Effects of Hexosamines and Their Acetyl Derivatives on Aggregation of Rat Hepatoma Cells in Rotation Culture

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SUMMARY

The effects of hexosamines and their acetyl derivatives on the aggregation of 4-dimethylaminoazobenzene-induced rat hepatoma cells, dRLa-74 and dRLh-84, which have a low and high tumor-producing activity, respectively, were examined in rotation-mediated cell culture.

D-Glucosamine, D-galactosamine, and D-mannosamine had relatively little effect on aggregation of either strain of hepatoma cells at concentrations lower than 3 mM. At concentrations higher than 10 mM, some differential inhibitory effects on the aggregation of the two hepatoma cell lines were observed. D-Glutamine and D-galactosamine were more effective against the aggregation of dRLh-84 cells than in that of dRLa-74 cells, and the reverse was the case with D-mannosamine.

N-Acetyl-D-glucosamine, N-acetyl-D-galactosamine, and N-acetyl-D-mannosamine were relatively ineffective against the aggregation of dRLa-74 cells at all concentrations used. N-Acetyl-D-glucosamine had an inhibitory effect on the aggregation of dRLh-84 cells only at a concentration of 30 mM, while N-acetyl-D-galactosamine had a pronounced inhibitory effect at both 10 and 30 mM.

INTRODUCTION

It is well known that the mutual cohesiveness and sorting-out mechanism of constituent cells may play an important part in the process of histogenesis and organogenesis in the normal development of higher animals (7, 8, 28, 29). In the process of carcinogenesis or neoplastic transformation, alterations in 1 or more membrane systems of tumor cells are a regular and common feature. Examples of such alterations are: asocial tissue organization (1, 4, 6), alteration in cellular adhesiveness (1, 4, 6), defective “contact inhibition” of movement and growth (27), abnormal electrophoretic mobilities (5, 6), changes in antigenic properties (24), altered chemical composition (10), abnormal fusion capacity (23), and changes in cellular ionic communication (12, 15, 19).

Recently, it also has been found that carbohydrate-binding proteins and glycoproteins such as wheat germ agglutinin and concanavalin A brought about agglutination of tumor cells, but not of normal cells (2, 3, 11), and that the tumor-cell-specific agglutination was inhibited by N-acetyl-D-glucosamine (3) or α-methyl-D-glucopyranoside (11). This suggests that some changes in the glycoproteins, or their carbohydrate moiety, present on the surface membrane may take place following malignant transformation.

In the present experiments the effects of some hexosamines and their derivatives on the mutual cohesiveness of DAB-induced rat hepatoma cells with different tumor-producing activities were examined in rotation-mediated cell culture.

MATERIALS AND METHODS

Cells. Two rat hepatoma cell lines, dRLa-74 and dRLh-84, used in the present experiments, were provided by Dr. J. Sato, Okayama University. They were induced in 56-day-old Donryu rats that were fed 0.06% DAB for either 191 or 312 days. Livers macroscopically showing tumor morphology were taken out and cultured for more than 900 days with 40 subcultures. dRLa-74 cells had a low tumor-producing activity, and in 3 experiments they produced tumors in 4 of 9, 1 of 3, and 0 of 3 rats when the cells were inoculated into newborn rats i.e., i.p., and s.c., respectively. dRLh-84 cells had a high tumor-producing activity and produced tumors in 3 of 5, 5 of 8, and 2 of 3 rats when again inoculated i.e., i.p., and s.c. (26).

Both cell lines were maintained with their respective tumor-producing activities in stationary cultures in TD-40 flasks in Eagle's basal medium supplemented with 20% bovine serum and were subcultured every week after treatment with 0.25% trypsin (Difco Laboratories, Inc., Detroit, Mich., 1:250).

Rotation Culture. The dissociated cells obtained from monolayer cultures by trypsinization were rinsed 3 times with calcium- and magnesium-free Tyrode solution, dispersed in culture medium, and counted in a hemocytometer. Three-ml aliquots of cell suspension containing 3 X 10⁵ cells were distributed into 25-ml Erlenmeyer flasks, which were rotated on a gyratory shaker with a constant speed of 70 rpm at 38° for either 24 or 48 hr, as previously described (22). Each aggregate formed was photographed, and an average diameter was obtained from 2 diameters measured at right angles to each other. Aggregates of the dRLa-74 cells (24 or 32) chosen at random were measured (4 to 24 aggregates of the dRLh-84 cells) and the mean diameter and S.D. of the aggregates of...
each cell line was calculated. For comparison, the mean diameter of the aggregates formed in the presence of test substances was expressed as a percentage of that of aggregates formed in control normal medium.

Hexosamines and Derivatives. Three hexosamines and 3 N-acetylhexosamines were tested for their effects on the aggregation of rat hepatoma cells. These were D-glucosamine hydrochloride, D-galactosamine hydrochloride, D-mannosamine hydrochloride, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and N-acetyl-D-mannosamine (Nakarai Chemicals, Ltd., Kyoto, Japan). They were dissolved directly in culture medium, and the pH was adjusted to 7.2, sterilized through a millipore filter in a Swinney adaptor, and added to the culture media at concentrations of 0, 1, 3, 10, and 30 mM.

RESULTS

Effects of Hexosamines. When dRLa-74 cells in suspension were rotated in a gyrating flask in normal culture medium, they produced numerous cell clusters and established a characteristic aggregation pattern after 24 hr. Their surface was bumpy or rough (Fig. 1a).

When dRLa-74 cells were rotated in the presence of 1, 3, 10, or 30 mM D-glucosamine, the sizes of the aggregates formed after 24 hr were directly related to the concentrations of D-glucosamine added (Fig. 1, a to d). The smallest aggregates were found in the highest concentration of D-glucosamine. The mean diameters of aggregates formed in the presence of various concentrations of D-glucosamine are shown in Table 1.

When dRLa-74 cells were rotated for 48 hr, the characteristic pattern and the size of the aggregates were essentially the same as those obtained after 24 hr (Table 1). At 0, 1, and 3 mM concentrations of D-glucosamine, the mean diameter of the aggregates increased slightly; whereas in the presence of more than 10 mM D-glucosamine, the mean diameter decreased slightly.

On the other hand, when suspensions of dRLh-84 cells were rotated in normal medium, almost all cells in the suspension cohered with each other and formed large aggregates after 24 hr. Very few free cells were found to remain in the culture medium (Fig. 1f).

Addition of D-glucosamine resulted in smaller diameters than in the controls at concentrations of 1 and 3 mM and an increase in the number of free cells remaining in the culture medium (Fig. 1, g and h; Table 1).

At D-glucosamine concentrations higher than 10 mM, the cells formed small and numerous cell clusters, but not large aggregates. There was a reduction in size and an increase in number as the concentrations of D-glucosamine were increased (Fig. 1, i and j; Table 1). After 48 hr in rotation culture, the pattern and the size of the aggregates were also essentially the same as those obtained after 24 hr (Table 1).

Histological observation revealed that the aggregates of dRLa-74 cells consisted of cells arranged with some of the regularity of hepatic tissue (Fig. 2a), whereas those of dRLh-84 cells consisted of cells that were more closely arranged with less regularity than in those of dRLa-74 cells (Fig. 2b).

When the mean diameters of aggregates of dRLa-74 and dRLh-84 cells obtained after 24 or 48 hr in the presence of various concentrations of D-glucosamine were expressed as a percentage of those obtained in control normal medium (Chart 1), it was found that the effect of D-glucosamine on the aggregation of both dRLa-74 and dRLh-84 cells increased gradually at higher concentrations, although greater effects were produced on dRLh-84 cells at concentrations of 10 and 30 mM.

D-Galactosamine, when added to the culture medium at various concentrations, produced similar inhibitory effects on the aggregation of both dRLa-74 and dRLh-84 cells, although

<table>
<thead>
<tr>
<th>Hr in rotation culture</th>
<th>Concentration of D-glucosamine (mM)</th>
<th>dRLa-74</th>
<th>dRLh-84</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. measured</td>
<td>Diameter (mm) Mean ± S.D.</td>
<td>No. measured</td>
</tr>
<tr>
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<td>24</td>
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<tr>
<td></td>
<td>1</td>
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<tr>
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<td>3</td>
<td>32</td>
<td>0.154 ± 0.035</td>
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<tr>
<td></td>
<td>10</td>
<td>32</td>
<td>0.131 ± 0.029</td>
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<td>30</td>
<td>32</td>
<td>0.112 ± 0.027</td>
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<td>30</td>
<td>32</td>
<td>0.110 ± 0.024</td>
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</table>
Effects of Hexosamines on Hepatoma Cells

Chart 1. Effects of D-glucosamine on the aggregation of rat hepatoma cells, dRLa-74 and dRLh-84, in rotation culture for 24 or 48 hr. Cell suspensions, each containing 3 x 10⁵ cells in 3 ml culture medium containing 0, 1, 3, 10, or 30 mM D-glucosamine, were rotated on a gyratory shaker at 70 rpm at 38° for 24 or 48 hr. Harvested aggregates were photographed, and each was measured along 2 diameters at right angles. The means of these 2 diameters were calculated for either 24 or 32 aggregates for dRLa-74 cells and for a range of between 4 and 24 aggregates for dRLh-84 cells and were expressed as percentages of that in each control culture.

The effects were slightly less than those of D-glucosamine.

In the presence of D-mannosamine, a much smaller mean diameter of dRLa-74 cell aggregations was found at concentrations higher than 10 mM and of dRLh-84 cell aggregations at 30 mM, although little inhibitory effect was found at concentrations lower than 3 mM (Chart 2).

Effects of N-Acetyhexosamines. N-Acetyl-D-glucosamine added to the culture medium was ineffective or only slightly effective in altering the pattern of dRLa-74 cell aggregation at all concentrations tested up to 30 mM and only moderately effective against dRLh-84 cell aggregation at 30 mM (Chart 3).

N-Acetyl-D-galactosamine had a pronounced inhibitory effect on the aggregation of only dRLh-84 cells at concentrations higher than 10 mM, whereas no effect or sometimes only a slight effect was found at all concentrations tested against the aggregation of dRLa-74 cells (Chart 4).

N-Acetyl-D-mannosamine was the most ineffective among the hexosamines and their acetyl derivatives used in the present experiments against aggregations of either dRLa-74 or dRLh-84 cells. No noticeable decrease in the mean diameter of aggregates was found even in the presence of 30 mM N-acetyl-D-mannosamine.

**DISCUSSION**

Rat hepatoma dRLh-84 cells, which have a high tumor-producing activity, form aggregates larger in diameter than those of dRLa-74 cells, which have a low tumor-producing activity. This tendency towards an increase in the diameter of aggregates following neoplastic or malignant transformation coincides with the results that had been obtained previously with Rous sarcoma virus-infected chick cells, mouse mammary...
were rotated on a gyratory shaker at 70 rpm at 38° for 24 or 48 hr. The 48 hr. Cell suspensions, each containing 3 × 10⁶ cells in 3 ml culture medium containing 0, 1, 3, 10, or 30 mM N-acetyl-D-galactosamine, were rotated on a gyratory shaker at 70 rpm at 38° for 24 or 48 hr. The procedure for calculating the aggregate size was the same as that used in the experiment shown in Chart 1.

Chart 4. Effects of N-acetyl-D-galactosamine on the aggregation of rat hepatoma cells, dRLa-74 and dRLh-84, in rotation culture for 24 or 48 hr. Cell suspensions, each containing 3 × 10⁶ cells in 3 ml culture medium containing 0, 1, 3, 10, or 30 mM N-acetyl-D-galactosamine, were rotated on a gyratory shaker at 70 rpm at 38° for 24 or 48 hr. The procedure for calculating the aggregate size was the same as that used in the experiment shown in Chart 1.

gland tumor cells, and mouse plasma tumor cells, when these cell aggregates were compared in size with the respective control aggregates of normal cells in rotation culture (16, 17). Normal liver cells from younger chick embryos showed a tendency to form larger aggregates than did liver cells from older embryos (18). Larger aggregates consisted of more constituent cells which may have cohesive activity higher than those in smaller aggregates. The increase in aggregate-forming activity shown by hepatoma cells with higher tumor-producing activity may parallel the behavior of younger, less differentiated normal embryonic cells.

D-Glucosamine at a concentration of 0.25% (ca. 14 mM) inhibited the normal adhesion and histogenetic association of dissociated 8- and 10-day embryonic chick liver cells in rotation culture (9). In the present experiments 3 hexosamines, D-glucosamine, D-galactosamine, and D-mannosamine, had relatively little effect on the aggregation of either dRLa-74 or dRLh-84 cells at concentrations lower than 3 mM. At concentrations higher than 10 mM some differential effect on the aggregation of dRLa-74 and dRLh-84 cells was observed. At these concentrations, D-glucosamine and D-galactosamine were more effective against dRLh-84 cell aggregation than against dRLa-74 cell aggregation, whereas D-mannosamine was more effective against dRLa-74 cell aggregation than against dRLh-84 cell aggregation.

On the other hand, the 3 N-acetylhexosamines used were relatively ineffective against dRLa-74 cell aggregation at concentrations up to 30 mM. I observed an interesting effect for N-acetyl-D-glucosamine and N-acetyl-D-galactosamine on dRLh-84 cell aggregation; the former had an inhibitory effect at a concentration of 30 mM and the latter produced a pronounced inhibitory effect at 10 and 30 mM on dRLh-84 cell aggregation, whereas these acetylhexosamines were ineffective against dRLa-74 cell aggregation. For embryonic normal liver cells, N-acetyl-D-glucosamine at concentrations up to 0.75% (ca. 34 mM) had no effect on their aggregation (9). The differential inhibitory effects of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine may be specific for hepatoma cells with a high tumor-producing activity.

It has been shown that glucosamine-14C was incorporated in the aggregates of dissociated embryonic chick neural retina cells and that its incorporation was directly related to the degree of aggregation (25). In embryonic chick liver and cartilage cells changes in intercellular material in the process of cell aggregation were visualized by lanthanum staining under an electron microscope (14). This surface material was absent from cell surface in freshly isolated cells but appeared after 1 hr of cultivation and increased in its amount as the aggregation proceeded. Enzymatic treatment of aggregates suggested that the lanthanum staining material may be mucopolysaccharide. These results indicate that hexosamines or mucopolysaccharides may participate in the cellular cohesion in the process of aggregate formation.

Among the mucopolysaccharides present in a variety of tissues of higher animals, heparin contains D-glucosamine as a component sugar, chondroitin sulfates A, B, and C contain N-acetyl-D-galactosamine, and hyaluronic acid contains N-acetyl-D-glucosamine (13). The differential effectiveness in the present experiments of hexosamines and their acetyl derivatives against the aggregation of rat hepatoma cells with different tumor-producing activities may suggest that there has been a change in the amino sugar or mucopolysaccharide composition of the cellular membrane of hepatoma cells.

Recently, it has been found that in vitro both D-glucosamine and D-mannosamine at a concentration of 50 mM induce vacuolization of the cytoplasm and retraction of the cytoplasm around the nucleus in Ehrlich ascites carcinoma and Sarcoma 180 tumor cells (20). In the Walker carcinoïda, in the liver, and in the kidney of adult rats that were given with D-glucosamine, a slight dilation of the cisternae of the endoplasmic reticulum and Golgi sacs was observed (21). However, the concentrations of hexosamines and their derivatives used in the present experiments were lower than those used in the above experiments and in addition at concentrations both higher and lower than that of the glucose used in the culture medium (5.5 mM). It is uncertain whether the inhibition of aggregation by hexosamines and their derivatives is due to their competitive reaction with matching binding sites of the cell surface or to biochemical changes in the surface membrane through a metabolic pathway within the cells. The temperature dependence of glucosamine inhibition (9) suggests the latter possibility.

REFERENCES
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Fig. 1. a, aggregates formed in 24-hr rotation culture of rat hepatoma dRLa-74 cells in control normal medium; b, aggregates formed in 24-hr rotation culture of rat hepatoma dRLa-74 cells in the presence of 1 mM D-glucosamine; c, aggregates formed in 24-hr rotation culture of rat hepatoma dRLa-74 cells in the presence of 3 mM D-glucosamine; d, aggregates formed in 24-hr rotation culture of rat hepatoma dRLa-74 cells in the presence of 10 mM D-glucosamine; e, aggregates formed in 24-hr rotation culture of rat hepatoma dRLa-74 cells in the presence of 30 mM D-glucosamine; f, portion of an aggregate formed in 24-hr rotation culture of rat hepatoma dRLh-84 cells in control normal medium; g, portion of an aggregate formed in 24-hr rotation culture of rat hepatoma dRLh-84 cells in the presence of 1 mM D-glucosamine; h, portion of an aggregate formed in 24-hr rotation culture of rat hepatoma dRLh-84 cells in the presence of 3 mM D-glucosamine; i, aggregates formed in 24-hr rotation culture of rat hepatoma dRLh-84 cells in the presence of 10 mM D-glucosamine; j, aggregates formed in 24-hr rotation culture of rat hepatoma dRLh-84 cells in the presence of 30 mM D-glucosamine. × 120.

Fig. 2. a, section of an aggregate formed in 24-hr rotation culture of rat hepatoma dRLa-74 cells in normal medium; b, section of an aggregate formed in 24-hr rotation culture of rat hepatoma dRLh-84 cells in normal medium. × 600.
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