HISTOCHEMICAL STUDIES ON DEOXYRIBONUCLEASE ACTIVITY IN NORMAL AND AZO DYE-FED RAT LIVERS BY THE MODIFIED PHOSPHATASE METHOD

(Plates VII-IX)

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Synopsis

The deoxyribonuclease (DNase) activity in normal rat liver and 4-(dimethylaminoazo)benzene-induced hepatomas was studied histochemically by means of Gomori's acid phosphatase method. DNase activity was detected mainly in nuclei and was apparently associated with the nuclear membrane. The cytoplasm showed relatively weak activity.

Intense reactions were observed in normal liver, nodular hyperplastic and cholangiofibrotic lesion. The neoplastic cells were negative but degenerating cells in tumors exhibited moderate DNase activity.

INTRODUCTION

DNase activities in various tissues have been studied histochemically by the substrate film method\(^{10-15}\) and Gomori's acid phosphatase method.\(^{2,4,22}\) Although biochemical studies revealed a 10~30% decrease of DNase activity in cirrhotic and neoplastic livers as compared with normal one on a "per cell" basis,\(^9\) histochemical results obtained by the substrate film method revealed that DNase activity was actually present in the stroma and necrotic regions of experimental and human tumors but absent in the cancerous cells.\(^{12,14,15}\) In general, the substrate film method seems to be poor in its resolving power, although resolution in the cellular level can be obtained in some cases.\(^{15}\) On the other hand, better resolution can be achieved by another method, i.e., the modified Gomori's method for acid phosphatase. It was deemed of interest to investigate the intracellular distribution of DNase activity in normal and cancerous liver by the latter method.

MATERIALS AND METHODS

Animals

Male albino rats weighing 100~200 g were fed on a standard diet, CE–2 (CLEA Japan Inc., Tokyo), and water ad libitum, and used as normal rats. Some rats were fed a carcinogenic diet consisting of rice mixed with 0.5% of casein and 4-(dimethylamino)azobenzene (DAB) dissolved in olive oil to give a final concentration of 0.06%, during 165 days. Regenerating livers were obtained 17 or 48 hrs. after partial hepatectomy performed according to the method of Higgins and Anderson.\(^{18}\)

Preparation of Tissues

Rats were exsanguinated from the abdominal aorta under ether anesthesia. The tissues were excised with scissors, washed in distilled water, and

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blotted on a filter paper. Pieces of the tissue specimens from normal and DAB-fed animals were placed side by side on a stage of a microtome kept in a refrigerated cabinet at $-20^\circ$. Sections were cut at 12 $\mu$ thick, placed on a slide glass, and dried at a room temperature. Serial sections were used for the DNase reaction or stained with Hematoxylin and Eosin after fixation for 5 mins. in acetone: Formalin: water (50: 10: 40), Baker's calcium formol, neutral formol saline, or without fixation. In some cases, tissue specimens were fixed in these solutions and paraffin sections were prepared for the enzyme reaction or histological observation.

**Demonstration of DNase Activity** The tissue sections were incubated at 37$^\circ$ for 1~20 hrs. in a slightly modified Vorbrodt's medium, having the following composition.

- DNA .............................................. 20 mg
- Acid phosphatase .................................. 5 mg
- 0.2$M$ acetate buffer (pH 5.2) ..................... 12.5 ml
- 0.4$M$ lead nitrate .................................. 0.1 ml
- Distilled water ...................................... 38 ml

Acetate buffer solution of pH 5.2 was replaced in some experiments by 0.1$M$ Veronal buffer of pH 7.0, 0.05$M$ Tris buffer of pH 7.0, or 0.1$M$ cacodylate buffer of pH 4.6~7.5.

As a control, tissue sections were processed simultaneously with sections being used for demonstration of DNase activity under the conditions of omitting exogenous DNA or acid phosphatase from the incubation medium. As a DNase inhibitor, 1$M$ NaCl, 0.025$M$ MgSO$_4$, 0.001$M$ CuSO$_4$, or 0.005$M$ EDTA was added to the complete incubation mixture. As the other control, tissue sections were placed in a boiling water for 10 mins. before incubation in order to inhibit completely the enzyme activity.

The slides were washed briefly in distilled water after the incubation and placed in 0.05% yellow aqueous solution of (NH$_4$)$_2$S for 10 mins. After washing in distilled water, the tissue sections were fixed with Carnoy's fixative for 5 mins. if the materials had not been fixed before. After these procedures, tissue sections were counter-stained with 0.5% Fast Green, dehydrated in ethanol, cleared in xylene, and mounted in Canada balsam.

**Chemicals** Chemicals used in the experiments were of reagent grade. Herring sperm powder DNA and wheat germ acid phosphatase were purchased from the Sigma Chem. Corp., U.S.A.

**Results**

**Demonstration of DNase Activity by the Modified Gomori Method**

The best results were obtained with sections incubated in the medium described above and containing lead nitrate at a final concentration of 0.8 mM. Higher concentrations of lead nitrate, for instance, 2 mM, did not increase the reaction to a further extent. Fixation of tissues and paraffin embedding reduced the intensity of the reactions, while intense reactions were observed with fresh tissue sections. Preservation of fresh tissue sections were satisfactory after short incubation periods but fixation following the histochemical reaction processes was necessary after long incubation. This fixation did not change the intensity of the enzymic reaction.

Two kinds of DNase were differentiated biochemically from their pH dependency of enzyme action and sources of preparations, i.e., pancreas DNase I and thymus DNase
DEOXYRIBONUCLEASE ACTIVITY IN LIVERS

Table I. Effect of pH on the Staining Intensity due to DNase Activity after 5 Hours' Incubation

<table>
<thead>
<tr>
<th>Buffer solution</th>
<th>pH 4.6</th>
<th>pH 5.0</th>
<th>pH 5.2</th>
<th>pH 5.4</th>
<th>pH 5.6</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2M acetate</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>0.1M Veronal</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.05M Tris</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.1M cacodylate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tbody>
</table>

II having their pH optimum at 6~7 and 5.0, respectively. Maximum intensity of the histochemical staining was obtained with acetate buffer of pH 5.2 within a range of pH 4.6~7.5, using different buffer solutions (Table I). The reaction of DNase II, as observed at pH 5.2, became appreciable after 1 hr. of incubation. The intensity reached a maximum after 5 hrs. and remained constant thereafter. The activity of DNase I, i.e., reaction at pH 7.0, was weak using either Veronal or Tris buffer solution. The localization of DNase I was similar to that of DNase II.

The staining reaction for DNase was very weak if acid phosphatase was omitted from the incubation medium. No staining reaction was observed with heat-treated sections as well as those incubated without exogenous DNA. When the various inhibitors were added to the medium at the concentrations mentioned above, no staining occurred. When tissue sections were placed for 10 mins. in a medium containing sodium phosphate at a concentration of 0.2 mg/ml instead of DNA, the reaction products due to inorganic phosphate were evenly distributed over the tissue sections, in contrast to the localized staining as observed with the medium containing DNA. These results indicate that the staining reaction obtained under the conditions mentioned above are due to the histochemical demonstration of DNase activity in tissue section.

**Distribution of DNase Activity in Normal Tissues and Regenerating Liver**

In the spleen, intense reactions were found in the red pulp and in rings of cells outlining the lymphatic nodules. In the small intestine, the staining was most prominent in nuclei of epithelial cells. In the liver, nuclei of parenchymal and Kupffer cells were stained intensely while the reaction was weak in cytoplasm (Photos 2b and 4b). The connective tissue elements and the bile duct cells showed weak DNase reaction (Photo 4b). Cells of regenerating liver at 17 hrs. or 48 hrs. after operation did not show higher DNase activity than those of the normal liver.

**DNase Activity in Normal and Cancerous Rat Liver**

Sections of normal and cancerous livers stained with Hematoxylin and Eosin are shown in Photo 1a, and adjacent sections stained for the DNase histochemistry are shown in Photo 1b. Normal liver (L) showed intense DNase activity while no reaction was given by hepatoma cells (H). Strong activity was detected however in hyperplastic nodules (N). Moderate reactions were observed in surrounding area of cholangiofibrotic lesions (C). In the normal liver, intense DNase activity was detected in the nuclei of parenchymal cells and Kupffer cells but weak activity in the cytoplasm (Photos 2b and 4b). Connective tissues in normal liver (Photo 4b) and in the marginal zone of cholangiofibrotic lesion (Photo 2b), as well as blood vessels in normal liver (Photo 4b), did not
show any DNase activity. Strong or moderate activity of DNase in the hyperplastic nodule (Photo 3b), as well as in cholangiofibrotic lesions (Photos 2b and 3b), was shown to be localized mainly in the nuclei.

In the large vein, as indicated by arrows in Photo 1, there were certain number of degenerating hepatoma cells showing a moderate DNase activity. It should be noted that the moderate reactions were observed only in the degenerating hepatoma cells and not in the leucocytes.

**DISCUSSION**

Two different histochemical methods have been developed for detecting the DNase II activity. The substrate film method of Daoust\(^{10,11,13}\) can detect DNase activity which is active in tissue sections without any environmental change such as contact with incubation medium. On the other hand, the method of Aronson et al.,\(^{2,4,22}\) based on Gomori’s acid phosphatase procedure, can reveal intracellular localization of the DNase with high resolution. According to the previous reports\(^{2,4,22}\) concerning the intracellular localization of DNase II by the latter method, the DNase activity was found in the nuclei as well as in the cytoplasm. Since many studies have shown the presence of hydrolytic enzymes in cytoplasmic lysosomes,\(^{16}\) nuclear staining of DNase was suspected to be due to an artefact to some extent.\(^{2,4,22}\) Therefore, attempts have been made to find out suitable conditions for obtaining less intense nuclear staining and stronger cytoplasmic reactions by changing various factors involved in this histochemical procedure. The results of the present work, however, showed that strong reactions of DNase activity were associated with nuclei while weak reactions were observed in cytoplasm under various conditions examined so far. In the inhibiting and control experiments, no reactions were found at all. Furthermore exogenous inorganic phosphate was precipitated evenly over the tissue section by the method of DNase detection. Thus, it is clear that inorganic phosphate was not bound to the nuclear structure specifically during the histochemical procedures described in this paper, although many factors involved in Gomori’s method have been studied and discussed in detail concerning the possibility of artificial nuclear staining.\(^{17,19}\) Relatively high activity of DNase was found biochemically in nuclear fractions isolated with aqueous or non-aqueous media from different types of mammalian cells.\(^{3,7,8,21}\) These biochemical results support strongly a conclusion that DNase activity is associated with nuclear structures, although it still remains unsolved whether it is present inside the nuclear membrane or not. About the same level of DNase activity was observed in 17-hour and 48-hour regenerating livers as in the normal one. This result is different from that obtained by biochemical determinations.\(^{1,6}\) The distributions of DNase activity in spleen and small intestine observed in this experiment were the same from histological point of view as that obtained by substrate film method.\(^{10,11,13}\) Therefore, it can be concluded that the staining reactions obtained in this experiment represent the localization and the activity of DNase in the cells.

As shown in this paper, the DNase activity was not detected in hepatoma but intense in normal liver, strong in hyperplastic nodules, and moderate in cholangiofibrotic lesions. These results were in agreement with those obtained by substrate film method.\(^{12,14,15}\) It is interesting, however, that degenerating hepatoma cells showed a moderate DNase
DEOXYRIBONUCLEASE ACTIVITY IN LIVERS

activity. This finding suggests a possibility that masked DNase in the hepatoma cell may be activated during degeneration or that the active enzyme might come into degenerating hepatoma cells from somewhere. In connection with this problem it may be worthwhile to note that necrotic regions had a strong DNase activity as demonstrated by the substrate film method.\textsuperscript{12,14} The mechanism of the appearance of DNase activity in degenerating hepatoma cells is not understood yet.

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REFERENCES


EXPLANATION OF PLATES VII-IX

Photos 1~4. Photomicrographs of (a) were stained with Hematoxylin and Eosin, (b) demonstrating DNase activity by means of Gomori’s acid phosphatase method and counterstained with Fast Green.

1. Normal and cancerous livers. Frozen sections containing normal and cancerous livers. \times 30
   Upper right (L), upper left corner (H), lower center ovoid structure (N), and arrow correspond to normal liver, hepatoma, hyperplastic nodule, and large vein, respectively. The rest of the picture is occupied by cholangiofibrotic lesions (C). Strong reactions of DNase activity is found in normal liver and hyperplastic nodule, and moderate reactions in cholangiofibrotic lesions but no reaction in hepatoma and connective tissue elements. Within the vein, degenerating hepatoma cells indicated by an arrow showed moderate reactions.

Normal liver (L) in the upper half and cholangiofibrotic lesion (C) in the lower. Connective tissues are found in the surface of the cancerous tissue and infiltrated into cholangioma. No reaction in these connective tissues but intense or moderate reactions were found in the cells of normal or cholangioma.


Hyperplastic nodule (N) in the lower half surrounded by connective tissue. Strong reactions in hyperplastic nodule but not in connective tissue.


Intense nuclear and weak cytoplasmic reactions in normal liver at right half. Blood vessel and surrounding connective tissue in normal liver did not show any reactions. No reaction was found in hepatoma cells at left half.