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LIVER PLASMA MEMBRANES AND PROTEOGLYCAN PREPARED THEREFROM INHIBIT THE GROWTH OF HEPATOMA CELLS IN VITRO

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1. Plasma membranes from rat liver or kidney inhibited the growth of hepatoma (AH-130) cells in vitro. AH-130 plasma membranes or erythrocyte ghosts inhibited the growth of AH-130 cells less effectively. The inhibitory activity of liver plasma membranes was lost by heat treatment, or mild protease (papain or bromelin, but not trypsin or pronase) treatment, whereas it was retained after sialidase treatment or delipidation by ethanol/ether. 2. Proteoglycan (proteoheparan sulfate) prepared from liver plasma membranes inhibited the growth of AH-130 cells, but heparan sulfate was less active. The inhibitory activity of liver plasma membranes seemed, however, not to be ascribable solely to proteoheparan sulfate associated with plasma membranes. 3. Preliminary investigations suggested that the molecular weight 40 000 component may be a major inhibitory principle in liver plasma membranes.

Introduction

In the preceding paper [1] we showed that a purified proteoglycan (proteoheparan sulfate) preparation from rat liver plasma membranes is bound to the surfaces of trypsinized ascites hepatoma cells, and at the same time it inhibits the concanavalin A-mediated agglutination of the cells. It is known that the cell surface proteoglycan is temporarily lost at the time of cell proliferation [2,3], and that mild protease-treatment of cells liberating acid mucopolysaccharides (and perhaps some other components) from cell surfaces often initiates the cell proliferation in systems both in vivo and in vitro [3–6].

From the viewpoint that the cell surface proteoglycans may be responsible for modulating the dynamics of proteins in plasma membranes, and hence may affect lectin-mediated agglutination [1] and/or cell proliferation, we investigated the effects of liver plasma membranes and proteoheparan sulfate prepared therefrom on the growth of ascites hepatoma (AH-130) cells in vitro in the present study, finding that both liver plasma membranes and proteoheparan sulfate inhibit the growth of AH-130 cells in vitro, although they appear to inhibit the growth of hepatoma cells by different mechanisms.

Materials and Methods

Preparation of plasma membranes. Plasma membranes were prepared from the livers or kidneys of male Donryū rats (body weight 140–180 g) by the method of Ray [7], or from freshly isolated AH-130 cells by the method of Ray with a slight modification, using the homogenization medium containing 2.0 mM $CaCl_2$. Erythrocyte ghosts were prepared by treating erythrocytes (rat) with 20 vol. 5 mM Hepes buffer, pH 7.4, followed by repeated washing with the same solution. Plasma membranes sterilized with 70% ethanol or by ultraviolet irradiation were washed with sterile phosphate-buffered saline, and finally suspended in the culture medium.

Cell culture. Ascites hepatoma cells (AH-130) were harvested from the peritoneal cavities of male, Donryu rats (7 weeks old) 7–8 days after inoculation, freed from the blood cells by repeated washing with sterile phosphate-buffered saline, and finally suspended in Eagle's minimal essential medium with 10% fetal calf serum, sodium pyruvate (110 mg/ml), penicillin G (100 units/ml) and streptomycin (100 μg/ml).

A 0.6 ml aliquot of AH-130 cell suspension ($(2–3) \cdot 10^5$ cells/ml) with or without test material was cultured in a small pyrex tube at 37°C for 1–3 days without shaking under 5% CO_2/95% air. The medium was not exchanged except when otherwise specified. To follow the cell growth, the cells in each tube were spun down (1 000 rev./min, 10 min) at certain intervals of culture, suspended in 2 ml 1 M citric acid/0.05% crystal violet, and the number of stained nuclei was counted in a hematocytometer. Cells in 2–3 culture tubes were counted at each time. The cells with larger than 90% viability (trypan blue exclusion test) were used throughout the present study.

Pretreatments of plasma membranes. Sialidase digestion of plasma membranes was carried out by incubating plasma membranes (6.0 mg protein) with sialidase (50 units) in 1 ml of 0.1 M potassium acetate buffer (pH 5.5)/1 mM $CaCl_2$/30 μg/ml leupeptin [1] at 37°C for 2 h. Plasma membranes were then spun down in an Eppendorf centrifuge, washed with phosphate-buffered saline (pH 7.4).

Protease digestion was carried out by incubating plasma membranes with proteases in various concentrations (indicated in the text) at 37°C for 10 min either in 5 mM phosphate buffer (pH 7.4) (trypsin and pronase digestion) or in 50 mM phosphate buffer (pH 6.8)/20 mM mercaptoethanol/1 mM EDTA (papain and bromelin digestion). After digestion, membranes were washed as described earlier. Heparitinase digestion was carried out by incubating plasma membranes (6.0 mg protein) with heparitinase (20 units) in 0.5 ml of 0.1 M sodium acetate buffer (pH 7.0)/1 mM calcium acetate/30 μg/ml leupeptin/30 μg/ml antipain at 37°C for 3 h.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of plasma membranes. Plasma membranes (125 μg protein) were solubilized in 50 μl of 1% sodium dodecyl sulfate solution containing 1% mercaptoethanol by heating at 100°C for 30 min, and then subjected to polyacrylamide (7.5%) gel electrophoresis according to Weber and Osborn [8]. Protein bands were stained with Coomassie brilliant blue. For molecular weight standardization were used bovine serum albumin, ovalbumin and myoglobin.

Gel filtration of solubilized plasma membranes through Sephadex G-100. Plasma membranes (40 mg protein) were extracted with 5 ml of 8 M urea/50 mM $NaHCO_3$ (pH 10.0) as described earlier [1], and 5 ml of the extract were applied onto a column (1.1 × 90 cm) of Sephadex G-100. The column was washed with 2.0 M urea/0.5 M NaCl/10 mM phosphate buffer (pH 7.4), with collection of fractions every 3 ml effluent.

Preparation of hepatocytes from rat livers. Isolated hepatocytes were prepared according to the method of Seglen [9] with a slight modification [10].

Biochemical assays. Protein was assayed by the method of Lowry et al. [11] with bovine serum albumin as standard. Hexuronate was assayed by the method of Galambos [12] with D-glucuronic acid as standard.

Biochemicals. Eagle's minimal essential medium was purchased from the Daigo Eiyokagaku Co., Japan, and fetal calf serum was from the Microbiological Research Foundation, Japan. Heparan sulfate was from Upjohn, Kalamazoo, U.S.A. Sialidase (*Vibrio cholerae*, 500 units/ml) was from BDH, Poole, U.K. Trypsin (Type I) and bromelin (grade II) were from Sigma, St. Louis, U.S.A. Pronase E was from the Kaken Kagaku Co., Tokyo. Papain was from Worthington Biochemical, Freehold, U.S.A. Heparitinase from *Flavobacterium heparinum* was a gift from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. Sephadex G-100 was from Pharmacia, Uppsala, Sweden.

Results

1. Effects of plasma membranes (liver, kidney, erythrocytes, AH-130) on the growth of AH-130 cells in culture

The growth curves of AH-130 cells in the presence of rat liver plasma membranes added in various

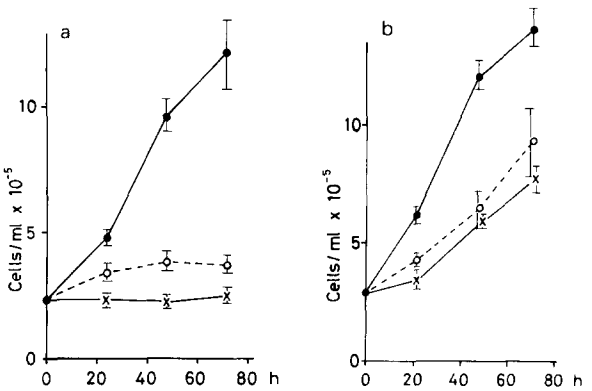

Fig. 1. Effects of plasma membranes prepared from rat liver and AH-130 cells on the growth of AH-130 cells in vitro. (a) Effect of liver plasma membranes. AH-130 cells were cultured in the absence (•) or the presence of liver plasma membranes at 0.1 mg protein/ml (○) and 1.0 mg protein/ml (X). (b) Effect of AH-130 plasma membranes. AH-130 cells were cultured in the absence (•) or the presence of AH-130 plasma membranes at 0.7 mg protein/ml (○) and 2.0 mg protein/ml (X). Ordinate and abscissa indicate number of cells (cells/ml X 10^{-5}) and time of incubation (h), respectively. Each point is mean ± S.D. from three tubes.

concentrations (0, 0.1, and 1.0 mg protein per ml) are shown in Fig. 1a, indicating that the rat liver plasma membranes inhibit dose-dependently the growth of AH-130 cells in culture. In the presence of 1.0 mg protein-equivalent liver plasma membranes per ml, the growth of AH-130 cells was inhibited completely. Fig. 1b shows the growth curves in the presence of AH-130 plasma membranes (0.7 mg and 2.0 mg protein/ml), indicating that the inhibitory activity of AH-130 plasma membranes is much smaller than that of liver plasma membranes. Erythrocyte ghosts were not effective, as was the case for AH-130 plasma membranes, whereas kidney plasma membranes inhibited the growth of AH-130 cells as much as liver plasma membranes (data not shown).

2. Effects of pretreatments of plasma membranes (liver) on the activity inhibiting the growth of AH-130 cells in culture

The activity of liver plasma membranes inhibiting the growth of AH-130 cells in culture was not affected by pretreatment with ethanol/ether (3 : 1, v/v) at room temperature for 1 h (delipidation), or with sialidase (50 units/ml) at 37°C for 2 h (data not shown). However, the inhibitory activity of liver plasma membranes appeared to be lost completely by heating the plasma membranes at 100°C for 10 min (data not shown).

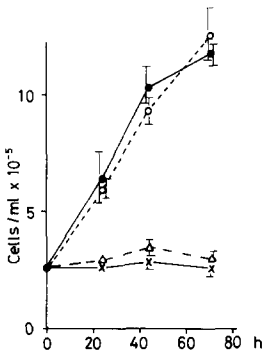

Fig. 2. Effects of liver plasma membranes pretreated with trypsin and papain on the growth of AH-130 cells in vitro. Rat liver plasma membranes pretreated with trypsin (100 μg/ml) (△) or papain (50 μg/ml) (○), or liver plasma membranes without protease-pretreatment (X) were added to AH-130 cell culture at a concentration of 1 mg protein/ml. The concentration of plasma membranes is expressed in terms of plasma membrane protein prior to protease treatment. • corresponds to the cell growth in the absence of plasma membranes. Each point is mean ± S.D. from three tubes. Ordinate and abscissa indicate number of cells (cell/ml X 10^{-5}) and time of incubation (h), respectively.

Pretreatment of liver plasma membranes with proteases resulted in either no appreciable change of activity or the complete loss of activity, depending on which protease was used. As shown in Fig. 2, trypsin-pretreated (100 μg/ml, 10 min) plasma membranes appeared to retain inhibitory activity, whereas papain-pretreated (50 μg/ml, 10 min) plasma membranes appeared to have lost inhibitory activity.

3. Apparent correlation between acid mucopolysaccharide release from plasma membranes (liver) and loss of inhibitory activity upon treatment with protease

Rat liver plasma membranes were pretreated with papain, bromelin, trypsin or pronase in various concentrations at 37°C for 10 min. Hexuronic acid (a component of acid mucopolysaccharides) released into the reaction medium (supernatant) and the inhibitory activity of protease-pretreated plasma membranes (pellet) were assayed.

As shown in Table I, acid mucopolysaccharides

TABLE I

HEXURONATE RELEASE AND LOSS OF INHIBITORY ACTIVITY UPON PROTEASE TREATMENT OF LIVER PLASMA MEMBRANES

Rat liver plasma membranes (1.4 mg protein per 0.5 ml incubation mixture) were treated with trypsin, pronase, papain or bromelin (concentrations are indicated) at 37°C for 10 min, and then spun down. Hexuronate released into the supernatant was assayed. The pellet was suspended in 1.0 ml water, and a 0.3 ml aliquot was assayed for protein. Plasma membranes in the rest (0.7 ml) were spun down, suspended in 1.5 ml of culture medium, and then mixed with an equal volume of AH-130 cell suspension in the culture medium. The whole mixture was incubated at 37°C for 2 days and the number of cells was counted. The number of cells (10^5 cells/ml) at zero time of incubation and after 2 days of incubation in the absence of plasma membranes were 2.49 and 8.66 ± 0.52, respectively.

Protease treatment		Plasma membranes [a] added (mg protein/ml)	Hexuronate release (μg)	Number of cells [b] (cells/ml) ($\times 10^{-5}$)	Increase (%)	Inhibitory activity
Protease	μg/ml					
None	0	0.25	0.0	4.00 ± 0.32	61	++
Trypsin	1	0.20	trace	3.24 ± 0.29	31	++
	10	0.11	trace	3.64 ± 0.06	46	++
	100	0.10	<0.5 [c]	4.79 ± 0.30	92	+
Pronase	10	0.09	<1.1 [c]	3.74 ± 0.25	50	++
	100	0.04	<1.1 [c]	7.81 ± 0.23	214	–
Papain	1	0.15	1.4	8.51 ± 0.69	242	–
	10	0.10	1.8	8.68 ± 1.12	249	–
	100	0.08	1.8	8.50 ± 1.22	241	–
Bromelin	1	0.14	1.1	4.65 ± 0.21	87	+
	10	0.11	1.4	8.68 ± 0.95	249	–
	100	0.08	1.7	8.35 ± 0.25	235	–

[a] Concentration is expressed in terms of protease-treated plasma membrane protein per ml.
[b] Mean ± S.D. from three tubes.
[c] Because of the interference by proteins in colorimetry, only the upper limits are indicated.

were released from plasma membranes (liver) much more sensitively upon treatment with papain and bromelin (SH-proteases) compared to trypsin and pronase. At the same time the inhibitory activity of plasma membranes appeared to be reduced more preferentially upon treatment with these SH-proteases. It should be noted that treatment of plasma membranes only with the medium for papain or bromelin digestion (blank digestion) did not affect the inhibitory activity.

4. Effects of proteoheparan sulfate and heparan sulfate on the growth of AH-130 cells in culture

The apparent correlation between acid mucopolysaccharide release from liver plasma membranes and loss of inhibitory activity of liver plasma membranes upon pretreatment with proteases led us to examining the possibility that proteoglycan (proteoheparan sulfate) or acid mucopolysaccharide (heparan sulfate) associated with liver plasma membranes may inhibit the growth of AH-130 cells in vitro.

Using purified preparation of proteoheparan sulfate from rat liver plasma membranes (prepared as described earlier [1]) at a concentration of 4 μg hexuronate-equivalent per ml (or 2.7 μg protein equivalent per ml), and heparan sulfate (freed from protein conjugate) at concentrations of 4 μg and 40 μg hexuronate-equivalent per ml, the effects of them on the growth of AH-130 cells in vitro were investigated.

As shown in Fig. 3, proteoheparan sulfate at 4 μg hexuronate/ml inhibited the growth of AH-130 cells a little more strongly than heparan sulfate at 40 μg hexuronate/ml, suggesting that the protein moiety of proteoheparan sulfate is required for the

maximal inhibitory activity much as in the inhibition of lectin-mediated cell agglutination by proteoheparan sulfate [1].

However, if we compare the inhibitory activity

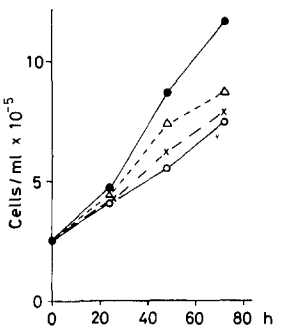

Fig. 3. Effects of proteohaparan sulfate prepared from liver plasma membranes and heparan sulfate on the growth of AH-130 cells in vitro. AH-130 cells were cultured in the absence (•) or the presence of proteoheparan sulfate at a concentration of 4 μg hexuronate-equivalent/ml (○), and heparan sulfate at concentrations of 4 μg hexuronate-equivalent/ml (△) or 40 μg hexuronate/ml (X). Ordinate and abscissa indicate number of cells (cells/ml X 10^{-5}) and time of incubation (h), respectively. Each point is mean from two tubes.

of proteoheparan sulfate with that of liver plasma membranes on the basis of equal hexuronic acid concentration, the inhibitory activity of proteoheparan sulfate appeared to be much smaller than that of liver plasma membranes, because the latter inhibited the growth of AH-130 cells almost completely at 1 mg protein/ml (Fig. 1a), which corresponds to about 1 μg hexuronate/ml.

5. Possibility of the presence of another inhibitory principle in liver plasma membranes

The proteoglycan (proteoheparan sulfate) inhibition of hepatoma cell growth was observed repeatedly using different batches of proteoheparan sulfate. However, as stated earlier, the inhibitory activity of liver plasma membranes appears to be too large to be explained only by proteoheparan sulfate associated with the plasma membrane preparations.

Preliminary investigations using $^{35}SO_4$-labelled liver plasma membranes prepared as described earlier [1] revealed that no appreciable radioactivity was transferred to AH-130 cells when the cells were incubated with the labelled plasma membranes for 2 days and then separated by centrifugation in a Percoll gradient (data not shown). Moreover, liver plasma membranes pretreated with heparitinase inhibited

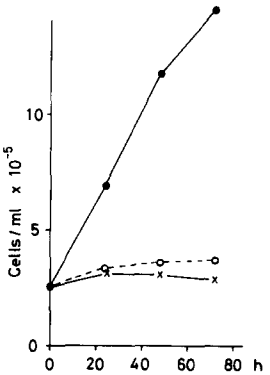

Fig. 4. Effect of liver plasma membranes pretreated with heparitinase on the growth of AH-130 cells in vitro. Rat liver plasma membranes pretreated with (○) or without (X) heparitinase (40 units/ml) at 37°C for 3 h were added to the AH-130 cell culture at a concentration of 1 mg protein/ml. • corresponds to the cell growth in the absence of plasma membranes. Ordinate and abscissa indicate number of cells (cells/ml X 10^{-5}) and time of incubation (h), respectively. Each point is mean from two tubes.

the growth of AH-130 cells much as the untreated liver plasma membranes as shown in Fig. 4, suggesting that the potent inhibitory activity of liver plasma membranes may be due to a component other than proteoheparan sulfate.

The possibility that the inhibitory activity of liver plasma membranes may be due to a protein component with 40000 molecular weight was raised from the following experimental results.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of liver plasma membranes pretreated with proteases (papain, bromelin, trypsin and pronase)revealed that one of the protein bands (the band 9 from amongst altogether more than ten detectable bands) was deleted commonly in the plasma membranes which were treated with papain or bromelin and thus lost the inhibitory activity, as shown in Fig. 5.

The band 9 protein was isolated from the 8 M urea/50 mM $NaHCO_3$ extract of trypsin-treated plasma membranes by gel filtration through Sephadex G-100 (the protein band corresponding to mole-

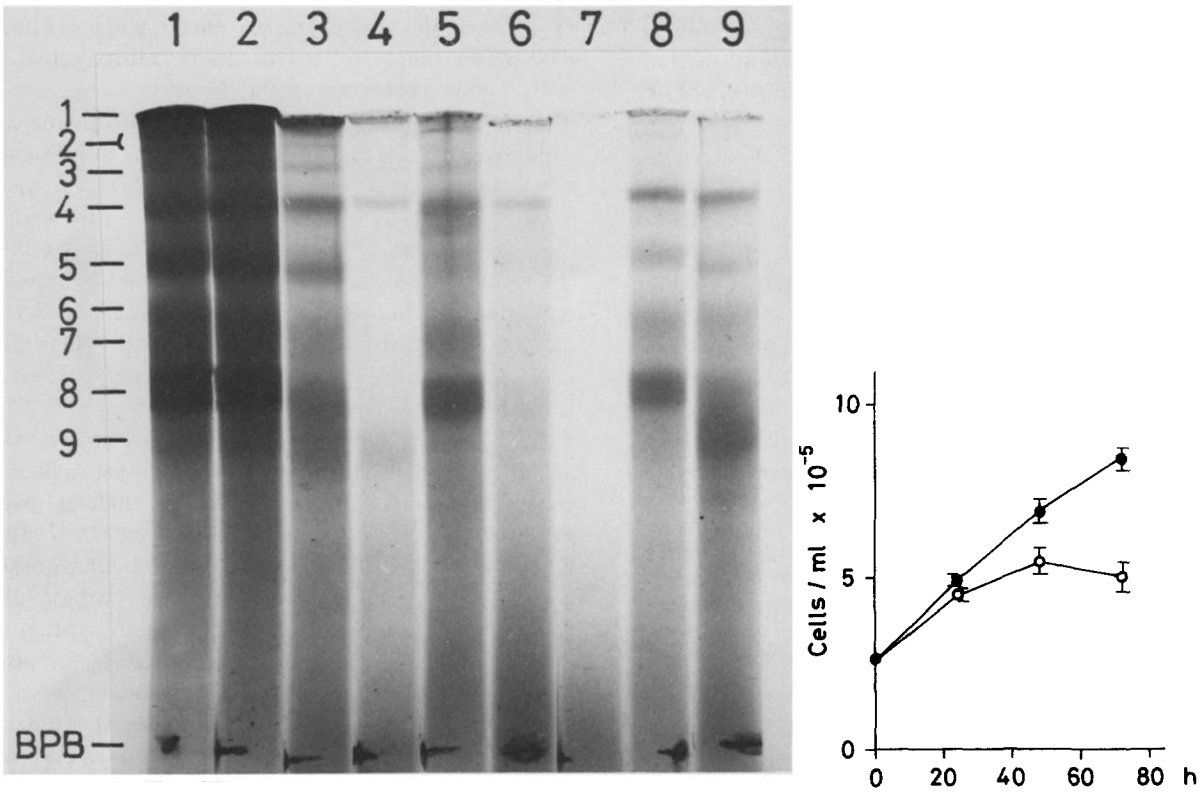

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of liver plasma membranes treated with various proteases. Liver plasma membranes corresponding to 125 μg protein were treated with proteases at 37°C for 10 min, and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis at 8 mA for 4 h as described in the text. The column numbers from 1 to 9 on the top correspond to (1) untreated plasma membranes, or plasma membranes treated with (2) trypsin (1 μg/ml), (3) trypsin (10 μg/ml), (4) trypsin (100 μg/ml), (5) papain (1 μg/ml), (6) papain (10 μg/ml), (7) papain (100 μg/ml), (8) bromelin (10 μg/ml) and (9) pronase (10 μg/ml). The numbers from 1 to 9 on the left indicate protein bands in the order of increasing mobilities detected by Coomassie brilliant blue staining. BPB corresponds to bromophenol blue used as reference dye.

Fig. 6. Effect of a 40 000 molecular weight component (band 9) isolated from liver plasma membranes on the growth of AH-130 cells in vitro. Rat liver plasma membranes (40 mg protein) were extracted with 8 M urea/50 mM $NaHCO_3$ (pH 10.0) as described in the preceding paper [1], and the extract was subjected to gel-filtration through Sephadex G-100, collecting a protein fraction corresponding to 40 000 molecular weight. The fraction was sterilized by passing through a Millipore filter, and was added to the AH-130 cell culture at a concentration of 50 μg protein/ml (○). • corresponds to the cell growth in the absence of the fraction. Each point is mean ± S.D. from two tubes. Ordinate and abscissa indicate number of cells (cells/ml × 10^{-5}) and time of incubation (h), respectively.

cular weight 40 000 was collected and confirmed to contain the band 9 as a major component by sodium dodecyl sulfate polyacrylamide gel electrophoresis), and then assayed for inhibitory activity. As shown in Fig. 6, the partially purified band 9 protein inhibited the growth of AH-130 cells considerably at a concentration as low as 50 μg protein/ml. The band 9 corresponding to 40 000 molecular weight (as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration) seems to be localized on the outer surface of liver plasma membranes because plasma membranes prepared from isolated hepatocytes preincubated with papain (100 μg/ml, 37°C for 10

min) inhibited the growth of AH-130 cells less effectively (data not shown).

The nature and the mode of action of the inhibitory principle in liver plasma membranes remains to be elucidated. Although actin (with a molecular weight of about 40000) is known to be associated with some plasma membranes [13,14], a reference preparation of actin at 50 μg/ml did not inhibit the growth of AH-130 cells (data not shown). Furthermore, preliminary investigations revealed that Eagle's minimal essential medium preincubated with liver plasma membranes (1 mg protein/ml) at 37°C for 1 day and then heated at 100°C for 10 min did not support the growth of AH-130 cells, in contrast to Eagle's minimal essential medium preincubated without plasma membranes and then heated at 100°C (data not shown), suggesting that the active principle in liver plasma membranes may destroy some essential component(s) in the culture medium.

Discussion

In the present study we showed that plasma membranes prepared from rat livers (or kidneys) inhibit the growth of ascites hepatoma cells (AH-130) in vitro. The biological implication of this finding seems to be a little different from the kind of model system for the contact inhibition of cell growth that has been reported for the growth inhibition of 3T3 cells (but not SV40-transformed 3T3 cells) by plasma membranes from 3T3 cells [15,16], because the cells, the growth of which is inhibited by liver plasma membranes, are not hepatocytes but are hepatoma cells. However, it seems interesting that the plasma membranes from AH-130 cells are less active compared to the plasma membranes from liver cells.

We have already reported that the proteoglycans in liver plasma membranes are mainly proteoheparan sulfate, whereas those in ascites hepatoma plasma membranes consist mainly of proteochondroitin sulfate [17]. Proteoheparan sulfate on liver plasma membranes appears to be susceptible to the hydrolysis by SH-proteases such as papain and bromelin but resistant to the hydrolysis by other proteases like trypsin and pronase as shown in the present stduy (Table I). Although proteoheparan sulfate itself exerts an inhibitory effect on the growth of AH-130 cells in vitro (Fig. 3), the proteoheparan sulfate in liver plasma membranes does not seem to be simply related to the inhibitory activity of liver plasma membranes (Fig. 4).

The band 9 protein corresponding to 40000 molecular weight appeared to be deleted in plasma membranes treated with SH-proteases (Fig. 5), and it inhibited the hepatoma cell growth in vitro (Fig. 6), suggesting that it may be a major inhibitory principle of liver plasma membranes. It should be noted that the proteoheparan sulfate molecule in liver plasma membranes showed a molecular weight of about 200000 [1], thus apparently being different from the band 9 protein.

The loss of inhibitory activity of liver plasma membranes by SH-proteases seems particularly interesting in relation to our earlier findings [4] as well as the finding by Kambara and Nohara [18]. We have already reported that an intraperitoneal injection of papain (but not trypsin, pronase or collagenase) gives rise to stimulated DNA synthesis in livers (rats and mice) which is preceded by temporary disappearance of cell coat acid mucopolysaccharides [4]. Furthermore, we have recently shown that the adenylate cyclase activity of liver plasma membranes (rats) is specifically stimulated by papain (but not trypsin) at low concentrations in vitro or after an intraperitoneal injection of papain (but not trypsin) [10]. Contrary to the case of liver plasma membranes, no stimulation of adenylate cyclase activity occurred in hepatoma plasma membranes upon treatment with either papain or trypsin in vitro (Koji, T. and Terayama, H., unpublished observations).

Mechanisms of proteoheparan sulfate inhibition of AH-130 cell growth in vitro are not entirely clarified in the present study. However, we have already reported that externally added proteoheparan sulfate is bound to hepatoma cell surfaces, and inhibits the concanavalin A-mediated agglutination of ascites hepatoma cells [1]. Proteoheparan sulfate introduced onto the hepatoma cell surfaces may modulate some enzyme activities relevant to the regulation of cell proliferation such as adenylate cyclase or others, probably through altered lateral movement of enzyme molecules and/or their modulatory components in plasma membranes, although other possibilities may not be excluded at the moment.

The results of present study indicated the possibility that the inhibitory activity of liver plasma membranes may not be ascribed to proteoheparan sulfate associated with the plasma membranes, but may be due to a 40000 molecular weight protein in liver plasma membranes. Although the protein appeared to be localized mainly on the outer surface of plasma membranes and to exert its effect by decomposing of some essential component(s) in the Eagle's minimal essential medium, the nature and mode of action of this interesting protein (or enzyme) in liver plasma membranes are now under investigation.

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