Serum Factors Affecting Cathepsin Release from Lysosomes

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Received April 1,1975

SUMMARY: Rat or bovine serum exhibited the inhibitory effect on cathepsin release from rat liver lysosomes in vitro. The inhibitory activity of serum was enhanced after dialysis. Heated (100° C, 3-5 min) serum showed the marked stimulatory effect on the cathepsin release. It was indicated that the adult and infant rat or bovine sera contain a large-molecular, heat-labile inhibitory factor and a small-molecular-heat-stable stimulatory factor. Fetal bovine serum, however, was found to be very poor in these factors. The activity of each of the serum factors was found to change characteristically within a short period after partial hepatectomy.

Although tissue regeneration is one of the most attractive subjects in biology, the real mechanisms for the homeostatic control of tissue cell proliferation have not yet been fully understood. In the preceding papers we have reported that temporary disappearance of cell coat, as detected by the cell coat acid mucopolysaccharide stain, seems to be intimately related with the initiation of cell proliferation (1, 2) and that the lysosomal cathepsin may be responsible for the initiation of liver regeneration since cathepsin inhibitors (leupeptin and/or pepstatin) injected during early periods of liver regeneration inhibited or retarded the nucleic acid (RNA, DNA) biosynthesis, mitosis as well as the temporary disappearance of cell coat accompanying the liver regeneration (3). In connection to our previous reports, the report by Adams (4) showing the elevation of some lysosomal enzyme activities (acid DNase, acid phosphatase, β-glucuronidase) soon after partial hepatectomy as well as that by Allison (5) showing a decrease in the number of lysosomal particles and migration of them from the deep inside to the periphery of liver

cells within a few hours after partial hepatectomy seem to be particularly interesting.

During the course of searching for possible humoral or cytoplasmic factor(s) responsible for the "lysosomal activation", we have found that rat or bovine serum contain factors affecting the permeability of lysosomal membranes against cathepsin.

MATERIALS AND METHODS

Lysosomes were prepared from the liver of male, Wistar rats weighing 100-150 g. A 1:8 (w/v) liver homogenate in 20 mM Tris (pH 7.2) - 0.25 M sucrose was subjected to the successive centrifugations, 750 x g for 10 min, 3,300 x g for 10 min, and then 15,000 x g for 20 min, to sediment lysosome-rich pellet, which was once gently rinsed. The lysosomal pellet derived from 10 g liver was suspended in 20 ml of 0.25 M sucrose - 1 mM EDTA, pH 7.2 (lysosomal suspension). Measurements of cathepsin release were carried out as follows. A 2.0 ml aliquot of the lysosomal suspension and a small volume (0.1-0.6 ml) of test material or 0.9% NaCl as the control were mixed and the mixture was incubated at 37° C for certain periods of time with gentle shaking, followed by centrifugation at 15,000 x g for 20 min. The pellet was suspended in 2.0 ml of 0.25 M sucrose - 0.2% Triton X-100 and the suspension was kept at 0° C for 60 min, followed by centrifugation at 15,000 x g for 20 min. The supernatant thus obtained was used for the assay of the "residual (unreleased) cathepsin D activity". The "total cathepsin activity" was measured by treating 2.0 ml of the lysosomal suspension directly with Triton. Assays for cathepsin D were carried out by the method of Anson (6). A 0.1 ml aliquot of supernatant

from the Triton-solubilized lysosomes was mixed with 1.9 ml of freshly prepared acid-denatured hemoglobin solution in 0.18 M acetate buffer, pH 3.2 and incubated at 37° C for 60 min. The reaction was stopped by the addition of 1.0 ml of 10% trichloroacetic acid. The supernatant containing acid-soluble hemoglobin hydrolyzates was subjected to colorimetry at 700 nm by the method of Lowry et al. (7). The percent release of cathepsin D was defined as 100 x (Total activity - Residual activity)/Total activity. The percent stimulation (or inhibition) was defined as 100 x (%Release in the presence of test material - %Release in the control)/%Release in the control : + sign for stimulation and - sign for inhibition. One unit of inhibitor or stimulator was defined as the activity to cause 1% inhibition or 10% stimulation, respectively. The more detailed conditions for the assay of the serum factors will be presented in the text. Partial hepatectomy was carried out by removing the median and left lateral lobes according to Higgins and Anderson (8). Newborn and adult rat sera were prepared from 5-day and 5-week old rats, respectively. Bovine sera (adult and calf) were from the Mitsubishi Life Science Research Institute and fetal bovine serum (Slow Laboratories, Rockville, U.S.A.) was a gift from Dr. Y. Mizuno in our Institute.

RESULTS AND DISCUSSION

In Fig. 1-a are illustrated the kinetics of cathepsin D release from rat liver lysosomes in the presence and the absence of adult rat serum. Inhibition of cathepsin release by the serum appears to begin at 2 h, become more and more marked as the incubation time increases, and finally become

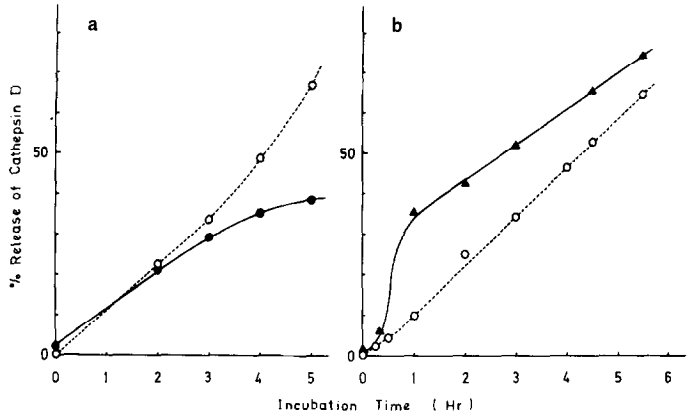

Fig. 1. Kinetics of cathepsin D release from rat liver lysosomes in the presence of rat serum (a) or of heated rat serum (b).

(a) 0.2 ml of freshly prepared adult rat serum was added to 2.0 ml of lysosomal suspension. As the control, 0.2 ml of 0.25 M sucrose-1 mM EDTA was added. (b) Adult rat serum was mixed with an equal volume of 0.9% NaCl, heated at 100°C for 5 min, cooled in ice-water and centrifuged. 0.6 ml of the supernatant (equivalent to 0.3 ml of the original serum) was added to 2.0 ml of the lysosomal suspension. As the control, 0.6 ml of 0.9% NaCl was added. ○ : control, ● : adult rat serum, and △ : heated adult rat serum.

almost complete at 4-5 h of incubation, while the release in the control appears to proceed almost linearly with time. Therefore, in the following experiments, 4.5 h incubation was adopted for assaying the serum inhibitory factor. As shown in Table I, the adult rat and bovine sera as well as the newborn rat and calf sera can inhibit the cathepsin release, while the fetal bovine serum inhibits only slightly. Furthermore, the inhibitory activity of serum was found to be increased after dialysis, suggesting that the sera may contain a small-molecular factor, which may counteract the effect of the large-molecular inhibitory factor. It should be noted that the inhibitory activity of dialyzed serum appears to increase as the age of donor animals becomes older

The presence of a stimulating factor in the serum was more directly

Table I. Inhibition of cathepsin D release from lysosomes by various sera and dialyzed sera.

0.3 ml of various sera or dialyzed sera (see Fig. 2) from different donors was added to 2.0 ml of lysosomal suspension. As the control, 0.3 ml of 0.9% NaCl was added. The mixtures were incubated at 37° C for 4.5 h.

Exp. No.	Addition	% Release	% Inhibition
I	None (control)	50.7	----
	Adult rat serum	36.7	27.6
	Newborn rat serum	36.2	28.6
	Bovine serum[a)]	34.5	32.0
	Calf serum	36.5	28.0
	Fetal bovine serum	46.1	9.1
II	None (control)	44.4	----
	Adult rat serum	32.5	26.8
	Dialyzed adult rat serum	26.8	39.6
	Bovine serum	30.4	31.5
	Dialyzed bovine serum	15.7	64.6
III	None (control)	55.7	----
	Dialyzed adult rat serum	33.0	40.7
	Dialyzed newborn rat serum	41.7	25.1
	Dialyzed adult bovine serum[a)]	37.7	32.3
	Dialyzed calf serum	41.8	24.9
	Dialyzed fetal bovine serum	47.3	15.1

a) Immobilized serum was used.

evidenced by observing the effects of heated sera. In Fig. 1-b we show the kinetics of cathepsin release in the presence and the absence of heated adult rat serum. In contrast to the inhibitory effect of whole serum or serum dialyzate, the stimulatory effect of heated serum seems to be most evident after 1 h of incubation and to become less marked as the incubation time is prolonged. Therefore, in the following experiments the stimulatory factor

Table II. Stimulation of cathepsin D release from lysosomes by various heated sera.

Heated sera were prepared from various sera from different donors as described in Fig. 1-b. 0.6 ml of heated serum (equivalent to 0.3 ml of original serum) was added to 2.0 ml of lysosomal suspension and the mixture was incubated at 37° C for 1 h. As the control, 0.6 ml of 0.9% NaCl was added.

Addition	% Release	% Stimulation
None	8.2	---
Heated adult rat serum	25.9	210
Heated newborn rat serum	21.4	161
Heated bovine serum[a)]	22.9	179
Heated calf serum	21.4	161
Heated fetal bovine serum	8.7	6

a) Immobilized serum was used.

was assayed at 1 h of incubation. In Table II are compared the stimulatory activities of various sera heated at 100° C for 5 min. It appears that all of the sera except the fetal bovine serum contain the heat-stable stimulatory factor.

As shown in Fig. 2, almost linear dose-response relation can be observed for both the inhibitory factor in dialyzed serum and the stimulatory factor in heated serum within a range below 35% inhibition or 140% stimulation, affording a rational basis for the quantitative assay of the serum factors.

Based on the above dose-response relation, activities of the rat serum factors were measured at various times after partial hepatectomy. As summarized in Table III, the stimulatory factor was found to increase immediately after partial hepatectomy, reach a maximal level almost 2-fold higher than the initial level at around 2-3 h after partial hepatectomy and

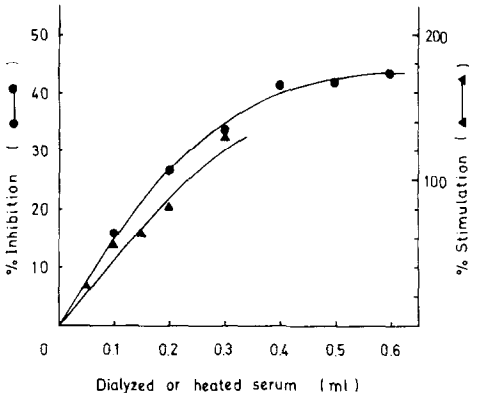

Fig. 2. Dose-dependent inhibition or stimulation of cathepsin D release from lysosomes by dialyzed rat serum or heated rat serum.

Various volumes of adult rat serum dialyzed against 0.9% NaCl at 4°C overnight or heated adult rat serum prepared as described in Fig. 1-b were added to 2.0 ml of lysosomal suspension. The final volume was adjusted to 2.6 ml by adding 0.9% NaCl. The mixture was incubated at 37°C for 4.5 h in case of dialyzed serum or for 1 h in case of heated serum. Percent inhibition or stimulation was measured as described in Materials and Methods.

Table III. Change of activities of the serum factors after partial hepatectomy.

Serum was prepared from 2 rats at various times after partial hepatectomy. The 0 h serum was prepared from 2 sham-operated rats. Dialyzed or heated serum was prepared as described earlier (Fig. 1, Fig. 2) and used for the assay of the inhibitory factor or the stimulatory factor, respectively. In each assay, two different volumes of serum (usually 0.1 and 0.2 ml) were added to 2.0 ml of lysosomal suspension in order to check the linear dose-dependency. The mixtures were incubated for 1 h or 4.5 h to measure the stimulatory factor or the inhibitory factor, respectively.

Time after partial hepatectomy (h)	Activity (units/ml serum) Stimulatory factor	Inhibitory factor
0	62	126
3	136	127
6	93	180

decline gradually thereafter. On the other hand, the inhibitory factor remained unaltered for the first few hour period, followed by the gradual

increase. Taking into considerations that the apparent lysosomal activation (5, 6) as well as the initiating stimulus for the regeneration (9) seem to be accomplished within a very early period after partial hepatectomy, the results in this paper showing the temporary surge of the serum stimulatory factor soon after partial hepatectomy seems quite interesting, although it may be still too early to conclude that this may be an actual chemical trigger for the liver regeneration. The nature as well as the mode of actions of these serum factors are now under investigations.

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