Effect of Hydrocortisone on Hela Cells with Special Reference to the RNA Metabolism*

HIDEO SUZUKI**, MOTOYASU OHSAWA AND HIROYOSHI ENDO

Department of Physiological Chemistry, Faculty of Pharmaceutical Science, University of Tokyo, Tokyo

Synopsis

Hydrocortisone (HC) inhibited the growth of HeLa cells at the concentration of 0.01 ~ 10 µg/ml but enhanced it at 0.001 µg/ml.

HC increased the β-glucuronidase and alkaline phosphatase activities of HeLa cells at 10 µg/ml in 4 days, although it did not affect the acid phosphatase activity.

Nucleic acids obtained from growing HeLa cells were fractionated by methylated bovine serum albumin column chromatography. Two peaks of rapidly labeled RNA were found in the chromatogram, and from the experiment of time course of 32P-labeling and base composition analysis, it was assumed that one was the precursor of r-RNA and the other d-RNA possibly containing m-RNA.

HC (10 µg/ml) inhibited the synthesis of nucleic acid in HeLa cells; in early period of HC treatment, only the d-RNA synthesis was slightly inhibited, while with longer treatment the synthesis of r-RNA precursor was most strongly inhibited in contrast with the weak inhibition of DNA and d-RNA syntheses.

From these data, the relationship between the catabolic action and enzyme-inducing action of HC was discussed.

It is known that glucocorticoids inhibit the growth of mammalian cells in tissue culture (Holden and Adams, 1957; Bass and Snell, 1961). On the other hand, glucocorticoids were found to increase alkaline phosphatase activity in cultured cells (Maio and Carli, 1963) probably due to the increase of de novo synthesis of the enzyme protein according to Nitowski et al., (1963) and Cox and MacLeod (1964).

It therefore seems to be worthwhile to examine the nucleic acid metabolism in such cells with concurrent growth inhibition and enzyme induction. In this paper, changes in growth, some enzyme activities (alkaline phosphatase, acid phosphatase and β-glucuronidase) and especially nucleic acid metabolism were studied in HeLa cells in relation to HC treatment.

Materials and Methods

Cultivation of HeLa cells and HC treatment

In all the experiments described in this paper, HeLa cells were cultivated at 37°C in the medium consisting of 0.4 % w/v lactalbumin hydrolysates (Nutritional Biochemicals Corporation), 20 % of inactivated bovine serum and 80 % of Hanks' saline in monolayer. No antibiotic was used. HC (generously supplied from Teikoku Hormone MFG Co., Ltd.) was added to the medium as ethanol solution.

Received for publication November 22, 1968.

* Dedicated to Professor Yosoji ITO in commemoration of his unselfish devotion for over fifteen years to the advancement of endocrinological science through the editorial management of Endocrinologia Japonica.

** Present Address: Institute of Applied Microbiology, University of Tokyo, Tokyo.
(final ethanol concentration in the medium was 0.2%, this ethanol concentration gave no effect on the growth of HeLa cells at all).

For growth experiment, simplified replicate tissue culture method (Katsuta et al., 1959) was employed. Two ml of HeLa cell suspension in medium containing HC at various concentration was inoculated in short test tubes to be cultivated at 37°C for 6 days. At 0, 2, 4 and 6 days the cell number was counted by citric acid-crystal violet-staining method. Average number of three tubes was employed.

**Enzyme assay**

All procedures were performed below 4°C unless otherwise stated. HeLa cells cultivated in HC-containing (10 mg/ml) or control medium in the 250 ml culture bottles for 2 or 4 days were harvested by addition of 0.02% EDTA solution in phosphate buffered saline (PBS), washed twice with cold PBS, and suspended in isotonic KC1 solution (5 x 10⁶ cells/ml). After addition of Triton X 100 (0.2%), they were homogenized in glass-homogenizer for 3 mins. and the homogenate was centrifuged at 6,000 g for 20 mins. The supernatant thus obtained was used as the enzyme solution.

β-Glucuronidase (β-Gase) assay was performed according to the method of Talalay-Fishman (Talalay et al., 1946); the reaction mixture consisted of 0.1 ml of enzyme solution (corresponding to 10⁶ cells), 0.1 ml of 0.005 M phenolphthalein glucuronide and 0.8 ml of 0.2 M acetate buffer (pH 3.6), was incubated at 37°C for 6 hrs. The reaction was stopped by the addition of 2.5 ml of 0.2 M glycine buffer (pH 10.5) and 1.5 ml of water to determine the optical density at 550 nm.

Acid and alkaline phosphatase (Pase) activity was determined using the method of Lowry (1946); the reaction mixture consisted of 0.1 ml of enzyme solution, 0.1 ml of 0.09 M p-nitrophenyl phosphate and 0.8 ml of 0.2 M acetate buffer (pH 5.8) or 0.04 M veronal buffer (pH 9.6) containing 0.005 M of MgCl₂. Incubation was continued for 30 mins. at 37°C and after addition of 0.1 M NaOH (4.0 ml) the optical density at 420 nm was measured. After enzyme activities were calculated as liberated phenolphthalein or p-nitrophenol/hr/10⁶ cells, HC-treated cells were compared with control. Average of 3 samples per a group was employed.

**32P-Labelling of HeLa cells**

HeLa cells grown in monolayers in 500 ml culture bottle with 20 ml of phosphate-free medium or that containing HC at the concentration of 10 μg/ml were labelled with 32P-inorganic phosphate of varying radioactivities (1 ~ 10 μc/ml), depending on the length of the labeling period. After labeling, the cells were quickly chilled in ice-water and harvested in the same way as mentioned in enzyme assay. The cell pellet was stored at -20°C.

**Extraction of nucleic acid**

The stored cells were thawed and suspended in 0.05 M Tris buffer (pH 7.6) containing of 0.02 M KCl and 0.01 M MgCl₂ and nucleic acids were extracted by the sodium dodecyl sulfate (1%) - phenol method at 4°C (Philipson, 1961; Yoshikawa-Fukada et al., 1965). Usually, for good extinction and radioactivity measurement about 3 x 10⁷ cells per a group were necessary. Under these conditions, more 90% of DNA and RNA could be extracted.

**MBSA column chromatography**

Nucleic acids were precipitated by addition of ethanol, collected by centrifugation at 10,000 rpm for 30 mins, dissolved in 10 ml of 0.3 M NaCl-containing 0.05 M phosphate buffer (pH 6.7) and applied to methylated bovine serum albumin column (Mandel and Hershey, 1960). Elution was done with linear gradient of NaCl from 0.3 to 1.7 M in 0.05 M phosphate buffer (pH 6.7) with a flow rate of 5 ml/tube/3.5 min. at 30°C. Optical density at 260 nm and radioactivity were examined in each fraction. The recovery of nucleic acids was usually about 100% in both extinction and radioactivity measurement.

**Base composition analysis**

The peaks in the chromatography were collected respectively and dialyzed against distilled water at 4°C for 24 hrs. In some cases yeast RNA was added as a carrier. After alkaline hydrolysis with 0.5 N KOH at 37°C for 16 hrs., resulting nucleotides were separated on a Dowex-1 formate column (Osawa et al., 1958). The radioactivity in the four nucleotide fractions was determined. Base composition analysis was performed twice independently and mean values were employed.

**Results**

**Effect of HC on the growth of HeLa cells**

As seen in Figure 1, HC inhibited the growth of HeLa cells at all the concentrations ranging from 0.01 to 10 μg/ml with progressively stronger degree. It has been reported that HC succinate inhibited the growth of HeLa cells at 50 μg/ml (Bass and Snell, 1961), but in this
work it was revealed that HC as low as 0.01 μg/ml was enough to inhibit the growth of HeLa cells, while the growth was rather enhanced at very low concentration (0.001 μg/ml) of HC. This enhancing effect was observed throughout the experiments, though the mechanism is still obscure.

**Effect of HC on enzyme activities in HeLa cells**

In the experiments on the relation between cell growth and enzyme activities, three enzyme activities (β-Gase, acid and alkaline Pase) per cell decreased in the early logarithmic phase, while they increased progressively as getting near the stationary phase (Fig. 2). Then effect of HC on these three enzyme activities was examined at two different growth phases, logarithmic and stationary phases. HC was added to the medium at 1 day after inoculation and after 2 or 4 days (3 or 5 days after inoculation, respectively) the enzyme activities were measured. In this experiment, 10 μg/ml of HC was used, where the growth of HeLa cells was markedly inhibited as shown in Figure 1.

As seen in Figure 3, no marked change was observed in all three enzyme activities at 2nd day of HC treatment, but β-Gase and alkaline
Pase activities were markedly increased at 4th day, though acid Pase activity was unchanged.

These results suggest that stimulatory effect of HC on enzyme activity is rather selective and a few days are needed for the appearance of the effects.

**Chromatographic profile of nucleic acids of HeLa cells**

The total nucleic acids extracted from HeLa cells after $^{32}$P-labeling were separated by MBSA column with linear gradient concentration of NaCl to several components that could be detected by ultraviolet absorption or radioactivity. The typical chromatogram of 30 mins. pulse-labeling is shown in Figure 4a. Peaks of unadsorbed, acid-soluble material (not indicated), s-RNA (eluting NaCl concentration; 0.4 M), DNA (0.6 M) and r-RNA (0.75 M) were distinguishable in the chromatogram by ultraviolet absorption, while two peaks of so-called rapidly labeled RNA were observed after r-RNA and designated as $q_1$ and $q_2$ ($q_1$ 0.80 M, $q_2$ 0.86 M). The eluting pattern might indicate larger molecular weight of $q_1$ and $q_2$ than that of r-RNA, in accordance with the report of Yoshikawa-Fukada et al., (1965) on nucleic acid pattern in FL cells obtained by MBSA column chromatography.

On longer labeling time (60 mins. Fig. 4b), the $q_1$ peak shifted toward r-RNA peak, although $q_2$ peak remained the same area as 30 mins. labeling. And the longer was labeling time, the smaller became the $q_2$ peak and the nearer shifted the $q_1$ peak toward r-RNA peak. With 24 hrs. labeling, the radioactivity profile almost coincided with the absorbance profile.

**Base composition of rapidly-labeled RNA**

To clarify the characteristics of rapidly labeled RNA, base composition of r-RNA, $q_1$ and $q_2$ peaks was examined according to the

<table>
<thead>
<tr>
<th>Table 1. The base composition of RNA and DNA in HeLa cells</th>
<th>mole %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td><strong>U</strong></td>
</tr>
<tr>
<td>r-RNA</td>
<td>18.4</td>
</tr>
<tr>
<td>$q_1$</td>
<td>21.6</td>
</tr>
<tr>
<td>$q_2$</td>
<td>30.2</td>
</tr>
<tr>
<td>DNA</td>
<td></td>
</tr>
</tbody>
</table>

* From Scherrer et al. (1963)
method of Osawa et al., (1958). As seen in Table 1, q₂ had very similar composition to r-RNA while q₂ was similar to DNA.

**Effect of HC on the nucleic acid metabolism in HeLa cells**

After treatment with HC (10 µg/ml) for 4 hrs., 2 or 4 days, HeLa cells were labeled with $^{32}$P for 30 mins. and total nucleic acids extracted were separated by MBSA column chromatography. In Figure 5 was shown the chromatogram of nucleic acid after 4 hrs. treatment with HC. The q₂ peak could not be seen so clearly as control (Fig. 4a), though s-RNA, DNA, r-RNA and q₁ peaks were similar to that of control. However, with HC treatment for 2 days the incorporation of $^{32}$P into DNA, q₁ and q₂ was all inhibited (s-RNA peak was too small to be compared). By calculation of inhibitory ratio (see the legend of Table 2) it was demonstrated that $^{32}$P-incorporation into q₁ was most greatly inhibited (Table 2). Further treatment with HC (4 days) resulted in almost complete disappearance of q₁ peak, though q₂ peak was still seen (Fig. 6).

**Discussion**

HC and other glucocorticoids are known to stabilize the lysosomal membrane (Weissman, 1964), possibly causing accumulation of lysosome particles and augmentation of lysosomal enzyme activities in the cells. But the fact obtained here that only β-Gase activity out of two lysosomal enzymes (β-Gase and acid Pase) was changed by HC, might suggest that the effect of HC was not the simple stabilization of lysosomal membrane. And it has also been shown that HC induced enzyme activities in the liver (Feigelson and Greengard, 1962; Schimke et al., 1965) and cultured cells, in which the induction of alkaline Pase was well known (Nitowsky and Herz, 1963; Cox and MacLeod, 1964). The increase of β-Gase and alkaline Pase activity seen at 4 day after HC treatment (Fig. 3) may therefore represent the result of the enzyme induction.
As shown in Figure 4a, two rapidly labeled RNAs, q₁ and q₂, were obtained by MBSA column chromatography after ³²P-pulse labeling. Scherrer et al., (1963) showed by sucrose density gradient centrifugation that the rapidly labeled RNA of growing HeLa cells in suspension culture consisted of two components with 35 S and 45 S, and by chase experiment and base ratio analysis they concluded that these rapidly labeled RNAs were both the precursor of r-RNA. On the other hand, Yoshikawa-Fukada et al., (1965) in FL cells, Ellem and Sheridam (1964) in L cells reported the presence of two rapidly labeled RNAs by MBSA column chromatography, concluding that one was the precursor of r-RNA and the other possibly m-RNA. In the present work, according to the chromatographic profile and base composition analysis, q₁ might reasonably represent the precursor of r-RNA and q₂ DNA-type RNA (d-RNA) possibly containing m-RNA, as reported by Yoshikawa-Fukada et al., (1965). Though discrepancy between Scherrer et al., (1963) and the present work could not be perfectly clarified, it may be due to the difference between the methods for separation of nucleic acids.

HC (10 µg/ml) inhibited the synthesis of rapidly labeled RNAs in different fashion, that is, only d-RNA synthesis was slightly inhibited in an early period, while with longer treatment period the synthesis of r-RNA precursor was most strongly inhibited in contrast with the weak inhibition of DNA and d-RNA synthesis. On the 4th day of HC treatment the synthesis of r-RNA precursor was completely stopped. The inhibition of the synthesis of r-RNA precursor may therefore represent another important key to explain the decrease of cell activity by HC, which had generally been thought to be due to acceleration of catabolism. It was known that low levels of levophenol was a selective inhibitor of r-RNA synthesis in HeLa cells (Simon and Praag, 1964) and dinitrophenol had the same activity in E. coli (Simon et al., 1966). In bacterial system, when a culture was shifted from nutriently rich to relatively poor condition (shift down) r-RNA synthesis was selectively inhibited (Rosset et al., 1966) as is well known. Thus, the fact that HC inhibited the synthesis of r-RNA precursor in HeLa cells selectively as shown in this work, may be the result of "shift down"-like condition prompted by the addition of HC. In fact, Mohri (1967) reported that HC decreased the free amino acids pools in HeLa cells. Since it is now evident that nucleolus is responsible for r-RNA synthesis, the effect of HC on r-RNA precursor synthesis observed here, may be explained in reference, to some changes in the fine structure of the cell.

Lang et al. (1968) observed that the m-RNA from HC-treated rat liver had the template activity for tyrosine transaminase when it was added to protein synthesizing system in vitro. The fact that the d-RNA or m-RNA was still to be synthesized and some enzyme activities increased within 4 days after HC treatment, in spite of perfect inhibition of the cell growth and synthesis of r-RNA precursor, might indicate the possibility that this remainder of d-RNA synthesis was responsible for the increase in those enzyme activities, though such possibility still remains to be proven.

References