EFFECTS OF PROTEASE INHIBITORS ON LIVER REGENERATION

Mizue Miyamoto[1], Hiroshi Terayama[1] and Takayuki Ohnishi[2]

1 Zoological Institute, Faculty of Science, University of Tokyo, Tokyo, Japan

2 Biol. Laboratory, Faculty of Science, Tokyo Inst. of Technology, Tokyo, Japan

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SUMMARY: Effects of microbial protease inhibitors, leupeptin and pepstatin, on rat liver regeneration were studied biochemically and histochemically. Leupeptin plus pepstatin (1 mg each) or leupeptin only (2 mg) given to rats by intraperitoneal injections four times every three hours after partial hepatectomy inhibited or retarded the surges of initial RNA synthesis as well as of subsequent DNA synthesis and mitosis in the regenerating livers. The results were discussed in relation to the possible involvement of lysosomal proteases (cathepsins) in initiating the liver cell proliferation.

INTRODUCTION

Although real mechanisms of the regulation of tissue cell proliferation have not yet been fully elucidated, intercellular adhesion with respect to the contact inhibition phenomenon (1-4) or some specific humoral factors stimulating or inhibiting the cellular proliferation (5-10) have been considered to be responsible for this important problem. In the preceding papers (11-14) we have reported that the cell coat acid mucopolysaccharides likely to be responsible for the intercellular adhesion disappear temporarily during a certain period of time preceding the mitotic surge in the liver of partially hepatectomized rats and mice as well as of the normal animals treated with intraperitoneal injection of crystalline papain. In the former case (liver regeneration) the disappearance of cell coat material has been speculated to be due to endogenous proteases (cathepsins)

which might be released or activated by some unknown mechanisms after partial hepatectomy. Activation of lysosomal enzymes prior to cell proliferation has been reported by Adams (15) or Allison and Mallucci (16). Recently some powerful protease inhibitors such as leupeptin (N-acetyl or N-propionyl-L-leucyl-L-leucyl-DL-arginal: Leup.) and pepstatin (isovaleryl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid: Pep.) were isolated from culture filtrates of various Actinomycetes (17-20). Leup. and Pep. are reported to inhibit rather specifically cathepsin B and D, respectively (21), which are considered to play a major role in the intracellular proteolysis in the liver (22).

With the aim to confirm the possible involvement of the lysosomal proteases in initiating liver cell proliferation, the effects of these protease inhibitors on nucleic acid (RNA and DNA) syntheses and mitosis in partially hepatectomized rat livers have been investigated in the present paper.

MATERIALS AND METHODS

Partial hepatectomy was performed by removing the median and left lateral lobes of male Wistar rats weighing 110-140 g according to the method of Higgins and Anderson (23). Leup. and Pep. were kindly provided by Dr. T. Takeuchi, Institute of Microbial Chemistry, Tokyo. Rats received four successive intraperitoneal injections of 0.2 ml of the Leup. plus Pep. solution (1 mg each in 0.2 ml dimethylsulfoxide) or 0.1 ml of the Leup. solution (2 mg in 0.1 ml physiological saline) with a 3-hour interval immediately after partial hepatectomy. As the control, partially hepatectomized rats received sham injections of the solvent under the similar conditions. At various times

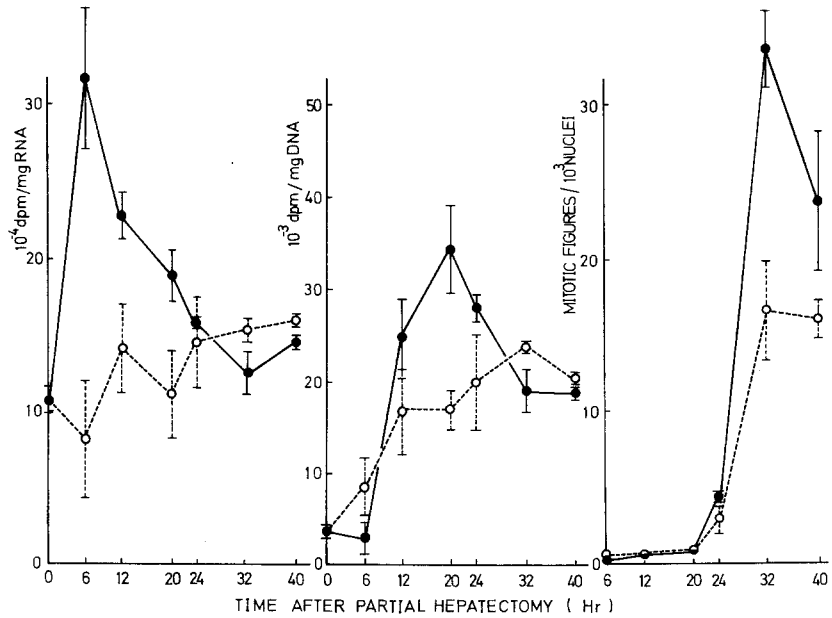

Fig. 1. Effects of Leup. plus Pep. on the activities of nucleic acid (RNA and DNA) syntheses and mitosis of regenerating livers of rats. Leup. and Pep. (1 mg each) dissolved in 0.2 ml of dimethylsulfoxide (experimental group) or 0.2 ml of dimethylsulfoxide without protease inhibitors (control group) were injected intraperitoneally four times every three hours immediately after partial hepatectomy. ^{14}C-Orotate (4 μCi) was injected intraperitoneally one hour prior to sacrifice to assay the nucleic acid syntheses. Parts of the livers were fixed in neutral 10% formalin and the sections were stained with hematoxylin to count mitoses. Each point indicates a mean value with an average deviation from 3-5 rat measurements. O------O ; Experimental, ●——● ; Control. (a) RNA synthesis, (b) DNA synthesis and (c) Mitotic index.

after partial hepatectomy 3-5 rats in each group received an intraperitoneal injection of 4.0 μCi of ^{14}C-orotate (Radiochemical Centre, Amersham, England; 62 Ci/mole) dissolved in 0.4 ml of physiological saline per 100 g body weight and the animals were killed 1 hour later. Portions of the livers were homogenized with 5 volumes of distilled water in a glass homogenizer and aliquots of the homogenates were treated according to the procedures of Schmidt, Thannhauser and Schneider (24) with a little modification to separate the RNA and DNA fractions. Radioactivities of the nucleic acid fractions were measured by

a liquid scintillation spectrophotometer with an external standard. RNA and DNA were assayed by the conventional colorimetric methods using orcinol (25) and diphenylamine (26), respectively, with yeast RNA and calf thymus DNA as standards. For histochemical staining of cell coat acid mucopolysaccharide, portions of the livers were fixed in 10% neutral formalin and the sections with 5 μ thickness were stained according to the method of Mowry (13, 14, 27). For measuring mitotic indices, the liver sections were stained with the Mayer's hematoxylin and numbers of mitoses per 10^3 nuclei were counted.

RESULTS AND DISCUSSIONS

Figs. 1 and 2 illustrate the time course changes in RNA synthesis, DNA synthesis and mitosis of regenerating livers of rats treated with Leup. plus Pep. (Fig. 1) or with Leup. only (Fig. 2) in comparison to those of rats treated with the solvent only (control). In accord with the already reported findings (28) RNA synthesis in the control groups increased immediately after partial hepatectomy, reached a maximal level within 6 hours and then declined. The increased RNA synthesis at the initial stage of liver regeneration is known to be responsible for the subsequently occurring biochemical events such as increased protein synthesis and DNA synthesis and mitosis (28). This initial increase in the RNA synthesis was found to be inhibited or retarded in the experimental groups treated with Leup. plus Pep. (Fig. 1-a) as well as in the experimental group treated with Leup. only (Fig. 2-a) though less markedly. The higher maximal level of RNA synthesis in Fig. 1 in comparison to Fig. 2 seems to be ascribed to the effect of dimethylsulfoxide as will be discussed later.

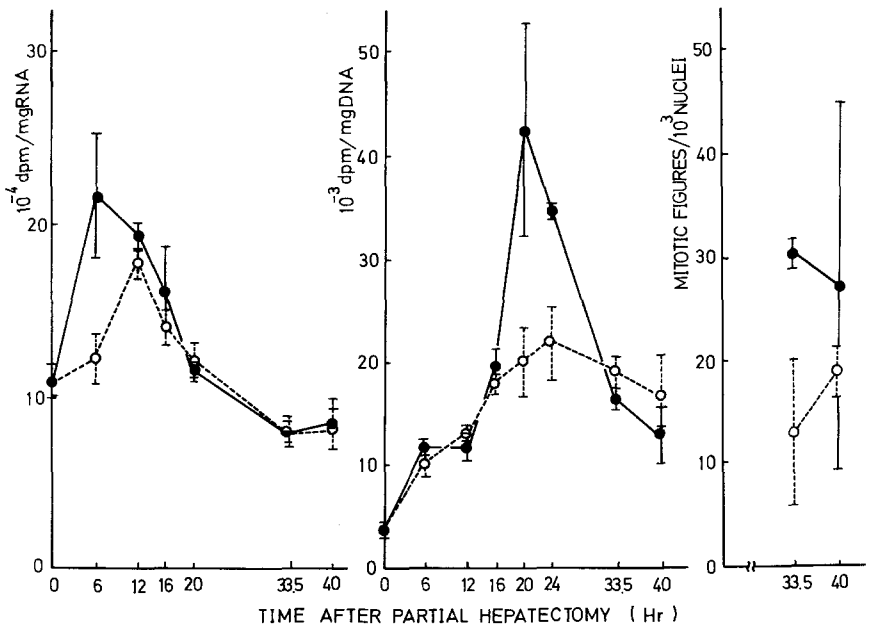

Fig. 2. Effects of Leup. on the RNA and DNA syntheses and mitosis in regenerating livers of rats. Two mg Leup. dissolved in 0.1 ml of physiological saline (experimental group) or 0.1 ml of physiological saline only (control group) were injected intraperitoneally four times every three hours immediately after partial hepatectomy. The nucleic acid syntheses and mitotic indices were measured in the same ways as in Fig. 1. O------O; Experimental, ●——●; Control. (a) RNA synthesis, (b) DNA synthesis and (c) Mitotic index.

DNA synthesis that is rather low in the normal liver is known to start to increase at around 12 h after partial hepatectomy, reach a maximal level at 22-32 h depending on the age of rats and decline later (29). The typical time course change in the DNA synthesis was observed for the control group treated with physiological saline (Fig. 2-b). The time course change of DNA synthesis in the control group treated with dimethylsulfoxide (Fig. 1-b) appears to be somewhat different, showing a premature increase in DNA synthesis. This premature increase in DNA synthesis seems to be also due to the effect of dimethylsulfoxide itself. In separate experiments we have observed the considerable increase not only in RNA but also in

DNA synthesis (60-70% over the control at 6 hours) of the normal liver of rats treated with dimethylsulfoxide under the similar conditions. Dimethylsulfoxide has been reported to increase the permeability of various cytomembranes and thus to activate the lysosomal enzymes in vivo (30). Treatment of partially hepatectomized rats with Leup. plus Pep. or with Leup. only appears to inhibit or retard the induction of DNA synthesis in accord with the similar effects of the antiprotease(s) on the preceding RNA synthesis. Similarly, mitotic figures which are known to appear at around 28 hours, rise steeply to a peak at around 28-30 hours, and fall off later were shown to become significantly smaller in the number in the experimental groups treated with the protease inhibitor(s) than in the control groups.

Histochemical examinations of the cell coat acid mucopolysaccharide (positive: blue, negative: without stain or faintly red) revealed that the cell coat acid mucopolysaccharides which disappeared almost completely in the control groups by 20 hours after partial hepatectomy were shown more or less to persist in the livers of experimental groups treated with the protease inhibitor(s) even at 20 hours, 24 hours or later. Details of the histochemical studies on the effect of the protease inhibitors on the pericellular construction in the regenerating livers will be reported separately.

The results presented in this paper seem to indicate that intraperitoneal injections of microbial protease inhibitor(s) can suppress or retard the sequential occurrence of key events related to the liver regeneration such as the surges of RNA synthesis, DNA synthesis and mitosis, supporting our hypothesis that the lysosomal protease may be involved in the initiation of liver cell proliferation.

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