Repression of Sialopeptidase Activity in HeLa Cells by Prednisolone*

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SUMMARY

HeLa cells incubated as stationary cultures in the presence of low concentrations of prednisolone (11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) have a reduced capacity to grow beyond the stage of confluence and usually do not form several layers of cell growth. This effect of the hormone analog seems to be associated with the accumulation of sialopeptides, normally released into the medium, at the cell periphery. Two such sialopeptide fractions have been isolated from the supernatant medium from cultures of the S3G strain of HeLa cells.

Treatment of plasma membrane preparations with supernatant fluid from high speed centrifugation resulted in the release of sialopeptides that had an elution pattern from Dowex-formate columns similar to the elution pattern of the sialopeptides released into the medium by intact HeLa cultures. This proteolytic activity was markedly reduced in preparations from HeLa cells grown in the presence of 4.1 X 10⁻⁵ M prednisolone.

The results give further support to the idea that surface glycopeptides are involved in the control of growth in cultures of epithelioid cells of neoplastic origin. They offer a possibility for studying such changes through hormonal manipulation of cell cultures.

In contrast to the studies of growth control of fibroblasts by cyclic adenosine 3′,5′-monophosphate (2-4), little information is available in the literature concerning regulation of growth in cultured cells of the epithelioid type. Such established cell strains, usually of neoplastic origin, lack density-dependent growth inhibition and, when grown in a perfusion system, form multiple cell layers (5). It would appear that in these cells the supply of nutrients and removal of wastes are the only factors affecting growth. However, when grown in the presence of relatively low concentrations of glucocorticoids, HeLa cells show a marked reduction in growth rate without any obvious signs of toxicity or cell degeneration. The decreased growth rate may be demonstrated under both intermittent (6) and perfusion (7) feeding schedules. The cells become enlarged because of an increased protein and RNA content (6) and, in some cases, they contain more sialic acid than untreated controls (8). Significantly, alkaline phosphatase, which frequently becomes elevated in HeLa cultures after treatment with glucocorticoids, contains large amounts of sialic acid (9) and is located at the cell periphery. Possibly, the increase in the amount of carbohydrate-containing material constituting the glycocalyx layer plays a role in restoring some degree of density-induced inhibition in HeLa cells. A model for the involvement of the cell “cover layer” in the regulation of growth has been developed by Burger and Noonan based on their work with virally transformed 3T3 line (10).

Recently we have reported that the accumulation of sialic acid residues at the periphery of HeLa cells treated with prednisolone is the result of reduced shedding of sialopeptides into the culture medium and is not a consequence of an increase in net synthesis of sialic acid (11). The present paper provides evidence that the effect is by repression, in cells grown in the presence of prednisolone, of an enzyme which releases sialopeptides.

MATERIALS AND METHODS

Cell Cultures and Media—A detailed description of the procedure for cultivating the HeLa S3G cells was provided in a recent paper (12). The original culture of this strain was a generous gift from Dr. M. J. Griffin from the Oklahoma Medical Research Foundation. The cells were grown in Eagle's minimal essential medium (MEM) purchased from Baltimore Biological Laboratories, Baltimore, Maryland. The medium was supplemented with 10% calf serum (Flow Laboratories, Rockville, Maryland), 100 units of penicillin per ml and 100 μg of streptomycin per ml. Unless otherwise stated, the cells were incubated either in the presence or in the absence of 2.0 μg (5.5 X 10⁻⁶ M) of prednisolone per ml for 72 hours at 37°, at which time the cells had reached confluence. They were harvested by scraping into cold (4°) 0.9% NaCl solution and were collected by centrifugation at 1,000 X g. The cells were then washed three times with 0.9% NaCl solution, resuspended, and then homogenized in a Dounce homogenizer. The disrupted cells were subjected to high speed centrifugation (100,000 X g for 1 hour), and the supernatant fluid was used as a source of proteolytic enzyme.

Preparation of 14C-labeled HeLa Cell Membranes—HeLa cell

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1 The trivial name used is: prednisolone, 11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione.
membranes were prepared in order to study the release of sialopeptides. The cells were grown for 72 hours in MEM containing 7.5 μCi of [14C]glucose per ml. Plasma membrane from such labeled cells was prepared as described by Brunette and Till (13). The method involves homogenization of cells in a hypotonic solution of ZnCl₂ followed by isolation of the plasma membrane fraction in a two-phase system consisting of polyethylene glycol 6000 and dextran 500 (Pharmacia, Stockholm). The plasma membrane preparation was monitored by phase microscopy. The final fraction contained approximately 150 cpm of protein per μg.

**Assay System for Sialopeptide-releasing Activity**—The release of sialopeptides from membrane preparations was followed by incubating approximately 200 μg of membrane protein with the 100,000 × g supernatant from HeLa cell homogenates, equivalent to 100 μg of protein. The incubation was carried out, unless stated otherwise, at 37° in the presence of 0.1 M Tris-HCl buffer, pH 7.5. After different incubation times, the reaction was stopped by 7-fold dilution of the reaction mixture and the non-solubilized material was separated by centrifugation at 12,000 × g for 5 min at 0°. The supernatant fluid was collected and was subjected to further analysis. This method of stopping the reaction was used in preference to heat or acid denaturation in order to prevent degradation of the sialopeptides.

**Isolation of Sialopeptides**—Sialopeptides were isolated by column chromatography as described earlier (11). A 1.5-ml portion of the supernatant material after incubation of the membranes was placed on a column (0.9 cm × 10 cm) of Dowex-formate and washed with 6 ml of distilled water. The adsorbed material was then separated by a stepwise elution with 3.0-ml portions (≈1.5 retention volumes) of increasing concentrations of formic acid. Of each fraction, 1 ml was evaporated to dryness and used for the determination of radioactivity. The protein content of the sialopeptide was determined by the method of Lowry et al. (14).

**Determination of Radioactivity**—For the determination of radioactivity by scintillation counting, the dried samples were dissolved in Soluene (Packard) and counted in the Packard Model 3375 Tri-Carb liquid scintillation spectrometer. The counting efficiency for 14C as determined by internal standard was approximately 75%.

**Chemicals**—d-[14C]Glucose (specific activity, 360 mCi per mmole) was obtained from Amersham-Searle Corp., Arlington Heights, Illinois. All other chemicals, obtained from several commercial sources, were analytical grade. Sephadex G-200 was purchased from Pharmacia, Uppsala, Sweden.

**RESULTS**

**Release of Sialopeptides from HeLa Cell Membrane Preparations**—We have reported previously (11) that sialic acid is released into the culture medium in the form of sialopeptides and that the accumulation of these sialopeptides in the medium is decreased when cells are grown in the presence of prednisolone. Therefore, attempts were made to identify the enzyme system which might be responsible for the release of the sialopeptide from the surface of the cultured HeLa cells. For this purpose, HeLa cell fractions were incubated with [14C]-labeled plasma membrane preparations and, after the reaction was stopped, the supernatant fluid was subjected to column chromatography on Dowex-formate. Fig. 1 shows a comparison of the elution profile of the reaction mixture supernatant fluid with the similar elution profile for the spent medium in which HeLa cells had been grown. It may be seen that portions of both elution profiles are similar. When the individual sialopeptides from the reaction mixture were eluted at either 0.10 M or 0.15 M concentrations of formic acid were mixed with corresponding eluates from the supernatant medium, they emerged as single peaks at the appropriate concentrations of formic acid. We have reported (11) that the sialopeptides isolated from the spent medium differ in their sensitivity toward trypsin, i.e. the sialopeptide eluted with 0.1 M formic acid is degraded by trypsin while the sialopeptide eluted with 0.15 M formic acid seems to be trypsin-resistant. A similar pattern of sensitivity to trypsin was evident in sialopeptides isolated from the reaction mixture. It is apparent that HeLa cell preparations contain an enzyme, tentatively named sialopeptidase, that liberates sialopeptides from plasma membrane preparations. The elution profiles of sialopeptides released from membrane preparations appeared to be qualitatively similar to those from the spent culture medium. However, it should be noted that the ratio of radioactivity of 0.15 M HCOOH to 0.10 M HCOOH eluate from membrane preparations was much higher than the corresponding ratio of the two eluates in the spent medium.

**Regeneration of Sialopeptidase Synthesis by Prednisolone**—To show the effects of prednisolone on the release of sialopeptides from membrane preparations, HeLa cells were grown for 72 hours in the presence of prednisolone as described under "Materials and Methods." As shown in Fig. 2, the activity of sialopeptidase was considerably reduced in cells grown in the presence of prednisolone. The effect could be increased further by growing cells in the presence of increasing concentrations of this steroid (Fig. 3). It is noteworthy that the concentration-dependent suppression of sialopeptidase activity was greater for the sialopeptide eluted with 0.15 M formic acid than for the 0.10 M formic acid sialopeptide fraction, although this difference could also be accounted for by variability in the distribution of label between the two fractions, by the heterogeneity of the sialopeptide fractions.
with either 0.1 M or 0.15 M formic acid were purified further by filtration on Sephadex G-200. The sialopeptide fraction eluted at 0.15 M formic acid seemed to be more heterogeneous than the one eluted with 0.10 M formic acid (Fig. 6A). Tentative average molecular weights calculated for the two sialopeptide fractions, determined on a Sephadex G-200 column, were 13,000 ± 3,000 for the 0.10 M formic acid fraction and 10,000 ± 3,000 for the 0.15 M formic acid fraction (Fig. 6B). Assuming that

or both. In terms of total label present in the membrane, the amount of radioactivity released in the two sialopeptide fractions was high. This could have been caused by the incorporation of label from glucose into other membrane components followed by its appearance in the released sialopeptides in the form of amino acid or lipid constituents. The problem of kinetics of membrane labeling and the distribution of label among membrane fractions will require a separate study.

To determine the minimum time necessary for the repression of sialopeptidase activity by prednisolone, HeLa cells were grown without steroid for 72 hours, at which time the medium was replaced by one containing the usual concentration of prednisolone. The incubation was continued for different times, the sialopeptidase activity became evident. The repression continued to increase up to 72 hours of incubation, at which time activity of the enzyme became stabilized.

**Fig. 2 (left). Kinetics of liberation of sialopeptides from 14C-labeled membranes from HeLa cell extracts prepared from cells grown in the absence or in the presence of 2 μg of prednisolone per ml for 72 hours. The cell extracts were prepared as described in the text. The 14C-labeled membranes (equivalent to 1 mg of protein and 12,000 cpm) were incubated at 37°C in 0.5 ml of 0.1 M Tris-HCl buffer, pH 8.2; with the cell extract (3 mg of protein) from cells grown either in the presence or in the absence of the hormone. Portions (0.1 ml) were withdrawn at the indicated times and were diluted to 0.7 ml. The diluted mixtures were centrifuged at 12,000 × g at 7°C, and the supernatant fluid was subjected to chromatographic fractionation to isolate the sialopeptides eluted with 0.15 M (---) and 0.10 M (----) formic acid (Δ, control extracts; O, extracts from cells grown with prednisolone).

Fig. 3 (right). Effect of increasing concentration of prednisolone on the enzyme system responsible for the liberation of sialopeptides from cell membranes. HeLa cells were grown for 72 hours at 37°C in the presence of 0, 0.5, 1.0, and 2.0 μg of prednisolone per ml. Cell extracts equivalent to 2 mg of protein were incubated with 0.5 mg of 14C-labeled membranes in Tris-HCl buffer at 37°C for 120 min. The reaction was stopped by dilution and, after centrifugation of the reaction mixture, the supernatant was fractionated on Dowex-formate to isolate sialopeptides.

**P pH Optimum of Sialopeptidase—**The pH optimum for the release of sialopeptides was around 8.2 (Fig. 5). In a series of experiments, Ca++, Mg++, and Mn++ were examined at several concentrations for their effects on the activity of sialopeptidase. No stimulation was evident.

**Characterization of Sialopeptides—**The sialopeptides eluted with either 0.1 M or 0.15 M formic acid were purified further by filtration on Sephadex G-200. The sialopeptide fraction eluted at 0.15 M formic acid seemed to be more heterogeneous
these estimates of the molecular weight are correct, the content of sialic acid would be $0.75 \pm 0.2$ mole per mole for the 0.1 m formic acid fraction and $1.2 \pm 0.2$ moles per mole for the 0.15 m formic acid-eluted sialopeptide. The reason for the large variation in the sialic acid content is not known at the present time, but it might reflect molecular heterogeneity of the fractions that could not be resolved with the present techniques.

Discussion

Based on the data from these experiments, there seems to be little doubt that the reduced release of sialopeptides into the medium is observed in prednisolone-treated HeLa cultures is the result of inhibition of sialopeptidase activity. That HeLa cells are capable of secreting glycoproteins into the medium is a well documented observation, dating back to the results of Kornfeld and Ginsburg (15), who showed that over two-thirds of labeled glucosamine taken up by HeLa S3 cells is ultimately excreted into the medium in the form of amino sugar-containing protein. Similarly, there is considerable information linking high rates of cell proliferation with the presence of proteolytic activity of either exogenous (16, 17) or endogenous (18, 19) origin. The growth-stimulating factor isolated by Rubin (20) from Rous sarcoma cells that promotes growth of chick embryo cells has many of the attributes of a proteolytic enzyme. Its growth-promoting activity can be duplicated by small amounts of pronase or trypsin. It is logical that the converse effect, i.e. blocking of unrestricted cell proliferation by a cell “coat,” would offer an attractive possibility for experimentally regulating the growth of neoplastic cells. Thus far, other workers have been concerned with density-inhibited cell cultures that lose this property after viral transformation (10). The phenomenon of restoration of some degree of density-induced growth control has not been studied extensively because of the lack of a suitable experimental system. Heteroploid (and presumably “transformed”) cells that assume a steady state growth (9) after continuous treatment with glucocorticoid could be developed into such laboratory models.

The major difficulty in following proteolytic activity in HeLa cell preparation is the lack of a suitable, well defined substrate. In the course of this study, unsuccessful attempts were made to follow the release of amino acids from casein, from fetuin, and from erythrocyte ghosts. Also we have searched in HeLa cell fractions for plasminogen activators, starting from the assumption that it is proteolytic activity in the serum supplement (21) that might be activated by the cells. The results were uniformly negative, necessitating the use of freshly prepared plasma membrane fractions, a procedure that does not lend itself as an assay for following enzyme purification. Our observation is in apparent disagreement with a study by Sylvén and Bois-Svensson (22), who studied proteolytic activity in tumor fluids in animals. They found considerable increases in acid proteinases, peptidases, and dipeptidases that were active against a variety of substrates. However, these workers were not dealing with a purely cellular system and could not determine what contribution to the total proteolytic activity was made by the interstitial fluid of the tumor tissue. Characterization of the sialopeptidase will have to depend on finding a suitable substrate which has a known chemical structure. Such an assay will also be necessary for the determination of the locus of the effect of steroid on the release of sialopeptide. Cells grown in the presence of prednisolone attach better to the glass surface (23). In HeLa cells, this increased adhesion coincides in time with the minimum exposure to the hormone required to decrease the shedding of sialopeptides. The relatively long time necessary to obtain these effects (12 hours) indicates that it might be a consequence of an earlier interaction of the steroid with the cell.

The molecular weights of two sialopeptides isolated by Dowex chromatography have been tentatively determined, but their amino acid and carbohydrate compositions remain to be elucidated. Their sialic acid content falls within the range obtained by Wray and Walborg (24) for the wheat germ lipase agglutinin and concanavalin A receptors isolated by papain digestion of Novikoff ascites tumor cells. It remains to be determined whether the isolated sialopeptide fraction can bind wheat germ agglutinin. It is known that sialic acid residues are needed for such activity and that agglutinability of HeLa cells by the wheat germ lipase agglutinin is increased in prednisolone-treated cells (25).

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