Changes in the glycogen phosphorylase isozyme patterns of AH130 during cell growth*1

Ichiro Hatayama and Kiyomi Sato
Second Department of Biochemistry, Hirosaki University School of Medicine*2

Changes in the glycogen phosphorylase isozyme patterns of AH130 and AH66F during cell growth were investigated immunochemically and electrophoretically. The content of the liver-like type increased as the cell growth in the ascites decreased, while the level of the fetal type (or prototype) remained approximately constant. The isozyme shift from the fetal type to liver-like type was more marked in subcutaneous solid AH130. In AH130 cells cultured in vitro under growth-inhibited conditions, the content of the hybrid between the above two types was increased, with increased amount of liver-like subunit. These results indicate that the phosphorylase isozyme patterns of Yoshida ascites hepatomas vary during cell growth under different conditions.

Key words: Yoshida ascites hepatoma — AH130 — AH66F — Culture — Glycogen phosphorylase — Isozyme

It has been accepted1,12,27) that phenotypes such as isozyme pattern in transplantable hepatomas are rather stable through generations of transplantation, although some phenotypes alter gradually or suddenly during successive transplantations.12,16,27) However, recently some factors purified from ascitic fluids or ascites tumors have been proved to induce differentiation of tumor cells or dedifferentiation of host liver cells.7,8,13) Therefore, we examined whether or not the isozyme patterns of glycogen phosphorylase (Ph) (EC 2.4.1.1), which seemed to be characteristic of Yoshida ascites hepatomas,19 actually fluctuate during cell growth in ascitic fluids in situ or in cultures under various growth conditions.

Materials and Methods

Chemicals Glucose[U-14C] 1-phosphate and DL-ornithine[I-14C] were obtained from New England Nuclear, Boston, Mass., U.S.A.; N6,O2-dibutyryl adenosine 3',5'-monophosphate (DBcAMP) from Daiichi Pure Chemicals Co., Ltd., Tokyo; Bactopeptone from Difco laboratory, Detroit, Mich., U.S.A.; and calf serum from Gibco, Grand Island, N.Y., U.S.A. Other chemicals were purchased from the sources mentioned in previous papers.19,20)

Sample Preparation Male Sprague-Dawley rats bred in our laboratory, weighing 200 to 250 g, were inoculated ip or sc with 1 x 107 cells of Yoshida ascites hepatoma AH130 or AH66F. The cells were collected at the indicated times and homogenized by sonication in the medium reported previously.19) The supernatant obtained by centrifugation at 600g for 10 min was used for assay of Ph. The supernatant obtained by further centrifugation at 105,000g for 45 min was fractionated with 30 to 70% saturation of ammonium sulfate at 0°C to achieve partial purification of Ph. In addition, AH130 cells were homogenized in the medium containing 10mM Na, K-phosphate buffer (pH 7.2), 0.1mM pyridoxal phosphate, 2.5mM dithiothreitol, 1mM EDTA and 250mM sucrose, and the supernatant at 105,000g for 45 min was used for the assay of ornithine decarboxylase (ODC) (EC 4.1.1.17).

*1 This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture.

*2 Zaifu-cho 5, Hirosaki 036 (雫山一郎，佐藤清美)
Culture Conditions AH130 cells (3 x 10⁶ cells/ml) were cultured in Eagle's essential medium (Nissui Seiyaku Co., Tokyo) containing bactopeptone (100 mg/liter), sodium pyruvate (110 mg/liter), glycine (10 mg/liter), L-serine (7.5 mg/liter) and 10% calf serum in glass vials (40 x 55 x 150 mm) (products of Ikemoto Co., Tokyo) under a humidified atmosphere of 5% CO₂ and 95% air at 37°C. DBcAMP, sodium butyrate, theophylline or dimethyl sulfoxide (DMSO) was added at various concentrations to the medium at the start of culture. The cells were cultured for 24 hr under the above-mentioned conditions, then collected, washed twice with 0.9% NaCl and homogenized as described above.

Enzyme Assays and Separate Determination of Ph Isozymes Ph was assayed as described previously. Activities of the liver-like and fetal types of Ph were measured as remaining activities upon inhibition by the specific antibody against the fetal type or the liver type, because the hybrid type is completely inhibited by excess amounts of both antibodies. The hybrid (FL) activity of Ph was estimated as the difference between the total activity (T) and the sum of activities of liver-like (LL) and fetal (FF) types as follows: FL = T - (LL + FF). ODC was assayed by the method of Ono et al., modified by using a volume of 0.5 ml of the total reaction mixture. One unit of Ph or ODC is defined as the amount of enzyme that catalyzes the incorporation of 1 μmol of glucose into glycogen per min or the release of 1 μmol of CO₂ from ornithine per min.

Polyacrylamide Disc Gel Electrophoresis The electrophoresis was carried out according to the method of Yonezawa and Hori.

Cell Count and Protein Determination Ascites hepatoma cells were counted with a hemocytometer. Protein concentration was determined according to the method of Lowry et al.

RESULTS

Enzyme Activities During Cell Growth in vivo After ip inoculation, AH130 cells proliferated logarithmically till the 7th day and then continued to proliferate with a gradually decreasing growth rate (Fig. 1). It was noticed that the total Ph activity increased significantly as the cell growth rate decreased (Table I), while ODC activity, which is known to be closely related to cell growth (it is a key enzyme of polyamine synthesis), decreased rapidly with decrease of the growth rate (Fig. 1). Glycogen synthase also increased markedly, and hexokinase, aldolase and pyruvate kinase also tended to increase slightly but significantly (data not shown).

Change of Ph Isozyme Pattern Of the Ph isozymes, FF activity changed little till the 7th day, but decreased at the 10th day (Table I). The activity of the LL type also did not change till the 7th day but markedly increased at the 10th day, when the growth rate declined. The activity of FL hybrid also increased remarkably after the 7th day and became four times higher at the 10th day than at the 3rd day. On the other hand, AH66F, an exceptional Yoshida ascites hepatoma strain which stores glycogen, contained high activities of LL and FL isozymes from the beginning of inoculation, although the total activities of AH66F were lower than those of AH130, and showed an increase of LL activity at the 7th day (Table I). We could not examine the pattern of AH66F at the 10th day owing to the poor survival rate of host rats.
GLYCOGEN PHOSPHORYLASE ISOZYMES OF AH130

Table I. Changes in Ph Isozyme Patterns of AH130 and AH66F during ip Cell Growth

<table>
<thead>
<tr>
<th>Cell</th>
<th>Days after inoculation</th>
<th>Ph activity (mU/mg proteins)</th>
<th>Total</th>
<th>FF type</th>
<th>LL type</th>
<th>FL hybrid type</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH130</td>
<td>3 (9)</td>
<td>22.89±3.20</td>
<td>15.86±1.29</td>
<td>2.04±0.64</td>
<td>4.99±1.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (9)</td>
<td>24.21±4.17</td>
<td>15.82±2.35</td>
<td>1.80±0.45</td>
<td>6.58±3.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 (9)</td>
<td>27.02±5.13</td>
<td>14.13±2.71</td>
<td>2.42±0.74</td>
<td>10.47±3.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 (8)</td>
<td>32.05±2.68</td>
<td>7.80±1.25</td>
<td>5.16±0.94</td>
<td>19.10±1.80</td>
<td></td>
</tr>
<tr>
<td>AH66F</td>
<td>4 (10)</td>
<td>11.42±1.30</td>
<td>2.78±0.60</td>
<td>3.63±0.69</td>
<td>5.01±1.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 (6)</td>
<td>13.10±2.43</td>
<td>2.78±0.81</td>
<td>5.44±1.00</td>
<td>4.89±1.59</td>
<td></td>
</tr>
</tbody>
</table>

The packed cell volumes of AH66F at the 4th and 7th days were 3.4±1.0 and 8.1±1.5 ml/rat, respectively. Values are expressed as means±SD. Assay numbers are indicated in parentheses.

To estimate which subunit of Ph increases during the growth of AH130 cells, the activity of FL hybrid was divided into halves, which were added separately to FF and LL activities. Because Ph is a dimer consisting of two homo- or hetero-subunits, the hybrid was inhibited by both antibodies to approximately half the extent of the homodimers (data not shown). The specific activities of purified FF and LL were approximately the same. Fig. 1 shows that the content of the L subunit, expressed as activity of the homodimer (LL), increased remarkably after the 7th day as the growth rate decreased but that of the F subunit changed little, suggesting that the L subunit was selectively induced during the late period of ip tumor growth.

Ph isozyme patterns of AH130 and AH66F on polyacrylamide disc gel electrophoresis are shown in Fig. 2. On the 3rd day, AH130 showed strong activity of FF, moderate activity of FL hybrid and only a trace activity of LL. However, on the 10th day the LL activity was clearly increased and the FF activity was decreased, although the FL hybrid was predominant. AH66F also exhibited a significant increase of LL at the 7th day, when the cell growth began to decrease. The small but significant amounts of FF determined immunochemically (Table I) were not evident in these electrophoretograms. These results suggest that the Ph isozyme patterns of some Yoshida ascites hepatomas fluctuate significantly during cell growth in the ascitic fluids in vivo, though the isozyme pattern characteristic of each strain is not completely lost. However, hexokinase, aldolase and pyruvate kinase of AH130 did not show any significant change in their isozyme patterns (data not shown). The isozyme pattern of AH130 cells inoculated sc and having a slow growth rate as estimated in terms of tumor growth (see footnotes to Fig. 2) was enriched with the LL type and became indistinguishable at the 12th day from that of AH66F ip at the 7th day (Fig. 2).
Ph Isozyme Pattern of AH130 Cultured in vitro

Preliminary experiments on cells cultured under various growth conditions were done to confirm results in vivo. Under the standard conditions described in "Materials and Methods," $3 \times 10^5$ AH130 cells/ml proliferated logarithmically for 24 hr and then the growth rate declined (no medium change) (Fig. 3). Two mM DBcAMP, 3% DMSO and 2 mM sodium butyrate which are known to induce the cell differentiation of mouse neuroblastoma and Friend leukemia cells, strongly inhibited the proliferation of AH130 cells for 72 hr so that there was no significant change in cell number. Furthermore, theophylline, which is known to inhibit cAMP phosphodiesterase activity and increase intracellular cAMP level, similarly inhibited the proliferation of the cells. Morphologically, swollen cells were observed, especially in the presence of DMSO or sodium butyrate. However, 2 mM cAMP, which is also known to inhibit the growth of malignant cells, did not show any marked effects on the proliferation and morphology of AH130 cells in our experiments.

AH130 cells cultured for 24 hr in the absence (control) and presence of the above-mentioned compounds were examined to compare the Ph isozyme patterns of the logarithmic and stationary growth states. Table II indicates that the activity of FL hybrid was clearly increased in the presence of sodium butyrate and DMSO, although the activity of the LL type did not change significantly. In the presence of DBcAMP and theophylline, the content of FF type was decreased and that of the FL hybrid increased. Under these conditions, it was estimated by the method shown in Fig. 1 that the level of the L subunit was always increased, though that of the F subunit was also increased in the presence of butyrate and DMSO (Table II). These results are consistent with our speculation (based on

Table II. Change in the Ph Isozyme Pattern of AH130 Cultured in vitro under Various Conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Ph activity (mU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FF type</td>
</tr>
<tr>
<td>Nontreated (10)</td>
<td>22.92±5.14</td>
</tr>
<tr>
<td>2mM DBcAMP (7)</td>
<td>17.18±1.25*$^*$</td>
</tr>
<tr>
<td>5mM theophylline (10)</td>
<td>17.86±4.64**</td>
</tr>
<tr>
<td>2mM butyrate (11)</td>
<td>21.44±3.22</td>
</tr>
<tr>
<td>3% DMSO (11)</td>
<td>22.27±6.58</td>
</tr>
</tbody>
</table>

Values are expressed as means±SD. Subunit amounts are expressed as in Fig. 1. Assay numbers are indicated in parentheses.

Significant differences from the values of the nontreated cells are indicated by * $P<0.005$ and ** $P<0.05$. 878 Gann
the results in vivo) that the decreased AH130 growth rate may be related to the increase of Ph L subunit content. The total Ph activities in vitro were higher than those in vivo, as is evident in Table I as compared with Table II, but the reason remains unknown.

**DISCUSSION**

The results reported in this paper indicate that the Ph isozyme pattern is not fixed even in the most malignant ascites hepatomas; it fluctuates during cell growth and the contents of the LL and FL types increase under growth-inhibited conditions.

It was reported that some cultured ascites hepatomas synthesize α-fetoprotein (AFP) in the G₁ and S phases of their cell cycles and secrete it into culture mediums, and that the amount of AFP secreted is enhanced when the growth of Yoshida sarcoma or ascites hepatoma AH66 is inhibited strongly by treatment with DBCAMP or cAMP under conditions similar to those used in this study. The isozyme pattern of alkaline phosphatase in HeLa cells is also known to vary, depending on the growth conditions.

Kozak and Murphy reported that the solid form of mouse anaplastic mammary carcinoma 15091A, which has the adult-type isozyme of L-glycerol-3-phosphate dehydrogenase, can be converted to the ascites form which has the embryonic-type isozyme, and both tumor forms are interconvertible. We also found that the solid form of AH130 cells inhibited in growth by sc inoculation has mainly FL and LL activities and shows a tendency for isozyme shift from the fetal type to the liver-like type (Fig. 2). The different Ph isozyme expressions of the ascitic and solid forms also seem to be related to the decreased tumor growth rate, although Kozak and Murphy considered that this phenomenon may be dependent upon the physical form of the tumor.

The liver-like type of Ph present in malignant hepatomas differs electrophoretically from the liver type in the adult liver although both types are indistinguishable immunochemically. The liver-like type is also present in fetal tissues, and we consider it to be one of the fetal types of Ph isozyme, which might appear chronologically between the prototypic brain type and organ-specific liver and muscle types.

Considering the various factors stimulating differentiation of mouse myeloid leukemia cells found in AH130 ascitic fluid by Hozumi et al., it is very interesting that the shift of Ph isozyme pattern actually corresponds to apparent differentiation during the growth of AH130 cells in the ascitic fluid, although the shift is not so extreme as to result in disappearance of the FF isozyme. The effects of these factors on the Ph isozyme pattern of AH130 are now being examined.

(Received June 18, 1980/Accepted Sept. 13, 1980)

**REFERENCES**