Tumor Res., **14**, 1-8, 1979

Effects of Insulin and Dexamethasone on Glucose-6-Phosphatase in Cultured Morris Hepatoma 7316A

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SUMMARY

Morris hepatoma 7316A cells maintained *in vivo* by serial transplantation in rats showed cytochemically a considerable degree of glucose-6-phosphatase activity; however, tumor cells maintained in a long-term culture with insulin-containing medium failed to show enzyme activity cytochemically. When these cells were transfered into dexamethasone-containing medium or hormone-free medium, the enzyme activity in the cells reappeared promptly. The cells cultured in dexamethasone-containing medium from the first, also showed considerable enzyme activity. The intensity of the activity was stronger in the cells cultured in dexamethasone-containing medium than in those cultured in hormone-free medium. These findings suggest that regulation of glucose-6-phosphatase by hormone in hepatoma cells is maintained even in a long-term culture.

INTRODUCTION

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase; EC 3. 1. 3. 9) is localized in the cisternae of endoplasmic reticulum and nuclear envelops, and is regulated by various hormones and nutritional conditions in the liver (1-4). In hepatomas, it was reported that the enzyme activity was significantly reduced (5-9), although being inducible by glucocorticoid (2), and was inversely proportional to the growth rate of the tumors (2, 7). With respect to cultured hepatoma cells, there is only one study that Chang hepatoma cells showed a decrease in G-6-Pase[1] activity ultracytochemically (10). Thus, it is worth-while to demonstrate G-6-Pase activity in other cultured hepatomas and also to investigate whether the enzyme activity is regulated in the cultured tumor cells as in the cells grown *in vivo*.

Abbreviations used are: G-6-Pase, glucose-6-phosphatase.

MATERIALS AND METHODS

Isolation of Tumor Cells

Morris hepatoma 7316A maintained in Buffalo rats were removed 3 weeks after transplantation. The tumor tissues were cut into small pieces after connective tissues and necrotic areas were removed. The tissue pieces were washed 3-times with cold Ca^{++}-free Hanks' BSS containing 1.5% bovine serum albumin fraction V, 0.5 μg/ml insulin, 0.04 mg/ml streptomycin, 40 IU/ml penicillin-G, and 50 IU/ml mycostatin. Then, they were minced with scissors and centrifuged at 50×g for 1 min. The pellet was transfered into a beaker, where the cells were dispersed in Ca^{++}-free Hanks' BSS, containing 120 IU/ml collagenase type I (Sigma Chemical Co.), 300 PU/ml dispase II (Godo Shusei Co., Japan), 0.5 μg/ml insulin, 0.04 mg/ml streptomycin, and 40 IU/ml penicillin-G, by bubbling with 95% O_2 and 5% CO_2 at 37°C for 15 min, and pipetting with a large-bore pipette. After 2-times filtration through sterile gauze, the cells were centrifuged at 50×g for 1 min. The cells were resuspended in 37°C-warmed Ca^{++}-free Hanks' BSS containing bovine serum albumin, insulin, streptomycin, penicillin-G, and mycostatin at the respective concentrations as mentioned above. The cells were washed in this solution by 3-times centrifugation at 50×g for 1 min, and suspended in 37°C-warmed culture medium: Ham's F-12 nutrient mixture supplemented with 10% fetal calf serum, 0.5 μg/ml insulin, 0.04 mg/ml streptomycin and 40 IU/ml penicillin-G. After centrifugation at 50×g for 1 min, the cells were resuspened in the culture medium and filtered 2-times through nylon mesh; the pore size of the mesh for the first filtration was 250 μm and the second was 60 μm. Viability of the cells was examined by trypan blue exculsion test. The inoculation size was 1×10^6 viable cells in a 60-mm plastic dish.

Maintenance and Subculture

The cultivation was made in Ham's F-12 nutrient mixture as mentioned above in a humidified atmosphere of 95% air and 5% CO_2. After reaching confluency, the cells were subcultured as follows: washing 3-times with Ca^{++}-free Hanks' BSS containing insulin, streptomycin and penicillin-G; treatment with this Ca^{++}-free Hanks' BSS supplemented with 750 PU/ml dispase II at 37°C for 5 min. Then the cells were harvested and subcultured in the following medium; 1) insulin-containing medium as mentioned above, 2) the same medium as 1) but containing dexamethasone (1 μM) instead of insulin, 3) the same medium as 1) but without insulin. Cytochemical examination was done 1, 2, 3 and 4 weeks after the transfer. Cultivation of the cells in dexamethasone-containing and hormone-free medium was also made using the cells cultured in the respective medium from the first.

Cytochemistry of G-6-Pase

The tumor tissues removed from rats were frozen in hexane cooled with dry ice and 6-μm-sections were cut with a cryostat. G-6-Pase activity was stained by Wachstein-Meisel's method (11).

The cells in culture in dishes were treated as follows: drying quickly; incubation in Wachstein-Meisel's medium at 37°C for 20 min; washing in distilled water for 30 min; 5-minute fixation in 10% buffered formalin; washing with distilled water quickly; short immersion in 2% ammonium sulfide solution.

For electron microscopy, the cells in culture in dishes were fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.2 M sucrose for 5 min, rinsed overnight in 0.1 M cacodylate buffer containing 0.2 M sucrose, pH 7.4. The cells were incubated in Wachstein-Meisel's medium at 37°C for 30 min. After quick washing with 0.1 M cacodylate buffer containing 0.2 M sucrose, the cells were postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, dehydrated through a graded series of ethanol and then embedded in Epon 812.

In control experiments, the cells were incubated in a substrate-free medium or in a medium added with 10 mM NaF. Examinations were also made using fructose-1, 6-diphosphate or glucose-1-phosphate as substrates confirming the substrate specificity of G-6-Pase.

Calculation of G-6-Pase-postive Cells.

G-6-Pase-positive cells were calculated as a percentage to the total cell number observed in each microscopic field with the use of 10× ocular and 10× objective. Two-3×10^2 cells were analyzed in each preparation.

RESULTS

The tumor cells *in vivo* showed cytochemically a considerable degree of G-6-Pase activity, although it varied from cell to cell (Fig. 1). In a large magnification, it was found that the activity was confined to cytoplasms of the cells but lacking in nuclei (Fig. 2). In most of the tumor cells maintained in insulin-containing medium over one year, the activity was not demonstrated cytochemically, although a weak activity was shown in a small proportion of the cells (Fig. 3).

When these G-6-Pase-negative cells were transfered to dexamethasone-containing medium or medium without either dexamethasone or insulin, the activity reappeared in one week after the transfer (Figs. 4, 5). Under a large magnification, it was found that the localization of the activity in the cells in culture was the same as that in the cells maintained *in vivo* (Fig.

Table 1. *Percentage of G-6-Pase-positive Cells*

Period	dexamethasone	hormone-free	insulin
1 w[a]	16.3	12.7	2.4
2 w[a]	62.7	30.5	—
3 w[a]	79.2	53.0	—
4 w[a]	64.4	44.8	10.4
60 w[b]	90.5	36.1	5.6

a : The cells were cultured in insulin-containing medium for 55 weeks, and then they were transfered to each medium.
b : The cells were cultured in each medium from the first.

6). As shown in Table 1, percentages of the cells positive for enzyme activity were increased promptly within one week after the transfer of the cells to dexamethasone-containing medium or hormone-free medium, and increased further at 2- and 3-week after the transfer. The increase was always higher in the cells cultured in dexamethasone-containing medium than in those cultured in hormone-free medium. Percentage of the activity-positive cells cultured in dexamethasone-containing medium from the first to the 60th week reached 90% and the percentage of those cultured in hormone-free medium was 30%, whereas it was only 6% in the cells cultured in insulin-containing medium. In cytochemical electron microscopy, the reaction products in the cultivated cells were seen localized in cisternae of endoplasmic reticulum and nuclear envelops (Fig. 7).

In control experiments, the cells were not stained cytochemically.

DISCUSSION

In normal liver, activity of G-6-Pase is well known to be regulated by various hormones and nutritional conditions ; for example, administration of glucocorticoid or starvation results in an increase and/or induction of the enzyme activity, while administration of insulin results in a decrease of the activity.

In hepatomas, it was reported that the endogeneous enzyme activity was inversely proportional to the growth rate of the tumors and it was induced by glucocorticoid in some hepatomas (2).

A number of studies have been reported on the regulation of various metabolic activities in hepatomas in culture, suggesting that some of the activities were well regulated (12-19). However, regulation of G-6-Pase in cultured hepatoma cells has not been reported as yet, although the enzyme

is one of representative metabolic activities of hepatocytes. The present study revealed that the enzyme activity examined cytochemically disappeared in the cells maintained in insulin-containing medium, while it reappeared promptly after the transfer of the cells to dexamethasone-containing medium or hormone-free medium. Eighty percent of the cells became positive for the enzyme activity in 3 weeks after the transfer to dexamethasone-containing medium, and 50% became positive in hormone-free medium. Furthermore, an immense number of cells cultured in dexamethasone-containing medium from the first showed positive activity, whereas only one third of the cells cultured in hormone-free medium were positive for the activity. In all events, the enzyme activity in cultured hepatoma cells was enhanced by dexamethasone and suppressed by insulin, as in the enzyme in normal liver, suggesting that the regulatory mechanism(s) involved in the expression of G-6-Pase is preserved in a well-differentiated hepatoma in culture even after more than one year of cultivation.

As to Morris hepatoma 7316A in culture, isozyme pattern of branched-chain amino acid transaminase was reported to shift from the pattern in well-differentiated hepatomas to that in poorly-differentiated tumors (20). Furthermore, there are reports suggesting that cells in culture undergo changes after a long term of cultivation (21–25). In the present observation, it was found that the growth rate, and the shape and size of the tumor cells were not altered even after more than one year of the cultivation (our unpublished data). One of the most noticeable difference between our culture condition and those hitherto reported was the use of dispase for harvesting cells from dishes. Since dispase was reported to be less injurious to cell membrane than trypsin (26, 27), the use of disapase may be favourable for preservation of regulatory mechanism(s) of G-6-Pase particularly in a long-term culture.

ACKNOWLEDGEMENTS

We are indebted to Miss N. Takeuchi and Miss Y. Takahashi for their skillful technical assistance. Our thanks are also due to Dr. Harrold P. Morris, Howard University, who kindly provided Morris 7316A hepatoma. This work has been supported in part by a Grant-in-aid for Scientific Research from Ministry of Eduction, Science and Culture Nos. 448143 and 337013, and by a Grant-in-aid from Hokkaido Geriatrics Research Institute.

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Figure legends

Fig. 1. G–6–Pase in Morris 7316 A hepatoma *in vivo*. ×112

Fig. 2. G–6–Pase in Morris 7316 A hepatoma *in vivo*. ×459

Fig. 3. G–6–Pase in Morris 7316 A hepatoma cells, in insulin-containing medium for 3 weeks. ×112

Fig. 4. G–6–Pase in Morris 7316 A hepatoma cells, in dexamethasone-containing medium for 3 weeks. ×112

Fig. 5. G–6–Pase in Morris 7316 A hepatoma cells, in hormone-free medium for 3 weeks. ×112

Fig. 6. G–6–Pase in Morris 7316 A hepatoma cells, in dexamethasone-containing medium for 3 weeks. ×459

Fig. 7. Ultracytochemistry of G–6–Pase in Morris 7316 A hepatoma cells in dexamethasone-containing medium for 3 weeks. ×14000


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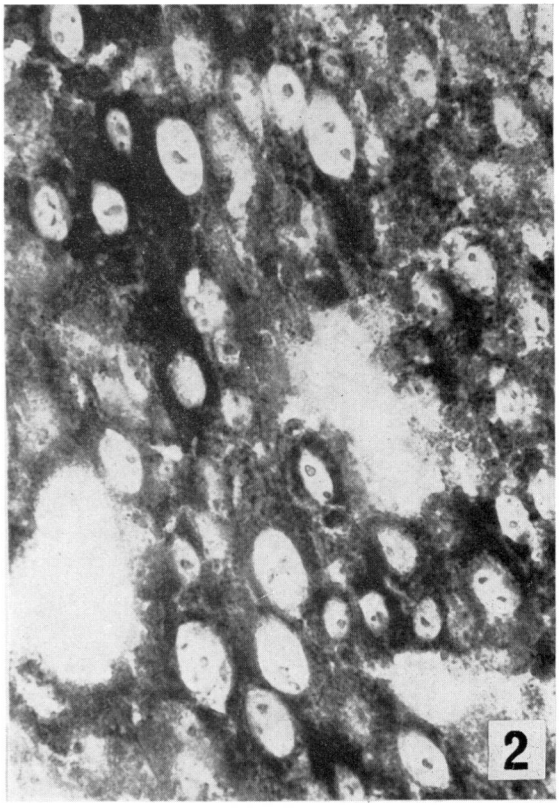
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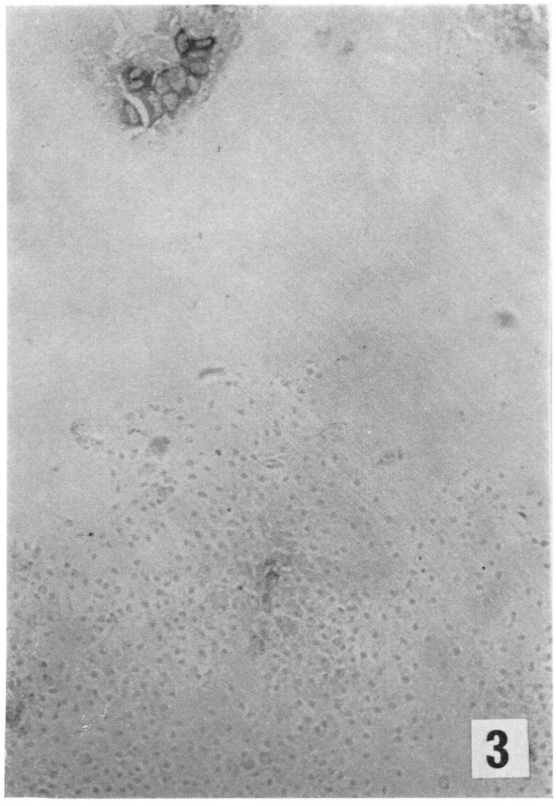
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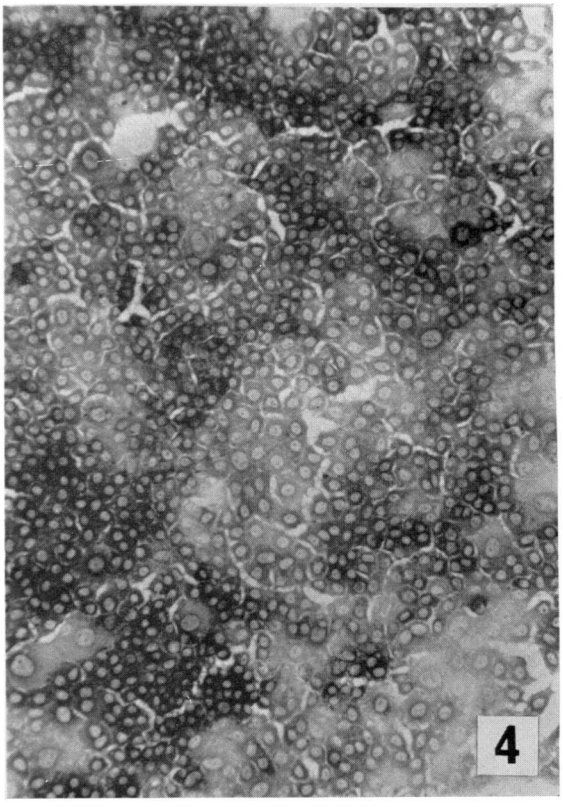
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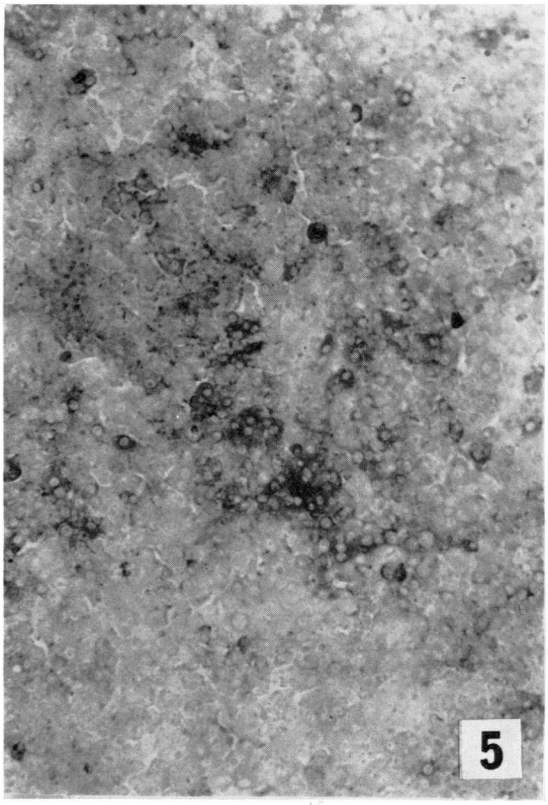

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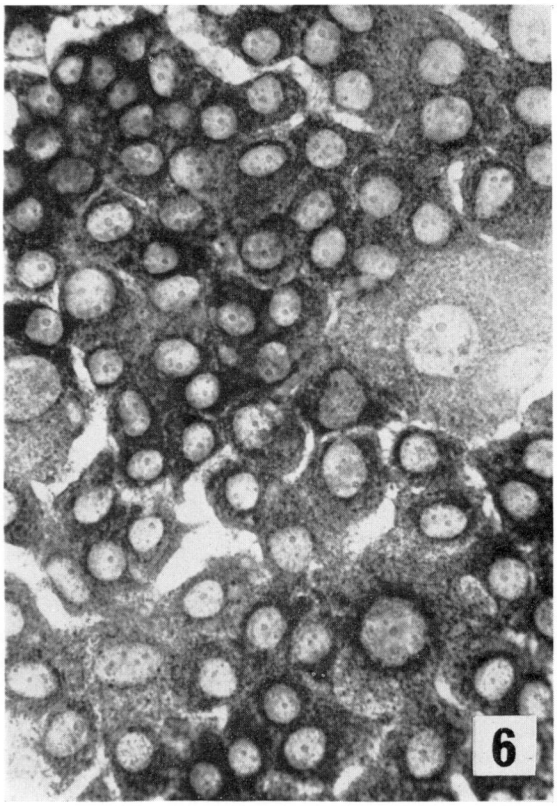

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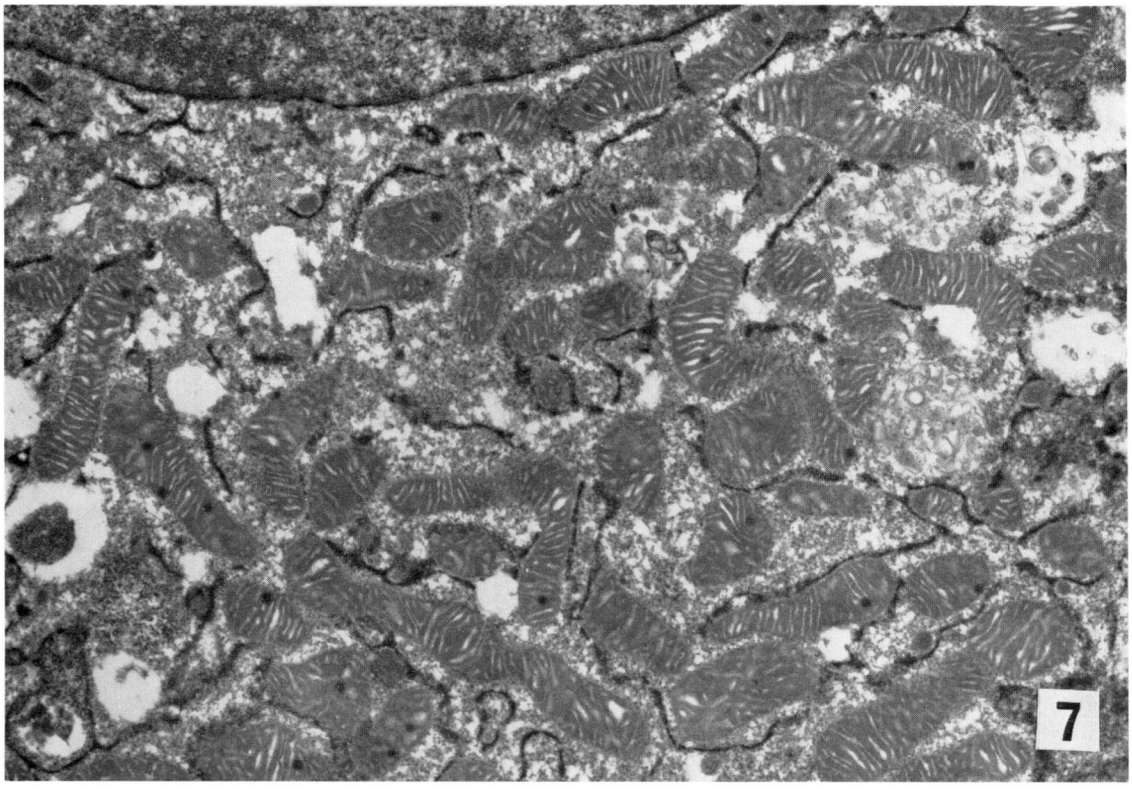

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