SIALIC ACID IN PREDNISOLONE TREATED HELA CELLS; SUGGESTED MECHANISM FOR ELEVATED LEVELS AT THE CELL SURFACE

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SUMMARY. The accumulation of sialic acid at the periphery of HeLa cells grown in the presence of prednisolone was demonstrated to be the result of a decreased shedding of sialopeptides by the treated cells. Two such sialopeptide fractions were isolated from the spent medium in which HeLa cells had been grown. Such compounds could be responsible for the apparent increase in cell density-induced growth inhibition which can be demonstrated in heteroploid cells grown in media that contain low concentrations of glucocorticoids.

INTRODUCTION. Elevated levels of sialic acid in cells grown in the presence of glucocorticoids [1,2] constitute only one of many observations relating the effects of these hormones to alterations in biochemical properties of the cell surface. The importance of such observations lies, mainly, in the relationship to similar changes taking place in a variety of cells undergoing transformation with oncogenic viruses. The difference between the two effects is that, usually, the transformed cells seem to be deficient in sialic acid while at the same time losing the density-induced growth inhibition [3,4], whereas, the steroid treated heteroploid cells normally having the tendency to form multicellular aggregates become somewhat "density inhibited" [5,6].

In this communication we report on the results of experiments that offer an explanation for the accumulation of sialic acid in HeLa cells grown in the presence of low concentrations of prednisolone.

MATERIALS AND METHODS. HeLa cells were obtained from Dr. Martin Griffin of

the Oklahoma Medical Research Foundation. They were grown as stationary cultures in one liter Blake bottles containing 100 ml of Eagle's Minimal Essential Medium supplemented with 10 per cent calf serum, 200 units/ml penicillin and 200 μg/ml streptomycin. Different amounts of prednisolone (11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) in ethanol, or ethanol alone, were added to the cultures. After incubation at 37°C for 72 hours the cells were harvested by gentle scraping with perforated cellophane strips, collected by centrifugation at 0°C and washed six times with physiological saline. In radioisotope experiments D-glucose-U-^{14}C was used at the level of 7.5 μCi/ml of medium.

Sialic acid content of whole cells was determined by column chromatography on Dowex-50 (H^+) and Dowex-formate [1], followed by the thiobarbituric assay. Whole cells (20-25 mg of cell protein) were suspended in 3 ml of physiological saline and were homogenized in a Dounce homogenizer with a tight fitting pestle. The pH of the disrupted cell mass was adjusted to 1.0 with 3.0 N sulfuric acid and the homogenate was heated for one hour at 85°C in a sealed tube. The hydrolyzate was cooled to room temperature and the supernatant fluid was collected by centrifugation at 4340 x g. The supernatant fluid was neutralized with barium hydroxide and the precipitated barium sulfate was removed by centrifugation. A one ml portion of the supernate was applied to a Dowex-50 (H^+) column (0.5 x 10 cm) and the sialic acid was eluted with 10 ml of water. The eluate was concentrated by lyophilization to 1.0 ml and was then chromatographed in a Dowex-formate column (0.5 x 10 cm). After washing the column with distilled water the adsorbed material was subjected to a stepwise elution with 3.0 ml portions of increasing concentrations of formic acid. Sialic acid was eluted with 0.35 N formic acid. Such fractions were pooled, formic acid was removed by lyophilization and the sialic acid concentration was determined with thiobarbituric acid.

For the determination of sialic acid released into the medium by HeLa cells, D-glucose-U-^{14}C was included in the medium as described earlier. After

removing the cells by centrifugation, the medium was subjected to chromatography on Dowex-formate columns as described. Portions (0.5 ml) of the radioactive medium were applied to such columns and the columns were washed with 6.0 ml of distilled water. The adsorbed material was eluted with different concentrations of formic acid as described. The pooled sialopeptide fractions were hydrolyzed with sulfuric acid and the released sialic acid was isolated by rechromatographing on Dowex-formate as already described. The elution pattern of the isolated radioactive material eluted from such columns with 0.35 N formic acid coincided with that of the authentic sialic acid. Fractions containing ^{14}C-sialic acid were evaporated to dryness and the radioactivity was assayed in Packard Tri-Carb Scintillation Spectrometer using toluene-based scintillator. In this system the counting efficiency for ^{14}C was approximately 75 per cent.

Sialic acid was removed from the whole cells by treatment with neuraminidase from *Clostridium perfringens* according to the method of Cassidy et al. [7]. The released sialic acid was determined by the method of Warren [8]. Protein was determined by the method of Lowry et al. [9].

Sialic acid and neuraminidase were purchased from Sigma Chemical Company, Saint Louis, Missouri and the D-glucose-U-^{14}C was obtained from New England Nuclear Corporation, Boston, Mass.

RESULTS. When HeLa cells were grown in the presence of different concentrations of prednisolone, the sialic acid content of the cells was increased (Table 1). Similar results have been reported by Carubelli and Griffin [1] who used cortisol in their studies. By increasing the concentration of prednisolone from 0 to 2 µg/ml of medium, a roughly proportional increase in sialic acid content was noted. Similarly, as shown also in Table 1, the amount of sialic acid released by neuraminidase was higher in the hormone treated cells than in the controls.

In order to determine the release of sialic acid into the medium, the

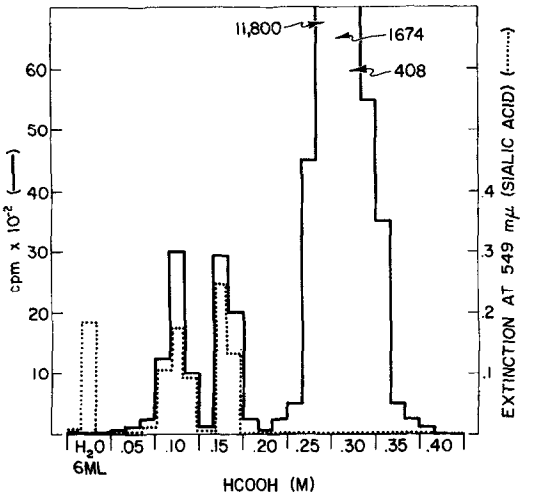

Figure 1. Elution profile of sialopeptides in the Dowex-formate column. HeLa cells were grown in Eagle's Minimal Essential Medium containing 7.5 μCi/ml of D-glucose-U-^{14}C. After 72 hours of growth at 37°C, 0.5 ml of spent medium was subjected to chromatographic fractionation in a Dowex-formate column (0.5 x 10 cm) using 3.0 ml portions of increasing concentration of formic acid as indicated in the figure. One ml of each fraction was collected and assayed for radioactivity and for sialic acid content. Solid line shows radioactivity and the broken line indicates sialic acid concentration in terms of absorbance at 549 mμ.

cells were labeled by growing them in media containing ^{14}C-glucose. However, chromatographic analysis of the unhydrolyzed medium revealed little or no free sialic acid in the medium. Attempts were then made to identify this amino sugar in its bound form. For this purpose the spent medium from cells grown in the presence of labeled glucose was chromatographed on a Dowex-formate column. The adsorbed material was eluted step-wise with different concentrations of formic acid as indicated in Figure 1. Each of the eluted fractions was tested for radioactivity and for the presence of sialic acid. The radioactive peaks at 0.1 M and 0.15 M formic acid showed the presence of sialic acid. The material from those peaks was pooled and lyophilized. It gave a positive reaction with both Lowry and biuret reagents and was precipitated with 10 per cent trichloroacetic acid. On this basis the material from both peaks was tentatively designated as sialopeptides. The two sialopeptide fractions differed

TABLE 1

Concentration of Prednisolone in Culture Medium (μg/ml)	*Sialic Acid (nanomoles/mg cell protein)*	
	Total	*Released by Neuraminidase*
0	4.9	4.1
0.5	5.7	5.4
1.0	6.4	6.0
2.0	6.8	6.5

Accumulation of sialic acid by HeLa cells grown in the presence of prednisolone; release of sialic acid by neuraminidase. Cells were grown at 37°C for 72 hours, harvested by scraping, homogenized and subjected to mild hydrolysis with sulfuric acid. Sialic acid was isolated by chromatography on Dowex-50 (H^+) and Dowex-formate columns. To release sialic acid from harvested cells by neuraminidase, the cells were suspended in saline containing 0.01 M acetate buffer, pH 4.5 and 0.2 mg crystalline neuraminidase from *Clostridium welchii*. Sialic acid was determined by the thiobarbituric assay of Warren [8].

TABLE 2

Concentration of Prednisolone in Growth Medium (μg/ml)	*Sialic Acid (cpm/mg cell protein)*		
	Released in Medium	*Retained by Cells*	*Total*
0	7697	991	8688
0.5	6243	1632	7875
1.0	5920	2944	8864
2.0	4220	3583	7803

Retention of ^{14}C-sialic acid by HeLa cells grown in presence of prednisolone. HeLa cells were grown in Eagle's Minimal Essential Medium containing D-glucose-U-^{14}C (7.5 μCi/ml) with indicated concentrations of steroid. Both cells and the spent medium were analyzed after 72 hours of incubation for radioactivity and for sialic acid as described in text.

in their properties. The sialopeptide eluted with 0.1 M formic acid was subject to degradation with trypsin, while that eluted with 0.15 M formic acid seemed to be resistant to tryptic digestion. By increasing the molarity of formic acid to 4.5 M, no other sialic acid-containing radioactive peak could be identified. When the two sialopeptides were subjected to mild acid hydrolysis with sulfuric acid, the radioactivity emerged in 0.35 M formic acid showing the elution pattern identical with sialic acid.

HeLa cells were then grown in different concentrations of prednisolone for 72 hours. The ^{14}C-labeled sialopeptides were isolated as described and the sialic acid content of the sialopeptides was determined. As shown in Table 2, treatment of HeLa cells during growth with prednisolone decreased the amount of sialic acid released into the medium. In a separate experiment the amount of sialic acid present in cells as well as that released as sialopeptides into the medium was compared in both steroid treated and in control cells. Total amount of ^{14}C-sialic acid synthesized by HeLa cells grown in the presence of different amounts of hormone for 72 hours was calculated by the summation of radioactivity present in whole cells and that being released into the medium. It was found that there was no net increase in sialic acid synthesized by the steroid treated cells as compared to the controls.

Moreover, as indicated in Table 3, it was found that not only the release of ^{14}C-sialic acid in its bound form was inhibited by the hormone, but the amount of radioactive sialopeptides accumulated in the medium was also decreased in the presence of the hormone. However, this decrease in sialopeptide material in the medium was not consistently proportional to the amount of prednisolone present in the medium.

DISCUSSION. These studies indicate that accumulation of sialic acid by HeLa cells cultured in the presence of prednisolone is due to the inhibition of shedding of surface sialopeptides into the medium. Total synthesis of sialic acid grown in the presence or absence of this steroid remains practically

TABLE 3

Concentration of Prednisolone in Growth Medium (μg/ml)	*Cpm in Sialopeptide Fraction/mg Cell Protein*	
	Fraction 1 (0.1 M formic acid)	*Fraction 2 (0.15 M formic acid)*
0	4852	5290
0.5	4635	3904
1.0	4554	3667
2.0	3632	3581

Accumulation of ^{14}C-sialopeptides by HeLa cells grown in the presence of prednisolone. HeLa cells were grown in the medium containing ^{14}C-glucose (7.5 μCi/ml) with indicated concentrations of prednisolone. After 72 hours of growth the spent medium (0.5 ml) was applied to a Dowex-formate column (0.5 x 10 cm). The column was washed with 6.0 ml of water and the adsorbed material was eluted with 3.0 ml each of 0.05, 0.1, 0.20, 0.25, 0.30, 0.35 and 0.40 M formic acid. One ml of each fraction was collected, evaporated to dryness and the radioactivity was determined. The radioactive peaks that contained sialic acid were eluted with 0.1 M and 0.15 M formic acid. The details of identification of sialic acid are given in text.

unchanged. The results of treatment of cells with neuraminidase indicate that the accumulation of sialic acid due to the presence of the hormone occurs mainly at the cell surface (Table 1).

Although the amount of sialic acid, presumably in the form of sialopeptides released into the medium decreased with the increasing concentration of prednisolone, the amounts of the sialopeptides themselves as measured in terms of radioactivity were not as consistent. Most likely, this is a reflection of the presence of other radioactively labeled materials present in these sialopeptide fractions.

It has been reported that surface components of cultured cells released into the medium are glycoproteins in nature [10,11]. The accumulation of this glycoprotein material has been related to various functional phenomena including the cell cycle and the regulation of growth [12,13]. It is possible that

HeLa cells grown in the presence of prednisolone develop a growth pattern resembling density induced inhibition as a consequence of the accumulation of surface glycopeptides that are retained at the cell periphery. The mechanism responsible for the decreased shedding of the cell surface components remains to be elucidated. However, preliminary results indicate that the surface associated proteolytic activity of prednisolone treated cells might be reduced.

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