Regulation on the Expression of Carcino-Embryonic Proteins in Newborn Rat Hepatocytes by Dexamethasone An Enzyme Histochemical and Immunohistochemical Study

By

Kohsuke CHIDA

Department of Pathology, Kitasato University School of Hygienic Sciences, Sagamihara, Kanagawa 228, Japan

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Summary: Dexamethasone (DEX) was administrated intraperitoneally to newborn rats at a dose level of 2 $\mu\text{g/g}$ body weight/day for four days, starting 4 days after birth. After administration of DEX, large quantities of glycogen granules accumulated in the cytoplasm of hepatocytes and the activity level of gamma-glutamyl transpeptidase (γ -GTP) in liver homogenate increased significantly, whereas that of alkaline phosphatase (ALP) exhibited no significant difference in comparison with the control group. In the histochemical analysis, after administration of DEX, a high level of activity of γ -GTP appeared along cell borders between adjacent hepatocytes in the peripheral portion of liver lobules. On the other hand, a low level of activity of ALP was seen along cell borders between adjacent hepatocytes in liver lobules. In the immunohistochemical analysis, no hepatocyte showing positive reactions to Alpha-fetoprotein (AFP) could be recognized after administration of DEX, and in livers of newborn rats receiving DEX, the number of hepatocytes which incorporated bromodeoxyuridine (BUdR) decreased. The present study showed in newborn rat that DEX induced the differentiation of hepatocytes and regulated the expression of carcino-embryonic proteins.

It is known that the expression of γ -GTP, ALP and AFP amplifies in the state of hepatocyte proliferation.^{1–15} In previous papers,^{16–17} the author reported that the expression of γ -GTP, ALP and AFP increased during the process of cell regeneration in galactosamine-injured rat liver. However, it is not still evident what kinds of factors regulate the expression of these carcino-embryonic proteins. Furthermore, no correlation between the expression of the carcino-embryonic proteins and the cell proliferation is obvious. Recently, it was reported that the expression of γ -GTP in newborn rat hepatocytes was induced by the intraperitoneal administration of DEX.¹⁸ In the present study, an enzyme histochemical and immunohistochemical method was used to examine how the expression of carcino-embryonic proteins in newborn rat hepatocytes was regulated by DEX.

Materials and Methods

Animals

Male and female Wistar-Imamitch rats were obtained from a commercial source. The vaginal smears of female rats were checked daily and pregnant rats were produced. Day 0 of the experiment was deter-

mined as the day the pregnant rats gave birth.

Experiment procedures

DEX dissolved in 0.01M phosphate buffer, pH 7.2, containing 0.85% NaCl (PBS) was injected intraperitoneally into newborn rats twice a day at a dose level of 1 $\mu\text{g/g}$ body weight for four days, starting 4 days after birth. In newborn rats of the control group, the same volume (about 80 μl /animal) of PBS was injected in the same manner. Rats were killed 24 hr after final injection. BUdR was injected intraperitoneally at a level of 50 mg/kg 1 hr before killing, and then livers excised.

Electron microscopy

Small fragments of livers were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, for 2 hr at 4°C. After washing with PBS, the fragments were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.4, for 1 hr at 4°C. Then the fragments were dehydrated in a graded series of ethanol and embedded in Quetol 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEM-100s electron microscope.

Enzyme histochemistry and Immunohistochemistry

Tissue preparation; Liver fragments were fixed in 4% paraformaldehyde in 30mM PIPES buffer, pH 7.2, overnight at 4°C. Frozen sections were cut into slices of 8 μ m in a cryostat and used for histochemical staining of ALP. Other liver fragments were fixed in periodate-lysine-paraformaldehyde (PLP) fixative¹⁹⁾ overnight at 4°C for the staining of γ -GTP and AFP, and in 70% ethanol overnight at 4°C for the staining of BUdR. The fragments were subsequently embedded in paraffin. Paraffin sections were cut into slices of 5 μ m and used for the stainings after being deparaffinized through xylene.

Enzyme histochemical staining; For the detection of ALP activity, frozen sections were immersed in the incubation medium of ALP by the azo dye method²⁰⁾ for 1 hr at room temperature. For the staining of γ -GTP, the sections were immersed in the incubation medium of γ -GTP, designed by Rutenberg *et al.*²¹⁾, for 20 min at room temperature. After the immersion in the incubation media, the sections were washed in distilled water and mounted in glycerin jelly after the nuclei were stained with 1% methylgreen.

Immunohistochemical staining; The sections were treated with 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature in order to inactivate endogenous peroxidase. For the staining of AFP, anti-rat AFP rabbit antiserum (Medical & Biological Laboratories, Co., Ltd., Nagoya) diluted 50-fold with PBS was placed on the sections and left for 30 min at room temperature. As a control, normal rabbit serum was used in place of specific antiserum. After the incubation, the sections were washed with PBS and then peroxidase-conjugated anti-rabbit IgG goat γ -globulin (Medical & Biological Laboratories, Co., Ltd., Nagoya) diluted 200-fold with PBS was applied in the same manner. For the staining of BUdR, the sections were hydrolyzed by being immersed in 4N HCl: 70% ethanol (1:1 v/v) for 30 min at room temperature and then neutralized by immersion in 0.1M Na₂B₄O₇ for 2 min. After washing with PBS, solution of monoclonal anti-BUdR antibodies (Takara Shuzo Co., Ltd., Kyoto) diluted 200-fold with PBS were mounted on the sections for 1 hr at room temperature. After washing with PBS, the sections were incubated with an avidin-biotin-peroxidase complex (ABC) kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). In order to detect peroxidase activity, the sections were immersed in diaminobenzidine (DAB) solution²²⁾ for 2–5 min at room temperature. Afterwards, the sections were washed with distilled water and mounted with glycerin jelly after the nuclei were stained with 1% methylgreen.

Enzyme assay; Newborn rat livers were homo-

genized in a Potter-Elvehjem homogenizer with 5 volumes of 0.02M Tris-HCl buffer, pH 7.2. The activity of ALP was determined by modifying the method of Komoda *et al.*⁶⁾. One enzyme unit is defined here as the amount of enzyme which hydrolyzes 1 μ mol of p-nitrophenyl phosphate in one minute. On the other hand, the method of Orlowski and Meister²³⁾ was modified for determining the activity of γ -GTP. One enzyme unit is defined here as the amount of enzyme which liberates 1 μ mol of p-nitroaniline from γ -glutamyl-p-nitroanilide in one min.

Protein concentration was determined by the method of Lowry *et al.*²⁴⁾. Experimental results were analyzed for their significance ($p < 0.05$) by Student's t-test.

Results

The body weight of DEX-injected rats was 8.0 ± 1.5 g (average $\pm$ standard deviation), which was the same as that measured 4 days after birth. Thus, scarcely any increase in body weight was recognized. On the other hand, the body weight of newborn rats in the control group increased to 15.5 ± 1.9 g (average $\pm$ SD).

In enzyme assay in rat liver homogenate, after administration of DEX, the activity level of γ -GTP increased rapidly, but that of ALP exhibited no significant change in comparison with the control group (Fig. 1).

Fig. 1. The activity of both γ -GTP and ALP in newborn rat liver homogenate after administration of dexamethasone. After administration of DEX, the activity of γ -GTP increases significantly ($p < 0.001$), but that of ALP exhibits no significant change in comparison with the control group. Bars are means $\pm$ standard deviation (SD). Light bars: The control group. Dark bars: The DEX-administered group.

Glycogen granules scattered in the cytoplasm of hepatocytes in the control rats were observed by electron microscope (Fig. 2A). However, large quantities of glycogen granules accumulated in the cytoplasm of hepatocytes after injection of DEX (Fig. 2B).

In the histochemical analysis, after administration of DEX, a high level of activity of γ -GTP appeared along cell borders between adjacent hepatocytes in the peripheral portion of liver lobules (Fig. 3b). Conversely, no activity of γ -GTP in the control group was observed in liver lobules (Fig. 3a). Moreover, a low level of activity of ALP was recognized along cell borders between adjacent hepatocytes in liver lobules after administration of DEX, as well as in the control group (Figs. 4a and 4b).

In the immunohistochemical staining, the positive reactions of AFP in control newborn rat livers were seen in the cytoplasm of small-sized hepatocytes which scattered in liver lobules (Fig. 5a). After administration of DEX, no hepatocyte showing a positive reaction to AFP was recognized (Fig. 5b). Furthermore, in the control group, hepatocytes whose nuclei showed positive reactions to BUdR were found scattered in the peripheral portions of liver lobules (Fig. 6a). After administration of DEX, the number of hepatocytes showing positive reactions to BUdR decreased (Fig. 6b).

Discussion

In the present study, the increase of body weight in newborn rats was suppressed by administration of DEX. This may suggest that DEX suppresses cell growth and proliferation. Moreover, after administration of DEX, large quantities of glycogen granules accumulated in the cytoplasm of hepatocytes. This indicates that glucocorticoids induce the synthesis of glycogen in liver. In a previous study,¹⁶⁻¹⁷ the expression of γ -GTP, ALP and AFP increased during the process of cell regeneration in galactosamine-injured rat liver. However, in the present study, the expression of these proteins by administration of DEX was different. By administration of DEX, the activity level of γ -GTP in newborn rat hepatocytes increased rapidly, but that of ALP was not induced. In addition, no hepatocyte containing AFP was recognized after administration of DEX. Cotariu et al. suggested that both RNA and protein synthesis were necessary for induction of γ -GTP by DEX in cultured fetal rat hepatocytes²⁵. Moreover, it had been reported that gene transcription of AFP in newborn rat liver was suppressed by DEX²⁶. Therefore, the expression of carcino-embryonic proteins by DEX in the present study is probably regu-

lated in the level of gene transcription.

In MCF-7 breast cancer cells, sodium butyrate was shown to enhance the activity of ALP and γ -GTP markedly²⁷. Sodium butyrate is a potent chemical inducer of differentiation of hematopoietic cell line²⁸⁻²⁹. Accordingly, the expression of the genes of ALP and γ -GTP in rat hepatocytes may amplify temporarily during the progress to a higher stage from a lower stage of differentiation of hepatocytes. Meanwhile, the gene of AFP may be expressed in the state of hepatocyte proliferation which shows a lower stage of differentiation of hepatocytes. In the present study, by administration of DEX, hepatocytes containing AFP disappeared and hepatocytes incorporating BUdR decreased. This may indicate that hepatocyte proliferation was suppressed and hepatocytes differentiated to a higher stage.

It was reported that the induction of γ -GTP activity in newborn rats by glucocorticoids was age-dependent and decreased progressively with increasing age¹⁸. This suggests that the inducibility of γ -GTP activity in newborn rats by glucocorticoids may be dependent on such factors as the quantity of the receptors of glucocorticoids present on hepatocytes or the regulation by the contact between hepatocytes. In addition, Sasaki et al. reported that hepatocytes containing AFP could not be recognized in rat liver lobules from 3 weeks after birth¹². This age coincides with that at which the inducibility of γ -GTP activity by glucocorticoids in rat liver is lost. This indicates that the inducibility of γ -GTP activity in newborn rat liver by glucocorticoids is relative to the state of hepatocyte proliferation.

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Explanation of Figures

Plate I

- Fig. 2. Ultrastructure of hepatocytes in newborn rat liver.
- A) The control group. Glycogen granules scatter in the cytoplasm of hepatocytes. $\times 6,270$
 - B) The DEX-administered group. Large quantities of glycogen granules accumulate in the cytoplasm of hepatocytes. $\times 9,810$

PlateII

Plate II

- Fig. 3.** Histochemical localization of γ -GTP in newborn rat liver.
a) γ -GTP activity in the control group. No activity is observed in liver lobules. $\times 325$
b) γ -GTP activity after administration of DEX. A high level of activity appears along cell borders between adjacent hepatocytes in the peripheral portion of liver lobules. $\times 190$
- Fig. 4.** Histochemical localization of ALP in newborn rat liver.
a) ALP activity in the control group. b) ALP activity after administration of DEX. A low level of activity is recognized along cell borders between adjacent hepatocytes in liver lobules. $\times 290$

Plate III

- Fig. 5. Immunohistochemical localization of AFP in newborn rat liver. $\times 290$
a) The control group. The positive reactions are seen in the cytoplasm of small-sized hepatocytes which scatter in liver lobules.
b) The DEX-administered group. No hepatocyte showing a positive reaction is recognized.
- Fig. 6. Immunohistochemical localization of BUdR in newborn rat liver. $\times 340$
a) The control group. Hepatocytes whose nuclei show positive reactions scatter in the peripheral portions of liver lobules.
b) The DEX-administered group. The number of hepatocytes showing positive reactions decreases.