. Giemsa staining. ×40

Photo. 10 The same colony as in Photo. 9. ×200

**Table 2** Plating Efficiency of Liver Cell Lines from Rats Fed with DAB

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Range</th>
<th>Mean*</th>
</tr>
</thead>
<tbody>
<tr>
<td>dRLN-53</td>
<td>17–25</td>
<td>24.0</td>
</tr>
<tr>
<td>dRLN-60</td>
<td>25–28</td>
<td>26.5</td>
</tr>
<tr>
<td>dRLN-61</td>
<td>31–33</td>
<td>31.7</td>
</tr>
<tr>
<td>dRLa-74</td>
<td>22–30</td>
<td>28.0</td>
</tr>
<tr>
<td>dRLh-84</td>
<td>39–41</td>
<td>40.0</td>
</tr>
</tbody>
</table>

* Average of 3 plates in each cell line.

3. Colony Size, Cell Density, and Piling-Up: In order to compare the colony size, cell numbers were counted in each colony. The longer DAB-feeding duration increased the incidence of large-sized colonies, i.e., few small colonies were observed in the cell line dRLN-61, dRLa-74, and dRLh-84. The incidence of dense colonies increased stepwise from the cell line dRLN-53 to dRLh-84. Concerning the cell density, not only the piled-up colonies but also the colonies forming the monolayer with closely packed arrangement were included. A clear-cut difference was observed between
the cell line dRLN-60 and dRLN-61, both in the incidence of dense and piled-up colonies. The relationship of the colony size to the rate of piled-up colonies was illustrated in Fig. 1.

**Table 3** Comparison of colony size, and incidence of dense and piled-up colonies in liver cell lines from rats fed with DAB

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Colony Size</th>
<th>Dense Colony</th>
<th>Piled-up Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VS*</td>
<td>S**</td>
<td>L***</td>
</tr>
<tr>
<td>dRLN-53</td>
<td>23</td>
<td>46</td>
<td>31</td>
</tr>
<tr>
<td>dRLN-60</td>
<td>2</td>
<td>10</td>
<td>56</td>
</tr>
<tr>
<td>dRLN-61</td>
<td>0</td>
<td>14</td>
<td>56</td>
</tr>
<tr>
<td>dRLa-74</td>
<td>0</td>
<td>4</td>
<td>66</td>
</tr>
<tr>
<td>dRLh-84</td>
<td>0</td>
<td>0</td>
<td>54</td>
</tr>
</tbody>
</table>

* Very small colony containing from 8 to 32 cells per colony.
** Small colony containing from 33 to 100 cells per colony.
*** Large colony containing from 101 to 1,000 cells per colony.
**** Very large colony containing more than 1,000 cells per colony.

4. Atypism and Pleomorphism of Cells in the Colonies: The colonies having the multinucleated giant cells or large nuclear cells were included in this classification. As shown in Table 4, even in the cell line dRLN-53, 3 percent of colonies was counted in this type, while in the cell lines dRLN-60, and dRLN-61, the percentage increased to approximately 40 and 50.

In the cell lines dRLa-74 and dRLh-84, the incidence increased to over 70 percent. The relationship between the colony size and the incidence of colonies of atypism and pleomorphism is illustrated in Fig. 1. The increase of the appearance of colonies containing atypical and pleomorphic cells was influenced partly by the colony size, and partly by the duration of DAB-feeding.

**Table 4** Incidence of colonies with atypism and pleomorphism of cells, and with cell migration in liver cell lines from rats fed with DAB

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Colonies with Atypism and Pleomorphism (%)</th>
<th>Colonies with Cell Migration (Grade I*)</th>
<th>Grade II**</th>
</tr>
</thead>
<tbody>
<tr>
<td>dRLN-53</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dRLN-60</td>
<td>48</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>dRLN-61</td>
<td>44</td>
<td>42</td>
<td>36</td>
</tr>
<tr>
<td>dRLa-74</td>
<td>74</td>
<td>14</td>
<td>68</td>
</tr>
<tr>
<td>dRLh-84</td>
<td>74</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

* Low grade of cell migration.
** High grade of cell migration.
5. Migration of Cells and Peripheral Areas of Colonies: Peripheral areas and the migratory phenomena were checked in the cell lines. The marginal lines of colonies derived from the cell line dRLN-53 were almost of regular circle, while those of dRLN-84 were mostly irregular, with migrating cells around them. The other cell lines, i.e., dRLN-60, dRLN-61, and dRLN-74, were situated between the two extremes, with the increasing percentage in proportion to the duration of DAB-feeding. The results are shown in Table 4.

DISCUSSION

SATO and YABE described the transitional changes of morphology among the cell lines in the mass cultures (17). In the present experiment, it was revealed that their observations were described more accurately as the changes in percentage of certain type of colonies such as dense and
Colonies of Cultured Rat Liver Cells

Piled-up, atypical and pleomorphic, and irregularly margined colonies with cell migration. In addition, growth potential of these cell lines were elevated in proportion to the duration of DAB-feeding. There still remain very important problems, such as (a) whether these differences of colonial morphology and growth patterns are attributable to the stage of carcinogenesis, or merely to the individuality of each cell line, indifferent to the stage of carcinogenesis, (b) to what extent, the problem of spontaneous malignant transformation of cells has to be considered after the long-term culture, and finally (c) to what extent the colony analysis of this kind will be applied to the in vitro azo dye-carcinogenesis using epithelial cells as liver cells. The following discussions deal with these problems.

1. Whether the present results can be attributed to the stage of carcinogenesis or to the individuality of each cell line, cannot be concluded from the present experiment using the limited number of cell lines. However, the results seem to be closely connected with the stage of carcinogenesis from two points, i.e., colonial growth patterns and colonial morphology of each cell line.

In general it is supposed that the increase of plating efficiency as well as the appearance of large sized colonies is indicative of an accelerated growth potential. The present results coincide with the experimental works of SATO. SATO reported that, while no outgrowth of liver cells was observed from the primary explants both of the liver tissues, and of regenerating liver tissues after partial hepatectomy (12), better outgrowth of liver cells was observed from the liver tissues of DAB-feeding adult rats, suggesting the enhancement of growth potential (17), though the origin of proliferating liver cells is a disputable problem, and further investigations have to be made biologically and biochemically.

From the aspect of colonial morphology, liver cell lines from the rats fed DAB for a longer period of days showed higher incidence of dense, piled-up colonies, irregularly margined colonies with migration of cells, and colonies with atypism and pleomorphism of cells. Piling-up of cells was explained as the loss of contact inhibition proposed by ABERCROMBIE et al. (21), and has been used as a marker of malignant transformation of cells in carcinogenesis in vitro with viral (22, 23, 24, 25), chemical (26, 27, 28, 29, 30), and other agents such as x-irradiation (31), as well as in vitro spontaneous malignant transformation (32, 33). In addition, the migration of cells and irregularity of marginal line of colonies seem to have some simulation to infiltrative invasion of cancer cells upon the surrounding normal tissues. Concerning the presence of pleomorphism and atypism of cells in colonies, it might not be due to the mixture of other clones,
because the plating was started as to have the single cell rate high enough to eliminate the contamination possibility. Thus the increase in percentage of colonies with atypism and pleomorphism of cells in proportion to DAB-fed feeding duration might be correlated with the higher incidence of abnormal mitosis in cancer cells.

From the foregoing discussion, again, the transitional changes in colonial morphology and growth patterns seem to reflect the different stage of in vivo carcinogenesis induced by DAB.

2. Since the first report of GEY (34), many investigators have reported the spontaneous malignant transformation of cultured cells of various species (35, 36, 37, 38, 32, 33). KATSUTA et al. reported that rat liver cell lines transformed morphologically in Nagisa culture (39). Following their report, SATO described that several rat liver cell lines underwent malignant transformation after about 850 days of culture (40). In the present experiment, the cell lines examined for colonial morphology and growth patterns were cultivated for the periods of from about 200 to 550 days as shown in Table 1. Though such periods of culture days were not long enough to induce malignant transformation of cells but enough to morphological transformation. As the result, the colonial analysis of these cell lines at an earlier period of culture might have revealed more distinct differences in colonial morphology.

3. Whether or not the colony analysis of this kind provides us with the criteria of malignancy in the in vitro azo dye carcinogenesis of liver cells has not been proved exactly. The characteristic colonies such as large-sized or small-sized, dense and piled-up or loose and monolayered, pleomorphic or uniform, and irregularly margined with cell migration or well margined colonies, have to be cloned and studied on the tumorigenicity.

NAMBA et al. succeeded in the in vitro malignant transformation of rat liver cells by 4-nitroquinoline-1-oxide (41). They further examined the correlation between the changes of colonial morphology and the acquisition of tumorigenicity of the 4NQO-treated cells, and showed a close correlation between the two events (42). Though their results could not be compared exactly with the present data, the appearance of the colonies with piling-up of cells and atypism or pleomorphism of cells may become one of indicators of the acquisition of malignancy even in the DAB-carcinogenesis in vitro.
SUMMARY

Morphological comparison at colonial level was made on a series of established liver cell lines derived from rats fed 4-dimethylaminoazo-benzene (DAB) for various periods of days for the purpose of elucidating more accurately the differences in morphology and growth patterns among these cell lines. Colonies of each cell line produced by the single cell plating technique were compared with regard to colony size, density and piling-up of cells, atypism and pleomorphism of cells, and the migration of cells from colonies. Plating efficiency of each cell line was also compared.

The cultured rat liver cells obtained from those rats fed DAB for a longer period of days showed higher plating efficiency, and increased the incidence of large-sized, dense, and piled-up colonies, of colonies consisted of cells having nuclear atypism and pleomorphism, and of irregularly margined colonies with migrating cells.

The correlation between the present results and the process of DAB-carcinogenesis is discussed.

ACKNOWLEDGEMENTS

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