Properties of Acidic Saccharides Produced by B16 Melanoma Cells Treated with 1-Methyl-3-isobutylxanthine

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SUMMARY

A cyclic nucleotide phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine, promoted the differentiation and maturation of B16 melanoma cells, phenomena associated with biological alterations in the surface properties of the cells. 1-Methyl-3-isobutylxanthine inhibited cell replication and increased the intracellular content of melanin and cyclic adenosine 3',5'-monophosphate. Significantly greater amounts of sialoglycoproteins were associated with 1-methyl-3-isobutylxanthine-treated cells. However, the total amount of [3H]glucosamine incorporated into anionic polysaccharides (both sialoglycopeptide and mucopolysaccharides) was not significantly changed.

INTRODUCTION

Present studies suggest that tumor cells may undergo morphological and functional changes which represent spontaneous maturational sequences (19). The final product of such changes is a highly differentiated, fully mature cell which often has lost replicative activity and some neoplastic traits. Such maturational sequences have been seen in both animal and human tumors and involve a variety of tumor types including leukemia (7), neuroblastoma (21, 22), seminoma (19), squamous cell carcinoma (19, 30), and melanoma (9, 27, 29). In some of these systems, the evidence supports the view that cyclic nucleotides may be involved in the regulation of tumor cell differentiation and maturation (11, 18, 33).

Murine melanomas have been especially useful model systems for the study of the role of cyclic nucleotides in differentiation. Melanogenesis in these systems is a process that involves the interaction of a single enzyme, tyrosinase, with its principal substrate, L-tyrosine (20). A number of studies in both the Cloudman S-91 melanoma and the B16 melanoma suggest an involvement of cyclic nucleotides in the activation of tyrosinase in the S-91 melanoma, treatment of the cells with dibutyryl-cAMP (12) or α-melanocyte-stimulatory hormones (35, 36) stimulates melanogenesis. Treatment with cAMP, dibutyryl-cAMP, or the phosphodiesterase inhibitors caffeine or theophylline accelerates melanogenesis in the B16 melanoma (13, 14).

Maturation in the B16 melanoma, whether induced by theophylline (J. W. Kreider, D. R. Wade, M. Rosenthal, and T. Densley. Maturation and Differentiation of B16 Melanoma Cells Induced by Theophylline Treatment, submitted for publication) or developing spontaneously in association with the attainment of confluency, is associated with an elevation in cAMP. Mature cells enlarge, become distended with melanin granules, and lose replicative activity and colony-forming capacity. Mature melanocytes in suspension attach to a plastic substrate more quickly than do immature cells, are more difficult to detach from the substrate, and are resistant to complement-dependent antibody cytolysis (W. Zajac, J. T. Flickinger, and J. W. Kreider. Modification of Membrane Properties of B16 Melanoma Cells, in preparation).

The synthesis of mucopolysaccharides is often viewed as a differentiated cellular function. A well-studied example of this is chondroitin sulfate production by chick chondrocytes (15, 28), which is inhibited by growth of the cells in the presence of 5-bromodeoxyuridine, commonly recognized as interfering with differentiated expression (1, 4, 28, 32, 34, 37). Cell attachment, adhesion, and antigen expression are all cell membrane functions that are dependent upon the quality and quantity of glycoproteins produced by the cells (5). We have previously characterized the anionic saccharides (sialoglycopeptides) and mucopolysaccharides synthesized by the B16 melanoma (24). A series of high-molecular-weight chondroitin sulfate molecules with varying sulfate content comprised a large amount (44%) of the total radioactivity incorporated from [3H]GluNH₂. These molecules were absent in a mouse iris melanocyte culture that produced primarily hyaluronic acid. 5-Bromodeoxyuridine treatment of the mouse melanoma cells resulted in inhibition of synthesis of the high-molecular-weight chondroitin sulfates, diminished sialoglycopeptide(s) synthesis, and induced synthesis of hyaluronic acid (26).

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2 Career Development Award, 7-K4-CA38,809.
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The purpose of the present study was to determine whether anionic polysaccharide synthesis was altered in B16 melanoma cells induced to mature as a result of treatment with a new and especially potent phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine (3).

MATERIALS AND METHODS

Cell Cultures. The B16 melanoma cell line studied was the 3rd clone sequentially isolated (B16C3). The cells were propagated in Eagle's minimal essential medium with Earle's balanced salt solution supplemented with heat-inactivated 10% fetal calf serum, sodium pyruvate, nonessential amino acids, twice the usual concentration of vitamins, 10 units penicillin G per ml, and 10 μg streptomycin sulfate per ml. All components with the exception of the antibiotic solution and the fetal calf serum were obtained from Grand Island Biological Company, Grand Island, N. Y. Cells were incubated at 36° in 5% CO₂; 95% air and were propagated in 16-oz glass prescription bottles. The cells became confluent at 1.5 to 2.0 × 10⁶ cells/bottle and were routinely subcultured to approximately 2 × 10⁶ cells/bottle by suspension with 0.02% EGTA in CMF-PBS solution.

Cultures were routinely tested for Mycoplasma contamination (24) and bacterial contamination by the use of thioglycollate and tryptose phosphate broth and for yeast and mold contamination by the use of Sabouraud’s media; no contamination was ever observed.

Reagents. Tritiated GluNH₂ (D-glucosamine[6-3H]hydrochloride, 7.3 Ci/m mole) and Na₂³⁵SO₄ (carrier free) were obtained from New England Nuclear, Boston, Mass. 1-Methyl-3-isobutylxanthine was dissolved in 5.0 mM CMF-PBS to the cells 15 mm before the glass beads were added, and the fractions were incubated for 24 hr at 37°. After dialysis, the EGTA-extractable fraction was adjusted to pH 6.5 and Pronase-digested (10 mg) as above. Following Pronase digestion, fractions were dialyzed against 3 changes of 0.2 N NaCl at 4° and against deionized H₂O for approximately 24 hr at 4°. The solutions were made 0.1 N in hydroxide ion concentration, and after 24 hr at room temperature (20–25°) they were neutralized and dialyzed for 24 to 48 hr against several changes of deionized H₂O. CPC, 2%, 1.5 ml in 0.03 M NaCl, was then added for each 100 ml of each fraction followed by 100 mg of washed Celite. These conditions allow approximately 95% recovery of the CPC-precipitable sialoglycopeptide(s) and mucopolysaccharides. The CPC-soluble fraction contains 75 to 80% of the incorporated tritium label present at this stage.

The precipitates were collected by centrifugation at 25,000 x g for 20 min and washed with the CPC solution until the radioactivity present in the wash was negligible; 3 to 4 washes were usually sufficient. The precipitate was then extracted 4 times with a total of 50 ml each with 0.4, 0.8, 1.2, and 2.0 N NaCl solutions; the 1st elution was with 20 ml and the other 3 were with 10 ml. All the elutions were carried out at 37° for 10 min except for 1 of the last 3 elutions which was for 18 hr at room temperature. These conditions were effective in removing 98 to 100% of the counts from the precipitate. The individual salt eluate fractions were filtered through Millipore HA filters under pressure and dialyzed at 43° to remove residual CPC. The dialyzed fractions were lyophilized and subjected to analytical evaluation.

Miscellaneous Assays. Cell counts were determined by triplicate counts with a hemocytometer. Viability was determined by trypan blue exclusion.

Cell volumes were measured with Model B Coulter Counter (Coulter Corporation, Hialeah, Fla.) calibrated with ragweed pollen grains. Cells were suspended in Isoton at approximately 80 × 10⁶ cells/ml and continually mixed during counting.

cAMP assays were performed by Dr. Lawrence Demers using the procedure of Steiner et al. (31) as modified by
D'Armento et al. (6). Chemical analysis of the salt eluate fractions used previously described methods (28). Exclusion chromatography on porous glass beads was performed by the general procedures described by Satoh et al. (24). Unlabeled reference compounds, prepared in this laboratory, were included in each chromatography. The recovery of labeled material after porous glass bead exclusion chromatography was routinely 90 to 100%. The radioactivity of individual fractions was measured in aquasol in an Intertechnique SL36 liquid scintillation spectrometer, and dual isotope calculations were performed with a standard program. Chromatography or dialysis was used to monitor the susceptibility of various peaks to specific enzymes. The activities of the neuraminidase and heparitinase were checked using ditrisialoganglioside (provided by Dr. C. Schengrund) or heparitin sulfate (9% sulfate, provided by Dr. A. Linker). Vibrio cholerae neuraminidase (Calbiochem B grade) used at 0.25 unit/assay and heparitinase (provided by Dr. A. Linker) were used for the determination of sialic acid and heparitin sulfate, respectively, in each sample.

The products released by V. cholerae neuraminidase were shown (24) to consist of N-acetylenuraminic acid:N-glycolyneuraminic acid (4:1). It was also shown that this treatment released all the available sialic acid when total sialic acid was determined by the method of Liu (16). The reaction mixture contained in a volume of 550 μl 0.25 unit V. cholerae neuraminidase, 0.1 N sodium acetate with 1 mM CaCl2 (pH 5.6), and substrate. Incubation conditions were for 24 hr at 37°. The enzyme was free of chondroitinase, hyaluronidase, and heparitinase activities as assessed using radioactive substrates.

The amount of heparitinase used was sufficient to render 200 μg of heparitin sulfate (9% sulfate) completely dialyzable. The products were characterized mainly as disaccharides by the increase in A235 with time. The heparitinase was devoid of chondroitinase and sialidase activity when assayed using labeled substrates isolated from previous experiments. The reaction mixture contained in a volume of 550 μl 0.1 N sodium acetate (pH 7.2), substrate, and the heparitinase. Incubation conditions were 24 hr at 37°.

RESULTS

Effects of 1-Methyl-3-isobutylxanthine on Cell Numbers, Size, and Melanin and cAMP Contents. Cultures treated with 0.1 mM 1-methyl-3-isobutylxanthine for 4 days showed a reduction in cell population density of 13 to 19% (Table 1), but the cell volumes of the 1-methyl-3-isobutylxanthine-treated cultures were somewhat greater than the controls (Chart 3). There were no effects of 1-methyl-3-isobutylxanthine on cell viability as estimated by trypan blue exclusion, nor did 1-methyl-3-isobutylxanthine irreversibly inhibit cellular replication (Table 2). The cAMP content of 1-methyl-3-isobutylxanthine-treated cells was increased greater than 2-fold over the controls (Table 3). Melanogenesis was markedly stimulated by 1-methyl-3-isobutylxanthine. Melanin production in the treated cultures was conspicuous on the 2nd day after addition, while the controls did not produce visible amounts of melanin until the 4th day. The melanin content of cells on the 4th day is presented in Table 4.

Anionic Saccharide and Mucopolysaccharide Assays. Porous glass bead chromatography of the salt eluate fractions from 1-methyl-3-isobutylxanthine-treated cells revealed no significant alterations on comparison with the control fractions; typical results are shown in Charts 1 and 2. The differences observed for the 0.8, 1.2, and 2.0 M salt eluates were not large and were minimized when the incorporated dpm were related to cell numbers. These fractions were a

Table 4
Melanin content of control and 1-methyl-3-isobutylxanthine-treated cells 4 days after exposure to 0.1 mM 1-methyl-3-isobutylxanthine.

<table>
<thead>
<tr>
<th>Cell no.</th>
<th>Melanin (µg)</th>
<th>Melanin (µg/cell × 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>2.96</td>
<td>360</td>
</tr>
<tr>
<td>1-Methyl-3-isobutylxanthine 1</td>
<td>2.49</td>
<td>954</td>
</tr>
<tr>
<td>Control 2</td>
<td>2.08</td>
<td>923</td>
</tr>
<tr>
<td>1-Methyl-3-isobutylxanthine 2</td>
<td>1.83</td>
<td>1134</td>
</tr>
</tbody>
</table>

renders these differences even less significant. The principal changes observed were in the 0.4 M salt eluate fraction.

The 0.4 M NaCl fractions were subdivided following porous glass bead chromatography as illustrated in Chart 1. Each combined peak was analyzed by chromatography for neuraminidase-released ³H dpm [assumed to be mostly N-acetylneuraminic acid (28)], amino sugar content, and heparitinase susceptibility. The results summarized in Tables 6 and 7 indicate that the sialoglycopeptide content of the experimental media fraction is reduced compared to the control fraction, whereas that of the EGTA-soluble fraction is significantly increased. The sialic acid content of the experimental cell-associated fractions was also increased compared to the control with the percentage increase rather large (~ 50), although the absolute magnitude was not as great as in the EGTA fraction. The amount of material rendered susceptible to neuraminidase was analyzed both by chromatography and by assaying dialyzable radioactivity following incubation with the enzyme; both procedures gave equivalent results. Since the total sialic acid values in the 0.4 M NaCl eluate fractions of the experimental and control were comparable, it appears that the sialic acid-containing glycopeptide(s) are not released to the medium from the experimental cells to the same extent as from the control cells. The amino sugar composition is similar to that of the controls, and the heparitin sulfate differences are small.

mixture of high-molecular-weight chondroitin sulfate and a lower-molecular-weight heparitin sulfate. There was an indication that the heparitin sulfate content was slightly increased in the 1-methyl-3-isobutylxanthine-treated cells, but the magnitude of the changes was slight. Data are summarized in Table 5. Since the volume of experimental cells was somewhat greater than that of the controls (Table 1; Chart 3), correction on the basis of cell volume (mass)
Table 5
Summary of CPC elution results

Data for the salt eluate fractions are expressed as dpm/10^6 cells. In a 2nd experiment utilizing larger quantities of isotopic label, substantially similar results were obtained. The percentage of 3H label was highest in the 0.4 M control media fraction (34.8) and the 0.8 M experimental media fraction (30.4).

<table>
<thead>
<tr>
<th></th>
<th>0.4 M</th>
<th>0.8 M</th>
<th>1.2 M</th>
<th>2.0 M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>Media 3H</td>
<td>93.0</td>
<td>24.9</td>
<td>68.3</td>
<td>64.5</td>
</tr>
<tr>
<td>35S</td>
<td>1.7</td>
<td>1.2</td>
<td>14.6</td>
<td>12.1</td>
</tr>
<tr>
<td>EGTA-soluble 3H</td>
<td>15.7</td>
<td>34.7</td>
<td>13.4</td>
<td>22.8</td>
</tr>
<tr>
<td>35S</td>
<td>0.3</td>
<td>0.3</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Cell-associated 3H</td>
<td>1.9</td>
<td>6.8</td>
<td>3.9</td>
<td>2.2</td>
</tr>
<tr>
<td>35S</td>
<td>0.2</td>
<td>0.2</td>
<td>0.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 6
Analytical data on 0.4 M salt eluate fractions

The data for the media and EGTA-soluble fractions refer to peaks isolated after porous glass bead chromatography as illustrated in Chart I. The cell-associated fraction contained insufficient radioactivity for chromatographic analysis, and the data are for the total fraction. The figures in each case represent percentage of 3H dpm within the given fraction: those for GluNH₂ and galactosamine were determined after hydrolysis by resolution on the amino acid analyzer, and those for sialic acid were determined by assessing dialyzable radioactivity after neuraminidase treatment.

<table>
<thead>
<tr>
<th></th>
<th>GluNH₂</th>
<th>Galactosamine</th>
<th>Sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
<td>III</td>
<td>II</td>
</tr>
<tr>
<td>Media</td>
<td>Control</td>
<td>5.5</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>13.1</td>
<td>17.3</td>
</tr>
<tr>
<td>EGTA-soluble</td>
<td>Control</td>
<td>4.8</td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>2.0</td>
<td>20.3</td>
</tr>
<tr>
<td>Cell-associated</td>
<td>Control</td>
<td>15.4</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>9.1</td>
<td>41.5</td>
</tr>
</tbody>
</table>

DISCUSSION

These studies demonstrated that 1-methyl-3-isobutylxanthine, an inhibitor of cyclic nucleotide phosphodiesterase, stimulated maturation in B16 melanoma in a manner similar to that of other phosphodiesterase inhibitors, e.g., caffeine and theophylline (13, 14). Although melanin and cAMP contents were enhanced by 1-methyl-3-isobutylxanthine treatment, no significant effects were observed in cell viability. The ability of the cells to replicate after 4 days of growth in the presence of 1-methyl-3-isobutylxanthine was unaffected as assayed by replacement with fresh B16 media (Table 2). Despite the alterations in surface properties, evident only in mature B16 melanoma cells, modest changes were evident in polysaccharides. The major effect on complex saccharide metabolism was on the release of sialoglycopeptide(s) to the medium. The rate of release was retarded by 1-methyl-3-isobutylxanthine treatment, and there was an increase in the amount of EGTA-extractable sialoglycopeptide.

The results suggest that the productions of mucopolysaccharide and melanin are controlled by separate mechanisms, a conclusion supported by our earlier observations with an amelanotic B16 line. An alternate explanation for the lack of dramatic effects on the polysaccharides might be that, at the point in time at which the controls were harvested, monolayer confluence had already been achieved. Our previous studies have indicated that an elevation of intracellular cAMP occurs in the postconfluency period (J. W. Kreider, L. M. Demers, and M. E.
Schmoyer. Alterations in Cyclic Nucleotides during Spontaneous Maturation and Differentiation of B16 Melanoma Cells in Culture, submitted for publication). However, this possibility does not seem likely since the cells in the 1-methyl-3-isobutylxanthine-treated cultures contained twice as much cAMP as the controls. Further, our earlier studies have not detected differences in anionic polysaccharides in replicating or post-confluent B16C3 cultures (J. Banks and E. A. Davidson, unpublished results).

In the current experiments, the radioactively labeled material not precipitated by CPC was also examined. This fraction contained sialoglycopeptides and glycopeptides that differed from the CPC-precipitable sialoglycopeptide in amino sugar content (75 to 80% GluNH₂), by electrophoretic mobility on cellulose acetate sheets, and by susceptibility to V. cholerae neuraminidase digestion. This digestion made the entire glycopeptide dialyzable (M. W. 12,000).

A comparison of the sialoglycopeptides found in the EGTA-extractable fraction with those cell surface components removed by proteolytic treatment or by proteolytic activity of transformed cells (10) may aid in understanding the mechanism(s) by which the sialoglycopeptide(s) are bound to the cells since covalent interactions appear to be ruled out by the dissociation conditions used.

Others have examined the effects of cyclic nucleotide phosphodiesterase inhibitors on complex saccharide synthesis. Goggins et al. (8) have examined the effect of simultaneous addition of dibutyryl-cAMP and theophylline on mucopolysaccharide synthesis of SV40- or polyoma virus-transformed 3T3 cells. If it is assumed that intracellular cAMP levels were elevated (this information was not given) and caused an elevation in the rate of mucopolysaccharide synthesis comparable to that of 3T3 cells, it would seem that the effects are quite different from those seen for the B16 mouse melanoma. The mucopolysaccharide dermanan sulfate was present but sialoglycopeptides were not studied. It has also been shown that SV40 transformation of hamster cells causes an increase in mucopolysaccharide synthesis (25). The particular cell line and/or transforming virus(es) studied may play some role in the nature of the anionic polysaccharides elaborated by the transformed cells and perhaps on the induced level of cAMP. Further study may indicate whether certain anionic polysaccharide compositions are characteristic of different types of tumors (17) and may be predictive of biological behavior.

The recent publication of Angello and Hauschka (2) raises questions concerning any differences observed in the glycopeptide(s) produced by transformed cells compared to their normal (untransformed) counterparts if these differences are of a small nature. The rigorous isolation and characterization methods utilized in our study eliminate the contamination problems described by them.

REFERENCES


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