In Vivo Effects of Proteases on Cell Surface Architecture and Cell Proliferation in the Liver

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SUMMARY

Effects of purified proteases such as trypsin, papain, pronase E, and collagenase injected i.p. on the cell surface architecture, as well as on the DNA synthesis and mitosis in the liver of rats and mice, were investigated biochemically and histologically. Among the proteases examined, only papain induced the stimulation of DNA synthesis and mitosis, as well as the alteration in the pericellular architecture. Histochemical staining showed that the pericellular acidic material in the liver, probably the cell coat acid mucopolysaccharide, disappeared temporarily prior to the stimulated DNA synthesis and mitosis in papain-treated animals.

Glucosamine-3H incorporations into the plasma membranes, as well as other subcellular fractions of the liver, were markedly reduced soon after papain injection, reaching a minimum at 12 hr but recovering to the normal level at 36 hr. On the other hand, the rate of removal of previously incorporated glucosamine-3H was increased soon after papain injection in the cytomembranes (microsomes plus plasma membrane fragments) but not in the cytosol.

INTRODUCTION

The proliferation of normal tissue cells in the animal body seems to be controlled by homeostatic mechanisms, details of which are not fully understood and which may differ to some extent from one tissue to another. For instance, in some tissues the cellular proliferation seems to be regulated at the G2 phase (G2 arrest) (14), while in many tissues it seems to be regulated at the G1 phase (G1 arrest) (3). In the latter case, the induction of mitosis under some physiological and pathological conditions is always preceded by stimulated DNA synthesis (5, 7).

As to the regulatory mechanisms of tissue cell proliferation, several lines of concepts have been presented. Probably the most popular one assumes some specific humoral factors, such as some serum factors (13, 27), hormones (8, 22), chalones (2, 8), and so on. In addition to the humoral factor theory, another major concept seems to be concerned with the cell to cell interactions or related to the cellular surface mechanisms, and it has been investigated mainly by tissue culturists (10, 11, 15, 25).

Recently, Kambara and Nohara (17) reported on the stimulated mitosis in the rat liver after i.p. injection of crude papain. Some proteases induce the cell proliferation in the confluent tissue cultures (10, 25), and the finding of Kambara and Nohara seems to suggest that a similar reaction may occur also in vivo. With the aim to elucidate the mechanism of papain-induced liver cell proliferation in vivo, the effects of i.p. injection of several purified proteases on DNA synthesis and mitosis have been investigated. Possible alterations in the pericellular architecture in the liver of papain-treated animals have been examined histochemically as well as biochemically.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 115 to 130 g and male ICR mice weighing 30 g were used. Animals were maintained in a constant temperature room (24°) and fed the semi-synthetic diet (Diet CE-2) (Nippon CLEA, Tokyo, Japan) ad libitum. For the biochemical experiments, rats previously fasted for 18 hr were used. Taking the diurnal rhythm into consideration, animals were sacrificed between 8:00 and 10:00 a.m. throughout the study.

Biochemicals. Proteases used in the present study were papain, twice crystallized, (Worthington Biochemical Corp., Freehold, N. J., and Sigma Chemical Co., St. Louis, Mo.), collagenase (Worthington), trypsin, twice crystallized (Sigma), and Pronase E (Kakken Industry Inc., Tokyo, Japan). Thymidine-3H [thymidine-6-3H, 20.2 Ci/m mole (Radiochemical Centre, Amersham, England)], and glucosamine-3H [D-glucosamine-1-3H, 30 Ci/m mole (Radiochemical Centre)] were used as labeled precursors.

Subcellular Fractionation Including the Plasma Membranes. Rats were sacrificed by decapitation and the liver was perfused in situ with cold 0.25 M sucrose. For routine subcellular fractionation without preparation of plasma membranes, the liver was homogenized with 3 volumes of cold 0.25 M sucrose containing 2 mM CaCl₂-20 mM Tris-HCl buffer, pH 7.6, in a motor-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1,000 X g for 15 min to precipitate the nuclear fraction. The supernatant was then spun at 15,000 X g for 20 min to precipitate the mitochondrial fraction. The postmitochondrial supernatant was centrifuged at 105,000 X g for 90 min to separate the microsomal fraction (precipitate) from the cytosol fraction (supernatant). The plasma membranes were prepared by the modified procedures of Takeuchi

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and Terayama (28) and of Berman et al. (4). The liver was homogenized with 9 volumes of 0.25 M sucrose containing 0.5 mM CaCl$_2$, 5 mM Tris-HCl buffer, pH 7.4, in a Dounce homogenizer with a ball-shaped Teflon pestle and a 0.34-mm clearance. About 50 manual up and down strokes of the pestle were sufficient to disrupt the liver cells. The homogenate was diluted with an equal volume of the homogenizing medium and filtered through 4 layers of gauze. The filtrate was centrifuged at 2,000 × g for 20 min to precipitate the crude nuclear fraction, in which the plasma membranes were grossly coprecipitated. The crude nuclear fraction was washed 3 times with an equal volume of the homogenizing medium and finally suspended in a small volume of the same medium. An aliquot of the suspension was layered over the following 4 discontinuous sucrose density layers: from the bottom to the top, 45 (d = 1.20), 41 (d = 1.18), 37 (d = 1.16), and 31% (d = 1.13) sucrose in the Ca$^{2+}$-Tris buffer, pH 7.4. The whole system was subjected to centrifugation in a centrifuge tube at 90,000 × g for 2 hr in a swinging bucket rotor, SW-25-1 or SW-25-2, with a Beckman-Spinco Model L2 ultracentrifuge. The pellet at the bottom consisted mainly of nuclei, and the pellet at the interphase between d = 1.20 and d = 1.18 seemed to be highly contaminated with large particulates. The pellet at the interphase between d = 1.18 and d = 1.16 consisted mainly of the plasma membranes with tight junctions (M$_1$), and the pellet at the interphase between d = 1.16 and d = 1.13 consisted of the plasma membranes rich in the microvilli (M$_0$) (12).

**In Vivo Incorporation of Thymidine-$^3$H into DNA in the Liver.** Thymidine-$^3$H, 35 μCi/100 g body weight, was injected i.p. 2 hr prior to sacrifice. The liver was perfused in situ with cold 0.25 M sucrose, excised, weighed, and then homogenized in a glass homogenizer with 3 volumes of cold 0.25 M sucrose. The homogenate was centrifuged at 1,500 × g for 15 min. The nuclear pellet was washed once with the same medium and then resuspended. An aliquot of the nuclear suspension was then subjected to the Schmidt-Thannhauser procedure (23). An aliquot of the hot trichloroacetic acid extract (DNA extract) was subjected to colorimetric DNA assay according to the diphenylamine method of Dische (24), with calf thymus DNA as standard. Another aliquot of the DNA extract was used for the measurement of radioactivity.

**In Vivo Incorporation of Glucosamine-$^3$H into Subcellular Fractions of the Liver.** Glucosamine-$^3$H, 30 μCi, was injected i.p. 2 hr prior to sacrifice. The liver was perfused in situ and homogenized, and the subcellular fractions were prepared as described above. An aliquot of each subcellular fraction was used for the assay of protein, according to the method of Lowry et al. (18), with crystalline bovine serum albumin as standard, and another aliquot was used for radioactivity measurement, which will be described later.

**Measurement of Radioactivity.** An aliquot of sample (consisting of DNA extract, subcellular fractions, etc.) was put into a vial and oxidized with a small amount of 70% perchloric acid and 30% H$_2$O$_2$ at 70° for 30 min (6). After cooling, 5 ml of ethyl Cellosolve and 10 ml of the scintillation fluid (6 g of PPO in 1 liter of toluene) (19) were added successively, and the radioactivity was measured by a liquid scintillation spectrophotometer, type LSC-501 (Aloka, Tokyo, Japan). Corrections for quenching were made by the conventional external method.

**Mitotic Activity Measure.** Freshly excised livers were fixed in neutral 10% formalin in 0.9% NaCl solution, embedded in Tissuemat (Fischer Scientific Co., Fair Lawn, N. J.), and then sliced into sections 5 μm thick. Sections were stained with Mayer’s hematoxylin-eosin, and mitotic figures were counted on 100 different fields of the peripheral area of the liver lobes. The mitotic activity in this paper was expressed in terms of the number of mitotic figures per 10$^3$ nuclei. Colchicine was not used in the present study.

**Histochemistry of Pericellular Acidic Materials and Cytoplasmic Carbohydrates.** The neutral formalin-fixed tissue or plasma membrane sections were subjected to the staining procedure of Mowry (21) to detect pericellular acidic materials with colloidal ferric hydroxide (blue) as well as cytoplasmic carbohydrate materials with PAS$^+$ reagent (red). The colloidal iron treatment has been carried out at a very low pH of 1.5 to detect preferentially the strongly acidic materials like sulfate-containing acid mucopolysaccharides.

**RESULTS**

Effects of i.p.-Injected Various Proteases on DNA Synthesis in the Liver and Kidney of Rats. Rats that had received an i.p. injection of 3 mg papain were sacrificed at various intervals of time. Thymidine-$^3$H was injected i.p. 2 hr prior to sacrifice, and the radioactivity incorporated into DNA in the liver and the kidney was measured. In Chart 1 the DNA-synthetic activities of the liver and the kidney after papain injection are shown.

The DNA synthesis in the liver of papain-treated rats begins to increase 24 hr after papain injection, reaching a maximal level (30 times greater than the control level) at 44 hr and then declining gradually. In contrast to the marked stimulation of DNA synthesis in the liver, the DNA synthesis in the kidney appears to be less sensitive to the effect of i.p.-injected papain.

So that we could examine whether or not the stimulation of DNA synthesis in the liver is specific to papain, rats received an i.p. injection of 3 mg each of papain, trypsin, and collagenase or 3.5 mg of Pronase E; the 2-hr incorporation of thymidine-$^3$H into DNA in the liver was measured at 44 hr after protease injection. The marked stimulation of DNA synthesis occurred only in the group of rats with papain injection (Table 1). Trypsin and collagenase stimulated only to a slight extent, while Pronase E caused almost no effect.

The above results seem to agree with the results of Kambara and Nohara (17), who reported the stimulated mitosis in the rat liver, which reaches a maximum at 48 hr after i.p. injection of papain. In the study of Kambara and Nohara, crude papain was used instead of crystalline and the effect of papain on the DNA synthesis was not investigated.

**Mitotic Stimulation in the Mouse Liver by i.p. Injection of Papain.** In order to confirm that the papain-induced mitotic stimulation is not limited to the rat liver, we examined the effect of i.p.-injected papain upon the mitotic index of the

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4The abbreviation used is: PAS, periodic acid-Schiff.
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mouse liver. As a result, the mitotic index increased only slightly at 24 hr after papain injection but was enhanced markedly at 48 hr (almost 5 times). Chart 2 summarizes the mitotic indices measured at 48 hr after i.p. injection of various amounts of papain.

Obviously, the dose-dependent mitotic stimulation with papain is observed in the mouse liver. The induced mitosis was observed only in the periphery of each liver lobe in contrast to mitosis in regenerating liver, in which more mitoses are observed in the periphery of each lobule instead of the lobe.

Temporary Disappearance of Pericellular Acidic Materials Prior to the Initiation of DNA Synthesis and Proliferation in the Liver. The fact that the stimulated mitosis is observed only in the peripheral area of the liver lobe suggests the possibility that i.p.-injected papain may penetrate through the capsule hepatitis, diffuse into each liver lobe from the periphery, and attack the pericellular architecture. For examination of this possibility, histochemical staining for the acid mucopolysaccharides was carried out according to the procedures of Mowry (21), with the mouse liver specimens prepared at 5, 20, and 48 hr after i.p. injection of 0.4 mg papain. It was found that PAS staining, which was more evident in the cytoplasm, remained unaltered throughout the entire period of observation, while the marked decrease in the blue staining, due to the colloidal iron binding to acidic material in the pericellular regions, was observed soon after papain injection in peripheral

![Chart 1](image1.png)

Chart 1. Change in the DNA-synthetic activity of the liver and the kidney of rats after i.p. injection of papain. Each of 5 to 6 rats in an experimental group (12 rats only in the control group) received an i.p. injection of 3 mg crystalline papain and, at various intervals, another i.p. injection of 35 μCi of thymidine-3H. Rats were killed by decapitation 2 hr after thymidine injection to measure the incorporation of thymidine-3H into DNA in the liver and the kidney. Abscissa, time of killing after papain injection. The value at zero time indicates the control group without papain injection. Each point represents the average value of all the individual rats in each group ± S.D. ○, liver; ●, kidney.

![Chart 2](image2.png)

Chart 2. Mitotic activity of the liver of mice at 48 hr after i.p. injection of various doses of papain. Each of 5 mice in a group received an i.p. injection of various doses of papain. Mice were killed 48 hr after papain injection, and mitotic figures per 10⁵ nuclei were measured as described in "Materials and Methods." With 0.5 mg papain, 2 out of the 5 mice died soon after injection.

<table>
<thead>
<tr>
<th>Protease</th>
<th>Amount of protease (mg)</th>
<th>No. of rats</th>
<th>Specific radioactivity (dpm/mg DNA)</th>
<th>DNA content (mg DNA/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Sham-injected</td>
<td>21</td>
<td>3,500 ± 380⁶</td>
<td>2.25 ± 0.20</td>
</tr>
<tr>
<td>Papain</td>
<td>3.0</td>
<td>6</td>
<td>88,700 ± 14,100</td>
<td>2.69 ± 0.17</td>
</tr>
<tr>
<td>Trypsin</td>
<td>3.0</td>
<td>5</td>
<td>5,970 ± 140</td>
<td>ND</td>
</tr>
<tr>
<td>Collagenase</td>
<td>3.0</td>
<td>5</td>
<td>5,800 ± 750</td>
<td>ND</td>
</tr>
<tr>
<td>Pronase E</td>
<td>3.5</td>
<td>5</td>
<td>3,130 ± 410</td>
<td>ND</td>
</tr>
</tbody>
</table>

⁶ Mean ± S.D.; ND, not determined.

Table 1

Effects of proteases on thymidine-3H incorporation into rat liver DNA

Rats in each group received i.p. injections of 3.0 to 3.5 mg of proteases and after a 42-hr interval another i.p. injection of 30 μCi of thymidine-3H per 100 g body weight. Rats were killed 2 hr later, and the radioactivities incorporated into DNA in the liver of individual rats were measured. The values in the table are expressed by the average of individual rat determinations in each group.
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parts of each liver lobe. Thus, the reduction in pericellular acidic material was already indicated 5 hr after papain injection and was more evident at 20 hr. However, the pericellular acidic material reappears later, showing the characteristic blue staining again at 48 hr after papain injection.

When other proteases (trypsin, Pronase E, and collagenase) were injected and the liver specimens were examined histochemically from time to time, the characteristic blue staining in the pericellular regions remained unaltered. The temporary disappearance of pericellular acidic material occurred similarly in the liver of papain-treated rats as well as in the mouse regenerating liver prior to the mitotic induction.

Contrary to the case of papain-treated rat or mouse liver, the cytoplasmic PAS staining (red) in the regenerating liver was reduced dramatically soon after partial hepatectomy. The blue staining in the pericellular regions in the regenerating liver disappeared at 20 hr after partial hepatectomy throughout the whole area in the lobe, but it became normal again at 48 hr, when vigorous mitosis took place.

Glucosamine-3H Metabolism in the Liver of Papain-treated Rats. As shown in Chart 3, soon after papain injection, the glucosamine-3H incorporation into each subcellular fraction of the liver is reduced markedly, reaching a minimum at 12 hr, but later the incorporation tends to recover; it becomes normal at 36 hr. The level of glucosamine-3H incorporation was highest in the plasma membranes as well as in the mitochondria.

The rates of release or breakdown of previously incorporated glucosamine-3H were compared between the cytosol and the microsome fractions. The microsomes used in the present experiments were isolated from liver homogenates prepared in a motor-driven homogenizer and therefore may have contained the plasma membrane fragments in addition to endoplasmic reticulum fragments. As shown in Chart 4, the rate of breakdown or release of previously incorporated glucosamine-3H in the cytomembrane (microsomes and plasma membrane fragments) fraction increases in papain-treated rats, while that in the cytosol fraction remained unaltered after papain injection.

DISCUSSION

In accordance with the results obtained with rats by Kambara and Nohara (17), the mitotic activity in the liver of mice was also stimulated by i.p. injection of purified papain. The papain-induced mitotic stimulation is dependent on the
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dose of papain and is more pronounced in the peripheral area of the liver lobes. The present finding that the stimulated DNA synthesis precedes the mitotic stimulation suggests that the papain effect may somehow be related to the release of G₁ arrest. It is surprising that other proteases such as trypsin, Pronase E, or collagenase were rather ineffective in vivo, showing a discrepancy between the in vitro and the in vivo effects of proteases on the cellular proliferation.

The in vivo effects of proteases on the DNA synthesis, as well as the cellular proliferation in the liver, may not be correlated with the acute toxicities of these proteases because all the rats that received 3 mg of papain or trypsin survived beyond 2 days, while one-third or two-thirds of rats that received similar doses of Pronase E or collagenase, respectively, died within several hr after injection. It seems more plausible that the specific effect of papain in vivo might be due to the greater permeability of the enzyme through the capsule hepati.

The in vitro studies with various proteases including papain have shown that the pericellular architecture is damaged with proteases, resulting in the release of sugar moieties of various sizes (29), the change of reactivity to some plant lectins (9), the loss of tissue-specific surface antigens (26), etc. Of course, to what extent the pericellular structure and also the cellular activity may be altered with proteases may depend on specificity of proteases, protease concentrations, and incubation periods, as well as types of cells. The damage of the liver cells in the present in vitro study does not appear to be severe because the alteration in the pericellular architecture is only temporary and limited to the peripheral area of the lobe. In fact, no necrotic lesions have been observed in the liver of papain-treated animals.

At the moment we cannot tell exactly whether the histochemically detected disappearance of pericellular acidic material may be due to the loss of cell coat acid mucopolysaccharide or plasma membrane-bound sialic acid-containing glycoprotein. However, as will be presented in a forthcoming paper, we could release an acid mucopolysaccharide from the isolated rat liver cells as well as the rat liver cell plasma membrane fractions by very mild papain treatment without paper, we could release an acid mucopolysaccharide from the hepatis.

The increase in the rate of breakdown of previously incorporated glucosamine in the cytomembrane fraction, but not in the cytosol fraction, seems to be in accord with the direct action mechanism of papain on the pericellular materials. On the other hand, the temporary decrease in the capacity to incorporate glucosamine-3H not only into the plasma membrane fraction, but also into the other subcellular fractions, suggests the possibility that papain may also affect the biosynthesis of glycopolypeptides and/or acid mucopolysaccharides in more general ways, probably as the result of altered permeability of glucosamine-3H or altered size of intracellular glucosamine pool. All these possibilities should be examined in future studies. The results of the present metabolic study seem to agree with the histochemical observation of temporary disappearance of pericellular acidic materials that are considered to contain or be derived from glucosamine.

As to the similar alteration of the pericellular architecture in the regenerating liver, the involvement of endogenous protease like cathepsin, which has been reported to be secreted from the liver cells (1) after partial hepatectomy, seems to be most plausible.

At the moment we cannot tell whether or how the temporary disappearance of pericellular acidic materials may be related with the initiation of cellular proliferation. On the other hand, the fact that the cytoplasmic PAS-positive material disappears temporarily in the regenerating liver but not in the papain-treated rat liver seems to suggest that the cytoplasmatic PAS-positive material (probably glycogen because the staining disappears after α- or β-amylase treatment) may not be related with the initiation of cellular proliferation.

REFERENCES


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