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STUDIES ON THE FIBRINOLYSIS IN TUMOR BEARING MICE

II. Appearance of Fibrinolytic Enzyme in Ascites after Tumor Cells Inoculation

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INTRODUCTION

Kinjo et al.[1] had reported previously that in the later stage after the intraperitoneal inoculation of Ehrlich ascites tumor cells, the antifibrinolytic activity in ascitic plasma had markedly decreased when examined with the streptokinase activation test. However, it has not been known whether this phenomenon was due to decreased antifibrinolytic substance or to relatively increased fibrinolytic enzyme. The following experiments were, therefore, carried out by the authors in order to obtain more precise information on the subject.

ANIMAL EXPERIMENTS

Both sexes of Swiss albino mice used, were bred in our laboratory, fed on a solid food (CLEA, Oriental commercial pellts for breeding purpose) and water ad libitum.

The Ehrlich ascites tumor cells used were obtained from the Growth Research Institute, Kobe Medical College. The tumor cells were propagated in both sexes of mice, 5-8 weeks of age, by intraperitoneal injection of 2×10^6 cells suspended in 0.5 ml of Ringer-Lock solution at every five days.

The mice used were received a single intraperitoneal injection of 2×10^6 cells suspended in 0.5 ml of Ringer-Lock solution. Cells used for the inoculation were obtained fresh from population in their 5th day of growth in the peritoneal cavity.

At different intervals after inoculation, blood sample of 1.0~1.5 ml was taken from the carotid artery under ether anaesthesia and placed in a test tube. Ascitic fluid was then obtained with a pipette and placed in another test tube. The samples obtained were immediately centrifuged for 10 min at 3,000 r. p. m. at 0°C and the resulting supernatants (the ascitic plasma and the blood serum) were employed for fibrinolytic assay.

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FIBRINOLYTIC ASSAY

A. *Materials*

1) Fibrinogen solution: At each experiments, 0.4% fibrinogen solution was freshly prepared by dissolving Cohn Fraction I (Armour Laboratory) in the borate saline buffer (pH 7.8) which contained 0.002 M of $CaCl_2$.

2) Thrombin solution: 0.9% saline solution containing 20 u. thrombin (Nippon Blood Bank) per a milliliter was used.

3) Plasmin inhibitor: Amino-methyl-cyclohexane-carboxylic acid (AMCHA) was synthetized by Nagasawa et al. in the Central Loboratories of Mitsubishi Kasei Kogyo Co., Tokyo. Epsilon amino-n-caproic acid (EACA) was furnished by Daiichi Seiyaku Co., Tokyo.

B. *Methods*

1) Preparation of euglobulin fraction: The supernatant was diluted twentyfold with distilled water, and was adjusted at pH 5.2 with 0.1% of acetic acid. Standing for 30 min in the refrigerator, the solution was centrifuged for 10 min at 3000 r.p.m. at 0°C. The resulting supernatant was decanted, and the precipitant was, then, dissolved with the borate saline buffer (pH 7.4) and a final volume was made as the same volume as the original plasma used.

2) Fibrin plate test: Astrup's method (standard plate method)[2] and Lassen's method (heated plate method)[3] were both used for the fibrinolytic assay and for the determination of the effect of EACA or AMCHA on the fibrinolytic activity of the euglobulin fraction of ascitic plasma. AMCHA or EACA was added to the fibrinogen solution before the formation of fibrin clot plate.

Estimation of the fibrinolytic activity was made after the usual way; but fibrinolysis was not typical in some cases; the surface of the fibrin clot was well dissolved yet the unsolved fibrin clot remained in the deeper layer. This kind of fibrinolysis was tentatively called "pseudo-fibrinolysis" in this paper.

RESULTS

Appearance of the fibrinolytic activity in the ascitic plasma after the inoculation

As shown in Fig. 1-a and b, fibrinolytic activity began to be observed in the ascitic plasma obtained in the later stage of tumor growth. Results had shown that, after 9th day of the inoculation, the distinct fibrinolysis was observed with standard fibrin plate in 38 cases out of 52; pseudo-fibrinolysis in 7 out of 52 and negative fibrinolysis in 7 out of 52: The distinct fibrinolysis was observed with heated plate in 36 cases out of 51; pseudo-fibrinolysis in 10 out of 51; and negative fibrinolysis in 5 out of 51.

However, before 9th day of the inoculation, the number of the cases of the distinct fibrinolysis shown on the both fibrin plate were far less decrease.

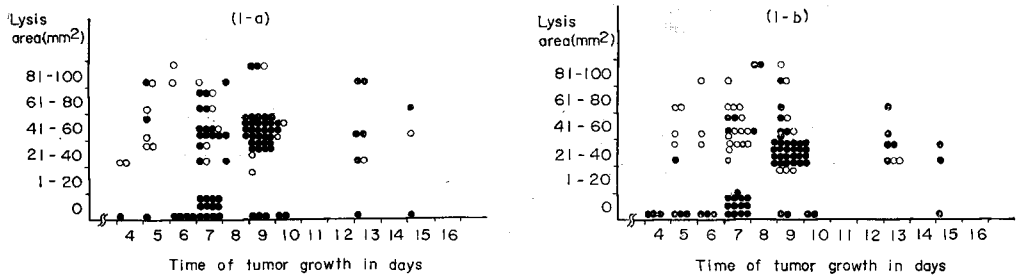

Fig. 1. The fibrinolytic activity of the ascitic plasma.
Fig. 1-a measured by standard plate test; Fig. 1-b, by heated plate test. Solid circles indicate fibrinolysis; open circles, pseudo-fibrinolysis.

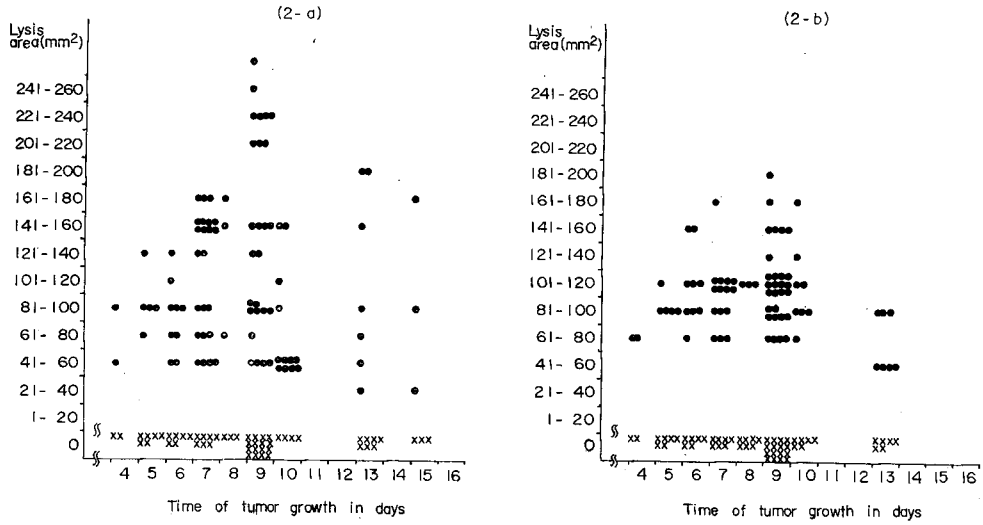

Fig. 2. The fibrinolytic activity of the euglobulin fraction of ascitic plasma.
Fig. 2-a, measured by standard plate test; Fig. 2-b, by heated plate test. Solid circles indicate the activity of the euglobulin fraction of ascitic plasma. Crosses, the activity of the euglobulin fraction of blood serum.

Appearace of the fibrinolytic activity in the euglobulin fraction of ascitic plasma after the inoculation

Results shown in Fig. 2-a and b indicated that, since 4th day after the inoculation, the euglobulin fraction of ascitic plasma showed stronger fibrinolytic activity than that of the natural ascitic plasma. Means and standard deviations of the activities on both kinds of fibrin plate were listed on Table 2. As to the activity of the euglobulin fraction of blood plasma, however, no appearance of the fibrinolytic activity was observed in the all of these experimental cases.

The effect of EACA or AMCHA on the fibrinolytic activity of the euglobulin fraction of ascitic plasma

To determine the nature of the fibrinolytic activity of the ascitic plasma, the effect of EACA or AMCHA on the fibrinolytic activity of the euglobulin fraction of ascitic plasma was examined.

As shown in Table 1, the fibrinolytic activity was completly inhibited at the concentration of 10^{-1} M of EACA, but AMCHA at 10^{-2} M completly inhibited the activity. Even at the concentrations as low as 10^{-3} M of EACA or 10^{-4} M of AMCHA the activity was considerably inhibited in both kinds of fibrin plate test.

Method	Conc. of AMCHA			
	10^{-4}M	10^{-3}M	10^{-2}M	10^{-1}M
Standard fibrin plate test	36%	80%	100%	100%
Heated fibrin plate test	50%	90%	100%	100%
Method	Conc. of EACA			
	10^{-4}M	10^{-3}M	10^{-2}M	10^{-1}M
Standard fibrin plate test	44%	60%	91%	100%
Heated fibrin plate test	10%	68%	95%	100%

Table 1. Percent inhibitory effect of AMCHA or EACA on the fibrinolytic enzyme of euglobulin fraction of the ascitic plasma.

DISCUSSION

Concerning the proteolytic enzyme associated with cancer, the reports have been relatively small in number. Winzler & Burk[4] in 1944 reported that, in rabbits, transplanted malignant tumors (V_2 and Brown-Pearce carcinoma) gave blood protease values several times greater than normal, but young homologouse benign tumors (Shope virus papillomas) did not. Using Rous chiken sarcoma cells, Fischer[5] in 1946 presented evidence that malignant tumor cells in vitro showed proteolytic activity. Tagnon et al.[6] in 1952 and Crane et al.[7] in 1955 respectively reported the increase of fibrinolytic activity in blood of the patient with metastatic cancer of the prostate. Cliffton et al.[8] in 1955

denoted that the potent fibrinolytic activity was found in some of the human cancer tissue obtained by surgical operation. Furthermore, in 1956,[9] a relation of the occurrence of metastasis to the fibrinolysin administered intravenously was studied by the same author.

The previous report by Kinjo et al. indicated that, in later stage after the intraperitoneal inoculation of Ehrlich ascites tumor cells, the antifibrinolytic activity in ascitic plasma had decreased when examined with SK activated test.

In this report authors had presented the evidence that the fibrinolytic enzyme existed in ascitic fluid in mice bearing Ehrlich ascites tumor. As shown in Fig. 2-a and b, the fibrinolytic activity of the euglobulin fraction of ascitic plasma was nearly the same order on both standard and heated fibrin plate. This activity continued fairly high from 4th day to 15th day after the inoculation. On the other hand, as shown in Fig. 1-a and b, the typical fibrinolytic activity of the natural ascitic plasma was observed only in a few cases untill 6th day after the inoculation. However, the fibrinolytic activity was more frequently appeared after the 7th day of the inoculation.

Time of tumor growth in day	Standard fibrin plate (mm^2)	Heated fibrin plate (mm^2)
4	60±24.0	71± 1.6
5	93±19.3	99± 8.2
6	86±34.0	91±38.1
7	92±51.9	78±25.0
8	124±44.2	94± 8.9
9	138±72.4	89±21.2
10	82±43.2	87±27.4
11		
12		
13	112±55.2	48±17.3

Table 2. Means and standard deviations of the fibrinolytic activities of the euglobulin fraction of ascitic plasma.

The difference of fibrinolytic activity in early stage between euglobulin and ascitic plasma would be reasonably explained as follows ; the fibrinolytic activity in the euglobulin fraction might be masked by an inhibitor in the ascitic plasma, and the antifibrinolytic activity in the latter was decreasing with course of the tumor growth. This explanation might be also well supported by the evidence presented by Kinjo et al. in the previous paper.

As shown in Table 1, the fibrinolytic activity of euglobulin fraction of ascitic plasma was inhibited completely at the concentration of 10^{-1} M of EACA or 10^{-2} M of AMCHA and fairly well, but not completely, even at the concentration as low as 10^{-3} M of EACA or 10^{-4} M of AMCHA. Basing upon the evidence reported by Okamoto & Okamoto[10] and Oshiba & Okamoto[11], it was suggested by the authors that the fibrinolytic activity of euglobulin fraction of ascites would be resulted from the fibrinolysin or plasmin in a narrow sense.

On the other hand, the evidence suggesting the hyperplasminic state in locus but not in circulatory blood was reported by Hatano et al.[12] and Sasaki et al.[13] respectively. This kind of local hyperplasminic state was experimentaly reproduced by the authors in the Ehrlich ascites tumor bearing mice.

Swiss albino mice and Ehrlich ascites tumor cells were employed in this experiments, but further detailed experiments would be needed by using other materials. As the ascitic plasma could not be collected untill 3rd day after the inoculation by the technique used, the studies about the fibrinolytic activity of ascitic plasma in such an ealier stage were not made.

As to the underlying mechanisms of the appearance of plasmin in ascites caused by Ehrlich ascites tumor cells, further experiments are in order.

SUMMARY

The fibrinolytic activities in ascites and blood of the Ehrlich ascites tumor bearing mouse have been studied.

1) Using both standard and heated fibrin plate test, the fibrinolytic activity was not infrequently observed in Ehrlich ascitic plasma in later stage after the inoculation. However, a strong fibrinolytic activity was constantly observed in the euglobulin fraction of the ascitic plasma.

2) In euglobulin fraction of the blood plasma taken from tumor bearing mice, however, any fibrinolytic activity was hardly observed by both plate tests.

3) The fibrinolytic activity of the euglobulin fraction of ascitic plasma was completly inhibited at various concentrations of amino-methyl-cyclohexane-carboxylic acid or epsilon amino-n-caproic acid. It is suggested that the fibrinolytic activity observed in the ascitic plasma resulted from the fibrinolysin or plasmin in a narrow sense.

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